DISSERTATION

# DROUGHT INFLUENCES THE PLANT MICROBIOME COMMUNITY STRUCTURE

Submitted by

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# ABSTRACT

## DROUGHT INFLUENCES THE PLANT MICROBIOME COMMUNITY STRUCTURE

Drought is predicted to increase in frequency and severity in many regions across the globe due to climate change. Plants must adapt to reduced water availability and increased temperatures, and much of this adaptation will be mitigated by the adaptations of the plant microbiome. Plant associated microbes provide a variety of beneficial functions to the plant host including the mitigation of plant abiotic stress. Given the relatively short timeframe by which microbes can evolve, we postulate that much of the rapid adaptation of the holobiont, the functioning unit of a plant and all its associated microbes, will be instigated by the microbes. In this dissertation, we demonstrate that (1) plant microbiome rhizosphere communities adjust in response to water deficit, (2) plant microbiome assembly in multiple niche compartments is influenced by water deficit, and (3) drought influences the plant microbiome in concert with AMF inoculation and the preexisting, native soil microbiome.

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# INTRODUCTION

## i.1. Drought is a significant challenge to agriculture and is getting worse with climate change:

Global food systems are threatened by drought which is becoming increasingly frequent and severe through anthropogenic climate change (Wheeler and von Braun 2013; Magnan et al. 2021). Droughts are projected to become five to ten times more frequent, even if aggressive climate mitigation strategies are taken (Naumann et al. 2018). The economic impact of even one year of severe drought can be severe. For example, the 2012 drought afflicted over 60% of the continental US, causing more than $40 billion in agricultural losses (Rippey 2015). Such losses will only increase in a future with more frequent and intensified drought (IPCC 2013; Melillo, Rhichmond, and Yohe 2014). In addition to causing crop losses, drought increases demand for irrigation, which already comprises 70% of US and global water consumption with an increase of 10% expected by 2050 due to climate change alone (Gupta, Rico-Medina, and Caño-Delgado 2020). In many parts of the world, water supply and demand deficits could increase by a factor of five (Naumann et al. 2018), meaning that sufficient irrigation to mitigate the increased drought stress will not be available. Given current population trajectories, which estimate a global population of 9 billion by 2050, 31% of the population, or 2.5 billion people, will be at risk for undernourishment without significant advancements to in agriculture (Dawson, Perryman, and Osborne 2016). When climate change is considered in the models, an additional 21% of the population or 1.7 billion more people are at risk (Dawson, Perryman, and Osborne 2016). Yield trial data from 1964-2010 for breeding wheat for drought prone areas indicates 0.7% to 2.75% increase in the rate of yield increase, which are insufficient to match the projected 70% rise in wheat demand by 2050 (Mwadzingeni et al. 2016). The efficiency of global food systems and their resilience to stressors exacerbated by climate change, like drought, must see significant advancement to address increasing food security challenges. Taken together, this means that dedicated research and innovation in the field of drought resilient agriculture is required.

### i.2. Drought is a significant stressor that plants have a variety of adaptations to combat

Numerous facets of plant physiology are negatively impacted by water stress, most notably photosynthetic capacity. Water stress mediated decreases in leaf water potential and stomatal opening lead to the down-regulation of photosynthesis-related genes and reduced availability of $CO_2$ (Osakabe et al. 2014). Plant growth and productivity are significantly reduced in drought. Drought stress affects the entire plant by desiccating the cells resulting in a loss of turgor pressure and osmotic homeostasis, interference with nutrient transport including the reduction of cation transport, and enzymatic inhibition, all resulting in a reduction of plant growth (Hussain et al. 2018; Yang et al. 2021; Osakabe et al. 2014). Drought leads to an accumulation of reactive oxygen species (ROS) that results in tissue damage and programmed cell death in addition to damaging nucleic acids and other molecules (Verma et al. 2019; Kaur and Asthir 2017). Complex physiological and biochemical adaptations have evolved in plants to help them cope with a variety of environmental challenges. Many studies have been conducted on the molecular and physiological mechanisms behind water-stress tolerance and water-use efficiency (Kang et al. 2021; Wang et al. 2017; Sekhon et al. 2010; Liao et al. 2022; Cardoso et al. 2020). These studies have revealed intricate regulatory network mechanisms that govern plant adaptation to water stress. Plants employ a range of molecular processes to enhance their stress tolerance, preserve proper hormone balance and responses, and shield themselves from excessive light damage. Research has demonstrated that intricate regulatory processes are regulated by abscisic acid (ABA) signaling, ion transport, and transcription factors (TFs) that control stomatal responses. These molecular networks are coordinated and allow plants to adapt and thrive (Kuromori et al. 2022; Chaves, Maroco, and Pereira 2003). Other signaling hormones involved in drought tolerance, including jasmonic acid (JA), salicylic acid (SA), ethylene, cytokinins, auxins, brassinosteroids, and gibberellins, can have synergistic or antagonistic interactions with ABA (Peleg and Blumwald 2011).

Water availability, and the lack thereof, is one of the most important drivers of plant evolution. Structural adaptations to dry environments, including deep tap roots, waxy leaves, and trichomes, are defining features of many plant species. Less obvious, but equally important, are inducible physiological responses that allow plants to adapt to the short-term fluctuations in water availability that occur in most climates. For example, many plants exhibit a degree of root plasticity that allows transient adaptation to environmental stresses. In response to drought, some plants regulate belowground C allocation by altering both the quantity and chemistry of root exudates (Williams and Vries 2020; Gargallo-Garriga et al. 2018; Lin et al. 2023). Plants may also up-regulate root exudation under moderate drought to release more mucilage, a polysaccharide that can ease the movement of roots through dry soil (Gargallo-Garriga et al. 2018; de Vries et al. 2019). Recent reports suggest that root exudates are central to the ecosystem responses to drought and therefore, understanding the dynamics of exudation patterns has potential implications for increasing the resilience of plants towards abiotic stresses. Despite considerable interest in the drought tolerance of crops, root responses have largely been overlooked and represent a key knowledge gap.

### i.3. Plant associated microbiome and their role in drought tolerance

Plants host diverse but taxonomically structured communities of microorganisms, the plant microbiota, that colonize every accessible plant tissue (Dini-Andreote and Raaijmakers 2018; Trivedi et al. 2020; 2022; Leach et al. 2017; Compant et al. 2024; Singh, Liu, and Trivedi 2020). Plant-associated microbiomes confer fitness advantages to the plant host, including growth promotion, nutrient uptake, stress tolerance and resistance to pathogens. It is now recognized that the ability of crops to face environmental stresses is partly mitigated by the microbiome inhabiting the soil, rhizosphere, roots, and other plant tissues. Understanding the dynamic relationships between soil microbes and plants during stress is essential for predicting and potentially managing plant-microbiome interactions to increase crop production's resilience to abiotic stresses (Rolli et al. 2015; Li et al. 2020; de Vries et al. 2020; Raghvendra Saxena 2019; Gagné-Bourque et al. 2015; Ali et al. 2022). Notably, the microbiome's beneficial traits strongly depend on the interplay between the soil nutrient status, environmental conditions, and the plant immune system (Singh, Liu, and Trivedi 2020; Trivedi et al. 2021; 2020). Thus, the successful implementation of microbiome-mediated improvement in plant adaptation and resilience to abiotic stress will depend on our mechanistic understanding of how the microbiome interacts with the plant hosts and one another in varied environmental settings. Despite the increasing recognition of the importance of the microbiome for plant growth and health, harnessing its genomic potential as a new platform for improved drought stress resilience of future crop production is a significant challenge for the coming decade.

### i.3.a. Drought impacts the assembly of plant-associated microbiome

Plant microbiome assembly is a complex ecological process driven by coevolution over millennia (Dini-Andreote and Raaijmakers 2018; Trivedi et al. 2022). It is proposed that the eco-evolutionary interactions between hosts and their associated microbiomes will play a key role in plant resilience to climate change (Trivedi et al. 2022) (See Appendix 1). In addition to the strong selection pressure exerted by the plant compartments for microbial assembly, ecological and evolutionary processes also play a role. For example, drought reduces the host impact on the assembly of the plant fungal community and increases the impact of stochastic forces (e.g. drift or stochastic dispersal) (Gao et al. 2020). Stress levels influence how microbial species interact during evolution. Microbes behave more competitively while under mild stress, while they behave more cooperatively or neutrally when under harsh stress (Gao et al. 2022). Models that forecast the eco-evolutionary dynamics of microbial communities can be created by deciphering the principles underlying the ecological and evolutionary characteristics of microbial communities. However, we have a limited understanding of the impact of drought on the community assembly processes and the influence of such changes in the plant's response to drought.

Drought can directly impact the assembly of the plant associated microbiome however such impact will be more pronounced in the plant surfaces (e.g. the phyllosphere), where environmental conditions fluctuate more rapidly as compared to the relatively stable internal plant tissue environments (i.e. the endosphere) (Trivedi et al. 2020). By changing the soil properties (e.g. soil moisture, redox potential, and soil C dynamics), drought alters the microbial community of the bulk soils that act as "seed banks" for the recruitment of plant-associated microbiome. The rhizosphere microbiomes are influenced not only by external climatic factors but also indirectly by host responses, which include changes in plant physiology, morphology, immune response and root exudation (Figure 1). Drought-induced changes in plant immunity may shape the plant–microbiome, particularly inside plant tissues. In addition, changes in the allocation or distribution of different classes of defense metabolites or signaling molecules under climate change have the potential to further impact microbiome assembly. Taken together, the mechanisms of drought-induced changes to the plant microbiome are numerous and the impacts of these changes on plant fitness in the face of drought stress are varied and difficult to predict.

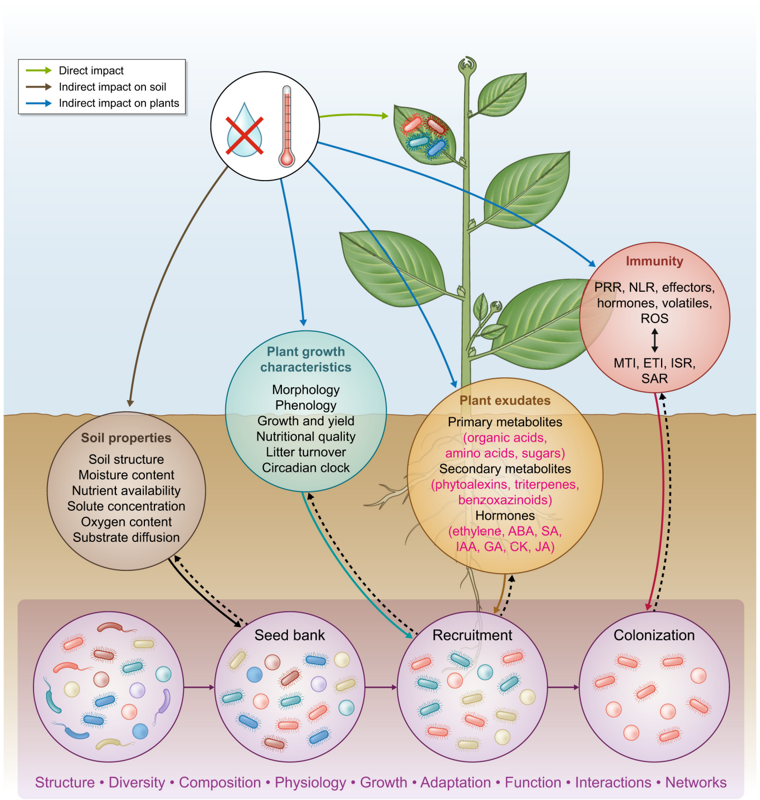

Figure i.1: Impact of climate change on the plant-associated microbiome. Climate change can have a direct (light green arrow) and indirect impact (brown and blue arrows representing the impacts on soils and plants, respectively) on the plant–microbiome. Different climate change drivers will have a variable impact on the microbiome. For example, the direct impact of warming is likely to be stronger on the aboveground epiphytic microbiome than drought. In comparison, drought will have a stronger indirect impact on the soil microbiome than warming. The indirect impact of climate change on the microbiome can be mediated by changes in soil properties (brown circle), plant growth characteristics (green circle), exudation (yellow circle) and immunity (pink circle). Climate change can impact the bulk soil ‘seed’ microbiome directly or indirectly via affecting soil properties (black arrow). These impacts will select tolerant (circular) and opportunistic (oblong) groups while reducing sensitive (curved) groups. As the initial soil microbiome is the source for the plant microbiome assembly, a variation in the composition and functioning of the soil microbiome can determine the outcomes of plant–microbiome interactions under climate change. Plants undergo a set of physiological responses that allow them to adapt to short- and long-term environmental fluctuations (green and yellow circles). Recent reports suggest that plant exudates (yellow circle) are critical to the ecosystem responses to climate change (Williams and Vries 2020). It is likely and equally important that the quantity and quality of root exudate directly affects climate resilience via its impact on the plant-associated microbiome. Growing evidence for the 'cry for help' hypothesis posits that plants recruit specific microbes that can alleviate plant stress in a given situation by regulating root exudation patterns. Changes in plant growth characteristics and exudation patterns will impact the microbial recruitment process (green and light brown arrows), selecting the microbial groups (green-, red- and yellow-colored ovals and circles) that can adjust to the new conditions and metabolize stress-induced communication signals. Plant colonization of microbes (red-colored circles and ovals) will be determined by changes in the plant immune responses (pink arrow) that are postulated to be significantly impacted by climate change drivers. Plant immune-mediated impact on microbial colonization will be stronger for endophytes than for epiphytes. Direct and indirect impacts mediated by climate change can alter many plant-associated microbiome features (purple text in shaded box). Climate change-induced alteration in plant-associated microbiome assembly will have a strong impact on many aspects of plant–microbiome interactions. These interactions will further influence plant growth characteristics, exudation patterns and immune response (dashed black lines). Successive changes in the microbial community composition are shown by purple arrows. ABA, abscisic acid; CK, cytokinin; ETI, effector-triggered immunity; GA, gibberellic acid; IAA, indoleacetic acid; ISR, induced systemic resistance; JA, jasmonic acid; MTI, microbe-associated molecular pattern-triggered immunity; NLR, nucleotide-binding domain leucine-rich repeat receptors; PRR, pattern recognition receptor; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic induced resistance (See Appendix 4) (Trivedi et al. 2022).

### i.3.b. "Cry for help" hypothesis

In addition to the direct effects of root exudate regulation on drought tolerance, the impact on the plant microbiome may be equally important. The emerging "cry for help" hypothesis posits that plants recruit specific microbes that can alleviate plant stress in a given situation (Rolfe, Griffiths, and Ton 2019; López-Ráez, Pozo, and García-Garrido 2011; Ait-El-Mokhtar, Meddich, and Baslam 2023). There is evidence to suggest that plants recruit specific beneficial microbes to their roots to promote drought protection (de Vries et al. 2020; Yandigeri et al. 2012). Upon perception of drought stress, plants adjust their root exudation profiles such that they can then serve as selective signals, chemo-attractants and/or nutritional sources to stimulate beneficial microbiome functions that the plant can leverage to cope with drought stress (López-Ráez, Pozo, and García-Garrido 2011; Rolfe, Griffiths, and Ton 2019; Chai and Schachtman 2022). For example, the glycolysis intermediate, glycerol-3-phosphate (G3P) is likely a key "cry" molecule triggering Actinobacteria enrichment as genes encoding ATP-binding cassette (ABC) transporters of G3P are frequently upregulated in monoderm organisms like Actinobacteria and G3P production has been shown to increase in the roots of drought stressed sorghum (Xu et al. 2018). Actinobacteria possess thick cell walls, meaning that G3P, a precursor to peptidoglycan biosynthesis and cell wall formation, is likely in high demand (Brown, Santa Maria, and Walker 2013; Yao and Rock 2013). Another set of molecules that could be involved in Actinobacteria enrichment are coumarins, which are secreted by plants in low iron conditions to attract siderophore producing microbes (Harbort et al. 2020). Given the evidence that a loss of iron-limitation in plant roots is the initial signal that leads to Actinobacteria enrichment and many strains of Actinobacteria produce siderophores, it is possible that coumarin secretion is one of the responses that triggers this enrichment, however further research is necessary to understand the drought response signaling that leads to the formation of the drought microbiome community. Interestingly, under drought conditions, transporters located in root epidermal cells can efflux ABA into the rhizosphere (Hartung et al. 1996). Several members of phylum Actinobacteria can catabolize ABA as a sole carbon source (Hasegawa et al. 1984; Belimov et al. 2014) suggesting that ABA may serve as a signaling molecule for drought induced microbiome recruitment. A recent study has demonstrated the role of drought induced pipecolic acid as a "cry" to recruit Actinobacteria in sorghum roots (Caddell et al. 2023). In addition to root exudates, plants can also alter the production of immune hormones (JA, and SA); abiotic stress-responsive hormones (ABA and ethylene (ETH)) and expression of gene regulators (MYB72, NRT1.1B, FERONIA, PHR1, F6'H1, HY5) to sculpt microbiota that can provide a novel layer of stress tolerance (Wang and Song 2022). Understanding how qualitative and quantitative changes in root exudation and specialized gene expression affect both competition for resources and cooperative relationships in the rhizosphere will illuminate the specific mechanisms underpinning these interactions, including how changes in microbial community interactions in response to shifting environmental regimes impact plant resilience against drought.

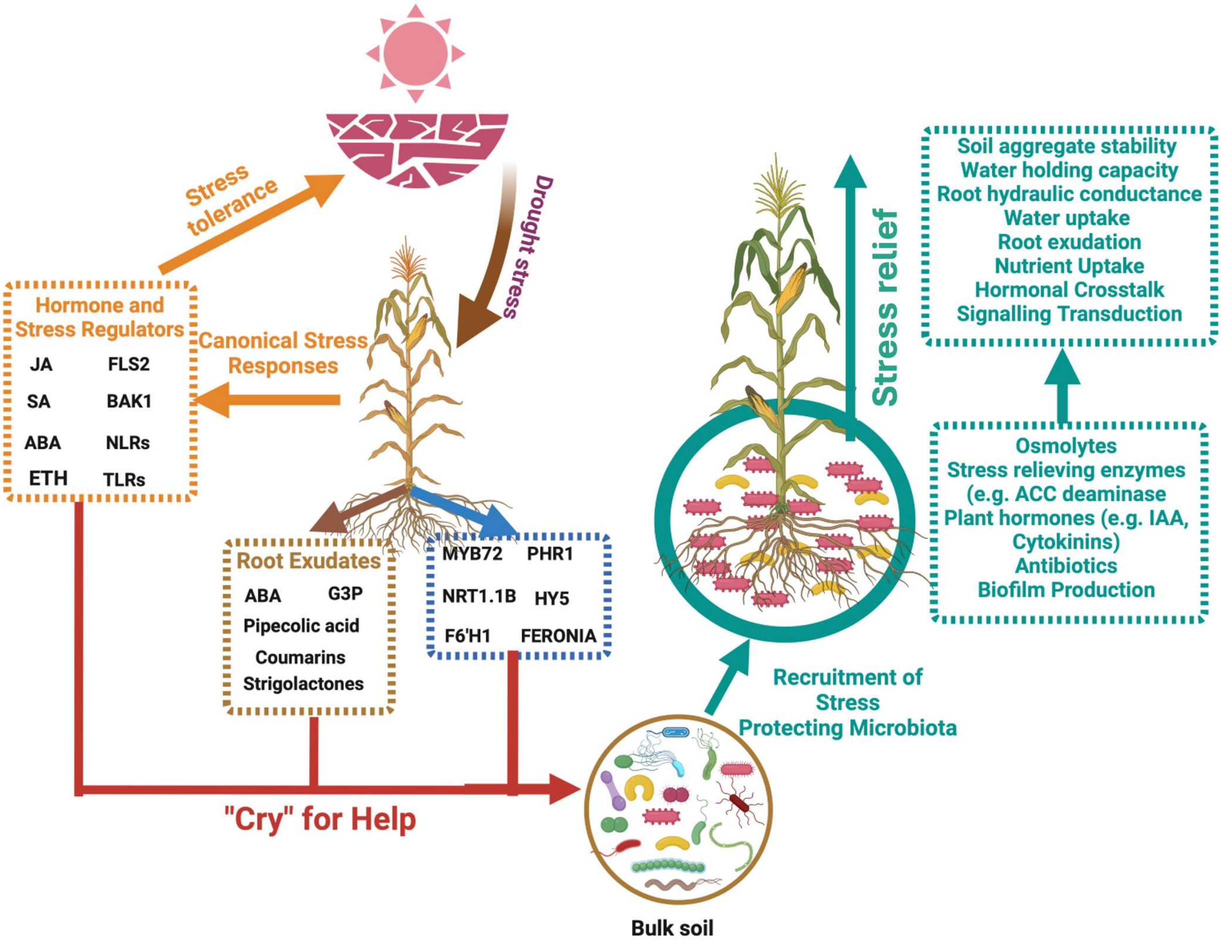

Figure i.2: Plants "cry for help" to reshaping a drought-alleviating microbiota. Under the drought stress plants change their root exudation patterns (brown box; ABA = abscisic acid; G3P = Glyceraldehyde 3-Phosphate ); production of hormones and stress regulators [orange box; JA = jasmonic acid; SA = salicylic acid; ETH = ethylene; plant membrane (FLS2, BAK1) or cytosol-localized immune receptors (NLRs, TLRs)]; and diverse plant genes (purple box; MYB72, NRT1.1B, FERONIA, PHR1, F6'H1, HY5). Drought induced activation of plant responses act as a "cry" to recruit "stress protecting microbiota" from the bulk soil (brown circle) to the plant associated environments (rhizosphere and roots; green circle). The drought recruited microbiota provides stress tolerance by producing osmolytes; stress relieving enzymes (ACC deaminase = 1-aminocyclopropane-1-carboxylate deaminase); plant hormones (IAA = indole acetic acid); antibiotics; or biofilm production (green box). The "cry for help" mediated microbial response increase drought resilience by various mechanisms (blue box).

### i.3.c. Conserved response of drought on the plant-associated microbiome

Drought induces a well conserved taxonomic response in the diversity and community composition of plant associated microbiome (Naylor et al. 2017; Naylor and Coleman-Derr 2018). Studies have suggested that fungi are more drought tolerant than bacteria (Evans and Wallenstein 2012; Bazany, Wang et al. 2022; Naylor and Coleman-Derr 2018; de Vries et al. 2018). The alpha diversity of bacteria present in rhizosphere and root compartments decreases with drought however there are only non-significant impacts on fungal diversity (Bazany, Wang et al. 2022). Although, drought significantly altered the overall bacterial and fungal compositions in the bulk soil, the greatest divergence from irrigated (or watered) controls was observed in rhizosphere and roots (Santos-Medellín et al. 2017; Wipf, Bùi, and Coleman-Derr 2021; Naylor et al. 2017). Drought imposes an overall consistent taxonomic response in different plant species and compartments wherein members of phylum Actinobacteria and Chloroflexi are enriched in response to drought while several members of Acidobacteria and Deltaproteobacteria are depleted (Naylor and Coleman-Derr 2018; Xu et al. 2018; Santos-Medellín et al. 2017). The observed relative enrichment is due to differing life strategies and cell wall architecture of the plant-associated microbiome (Naylor and Coleman-Derr 2018; Xu et al. 2018). Drought-enriched bacteria can form spores and have thick cell walls made of an outer layer of peptidoglycan. This ability to enter a stable, quiescent state during environmental stressors allows the bacteria to persist under drought conditions, while the abundance of less fit bacterial lineages decreases. The enrichment of drought-enriched bacteria is not only due to their ability to survive under stress conditions but is also mediated by the plant host. In addition to upregulating the exudation of exudates that attract certain beneficial taxa (i.e. “cry for help”), drought can deplete taxa that are frequently enriched under normal circumstances. For example, drought can also result in the increased production of reactive oxygen species (ROS) by plant roots, which have been shown to detrimentally affect Gram-negative bacteria to a greater degree than Gram-positive bacteria (Mai-Prochnow et al. 2016), which may contribute to the competitive advantage of Actinobacteria and other durable monoderms in root and root-associated compartments in drought.

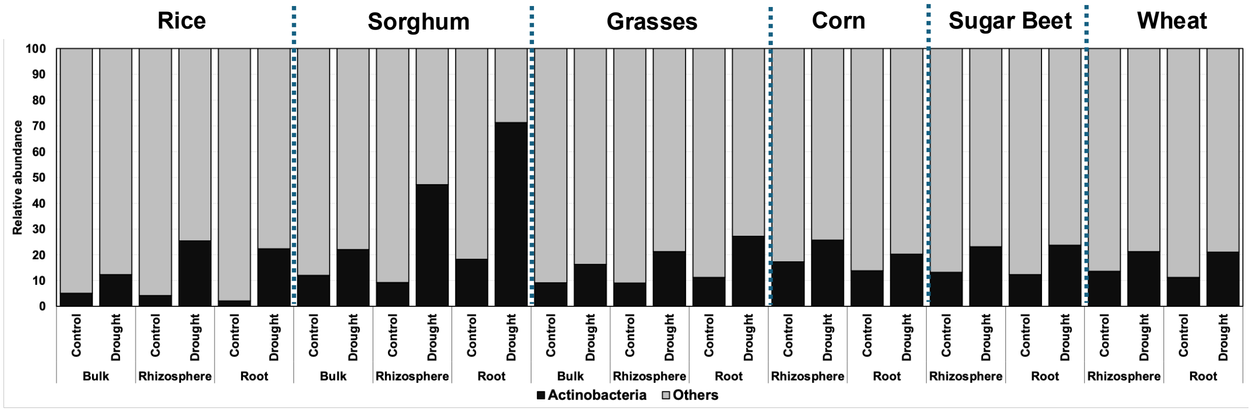

Figure i.3. The impact of drought on the relative abundance of Actinobacteria vs other bacteria phyla from different niches. Bar charts show proportional abundances for Actinobacteria vs other bacterial phyla detected in the bulk soil (Bulk), rhizosphere and root of rice (Santos-Medellin et al. 2017), grasses (Naylor et al. 2017), sorghum; (Xu et al. 2018), corn (Bazany et al. 2022 and this study), sugar beet (Bazany et al. 2022 and this study), and wheat (this study). Abundances were estimated by 16S rRNA gene sequencing. The selected studies used high-throughput sequencing to profile the bacterial communities in field conditions (for corn and sugar beet) and greenhouse conditions (for rice, sorghum, and wheat).

### i.3.d. Co-occurrence network analysis for evaluating microbial communities under drought stress

Co-occurrence networks have become an increasingly popular approach to evaluate microbiome structure in various habitats under different environmental conditions. There are some logical fallacies that may arise in the interpretation of networks. For example, some interpretations conflate positive co-occurrence correlations with evidence of symbiosis and negative correlations with evidence of competition between organisms. However, such interpretations must be based in the biology of the system and consider the scale of sampling as organisms can co-occur by chance (Hartman et al. 2018; Berry and Widder 2014). With careful interpretation, network analysis is a powerful tool in the interpretation of how the composition, structure, and patterns of co-occurrence in a system are impacted by environmental treatments by examining patterns of modularity (Zhou et al. 2011), centrality of certain members of the network (Martín González, Dalsgaard, and Olesen 2010), and the connectivity of the network as a whole (Banerjee et al. 2019). Multi-kingdom networks can allow researchers to examine how diverse taxa co-occur and how these patterns vary with given hosts environments, geographic locations, and experimental treatments (Hartman et al. 2018; Banerjee et al. 2019).

Drought frequently reduces the complexity and stability of the networks (Peng et al. 2024; Gao et al. 2022; de Vries et al. 2018). Networks in drought systems generally have fewer highly connected nodes than networks in unstressed systems (Debray et al. 2022). There is also generally a higher prevalence of positive correlations under drought, which is an indication that the networks are less stable overall (de Vries et al. 2018). Interestingly, when considering functional groups, for example arbuscular mycorrhizal fungi (AMF), networks were strengthened by drought (Gao et al. 2022) indicating that the interpretation of the community response can change given which microbial kingdoms are included, highlighting the importance of multi-kingdom inclusion in co-occurrence networks.

### i.3.e. Microbiome engineering for drought tolerance

Host-mediated microbiome engineering (HMME) is a strategy that utilizes the host phenotype to indirectly select advantageous microbiomes that can improve plant fitness and growth under environmental stress conditions. HMME has been successfully employed to enhance microbiome-mediated drought resistance in wheat (Jochum, McWilliams, Pierson, et al. 2019) and rice (Styer et al. 2024). In the last few years several bacterial isolates have been isolated and characterized for their ability to confer drought tolerance to plants by their ability to produce metabolites or other mechanisms. Microbes can produce a variety of osmoprotectants including proline (Wang et al. 2012), glutamic acid (Carlson et al. 2020), choline and glycinebetaine (Gou et al. 2015) that help protect the roots from desiccation. They can also produce biofilms which can help preserve moisture around the roots (Karimi et al. 2022). Plant-associated microbes can also produce or upregulate the production of a wide variety of hormones in the plant including ABA (Gowtham et al. 2021), indole-3-acetic acid (IAA), SA (Jochum, McWilliams, Borrego, et al. 2019), JA, gibberellic acid, cytokinin, brassinolides (Carlson et al. 2020) that can help the plant to better respond to osmotic stress and other associated stresses by initiating transcription changes, like priming the plant for future pathogen exposure (Egamberdieva et al. 2017). They can also increase 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, which lowers ethylene levels (Carlson et al. 2020). Microbes have been developed and applied in the form of bioinoculant of individual isolates or Synthetic Communities (SynComs) to increase plant growth under drought stress (Xu et al. 2018; Gao et al. 2024; Armanhi et al. 2021; Qi et al. 2022).

Arbuscular mycorrhizal fungi (AMF) are being investigated for their potential to mitigate drought stress in plants. AMF improves soil aggregate stability, soil water holding capacity, root hydraulic conductance, water uptake, plant water status, root exudation, root surface area, root development, uptake of nutrients and water and leads to a significant increase in drought tolerance (Tang et al. 2022). Moreover, AMF improves hormonal crosstalk and the accumulation of osmolytes and phenols. AMF also induces signaling transduction, maintains ionic homeostasis, increases intercellular $CO_2$ concentration, and increases the efficiency of Photosynthesis (PS-I, PS-II) resulting in improving plant growth and yield under drought stress (Liu et al. 2024; Zhang et al. 2019; Boutasknit et al. 2020). A "cry for help" response that is mediated by the release of strigolactones and other exudates is postulated to act as a signaling mechanisms to attract AMF under drought tolerance (López-Ráez, Pozo, and García-Garrido 2011). AMF inoculation has been reported to enhance biomass (Chandrasekaran 2022) root morphology (Liu et al. 2024) and nutrient uptake (Püschel et al. 2021) under drought stress.

Microbes have long been applied as inoculants for biocontrol or biostimulation in agricultural systems. However, their efficacy seems to vary with climate, soil type, and other environmental factors, and their benefits are often sub-par or do not persist, requiring repeat applications even within a growing season. It is now clear that the functionality and persistence of microbes are dependent on interactions with the environment as well as other microbes within a community. To effectively harness the microbiome, we need new approaches which recognize that those microbes living in natural and managed systems typically do so as communities, not populations of single organisms functioning alone. There is strong evidence that interactions of different organisms alter their function while also enhancing the persistence and resilience of microbial communities. For example, the suppression of disease (Bazany et al. 2022; Peralta et al. 2018) (See Appendix 5), degradation of contaminants (Haque et al. 2022; Álvarez et al. 2021), or mineralization of organic matter (Letian Wang et al. 2023) typically involve metabolic interactions between a diverse array of microbes living together. Fundamental ecological and design principles underpinning microbiome communities are in their infancy but are critical to the rational design and manipulation of microbiomes in agricultural systems.

### i.4. Knowledge gaps surrounding the role and response of the drought stressed plant microbiome

*Knowledge Gap 1:* We don’t know how drought impacts plant microbiomes in the field under varying conditions with different host crops and at different sites with different preexisting microbiomes.

Improving our understanding of how rhizosphere microbiomes are shaped by water deficit in the field will allow us to better predict how these communities will shift under climate change scenarios. The examination of bacterial, fungal, and protistan communities will clarify how these microbial kingdoms respond to drought and how their patterns of co-occurrence in the rhizosphere will be altered.

*Knowledge Gap 2:* We don’t know how drought will affect the community assembly processes driving microbial community assembly throughout the plant.

Furthering our understanding of the mechanisms by which microbial communities are formed in various compartments of the plant in different plants and under drought conditions will allow for more robust predictions on how perturbations will affect plant microbiomes and allow for more intentional and informed manipulations of plant microbiomes towards improving plant fitness during droughts. Increased knowledge of the ecological principles that govern the response of microbiome structure and function to climate change will advance our understanding of microbiome properties (e.g. resistance and resilience) that improve plant fitness under novel environmental conditions.

*Knowledge Gap 3:* We don't know how bioinoculants like AMF will impact the plant and the plant microbiome in different host genotypes and root accessions, under different drought conditions, and with different starting soil biochemistry.

AMF are obligate symbionts meaning that they cannot exist without a partnership with a plant, but plants will only form associations with AMF when under some form of stress, most commonly nutrient deficits (Frew, Heuck, and Aguilar-Trigueros 2023). Under drought conditions, there is some evidence that plants are more likely to invest in partnerships with AMF (Bahadur et al. 2019). While AMF are typically considered to be beneficial symbionts, the relationship between AMF and their plant hosts can range from symbiotic to parasitic, though the factors determining where an AMF-plant relationship will fall on this spectrum are largely unknown.

Improving our understanding of both what factors control AMF associations, and the nuanced implications of AMF association on their plant hosts, the plant microbiome as a whole, and the soil, would allow for more accurate predictions on how plants and AMF will respond to environmental perturbations in climate change. Understanding the mechanisms behind plant-AMF coevolution can help inform agricultural and other land management strategies to capitalize on plant-AMF associations for ambitious goals of plant stress relief management, soil management, and crop yield protection under drought.

To better understand how plants adapt to the impacts of global change, we need to understand the role of plant-associated microorganisms – the plant microbiome – in these mechanisms. In this dissertation, I seek to understand how water deficit stress impacts the bacterial, fungal, and protistan communities in corn and sugar beet rhizospheres (Chapter 1), how drought influences the community assembly processes of microbial communities throughout multiple plant niche compartments (Chapter 2), and how an AMF inoculant impacts the fitness of winter wheat and its associated microbiome in varying host genotypes, under different watering conditions, and with different starting soil microbial communities (Chapter 3).

# CHAPTER 1: Water deficit affects inter-kingdom microbial connections in plant rhizosphere

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## 1. Summary

The frequency and severity of drought are increasing due to anthropogenic climate change and are already limiting cropping system productivity in many regions around the world. Few microbial groups within plant microbiomes can potentially contribute towards the fitness and productivity of their hosts under abiotic stress events including water deficits. However, microbial communities are complex and integrative work considering the multiple co-existing groups of organisms is needed to better understand how the entire microbiome responds to environmental stresses. We hypothesize that water deficit stress will differentially shape bacterial, fungal, and protistan microbiome composition and influence inter-kingdom microbial interactions in the rhizospheres of corn and sugar beet. We used amplicon sequencing to profile bacterial, fungal, and protistan communities in corn and sugar beet rhizospheres grown under irrigated and water deficit conditions. The water deficit treatment had a stronger influence than host species on bacterial composition, whereas the opposite was true for protists. These results indicate that different microbial kingdoms have variable responses to environmental stress and host factors. Water deficit also influenced intra- and inter-kingdom microbial associations, wherein the protist taxa formed a separate cluster under water deficit conditions. Our findings

help elucidate the influence of environmental and host drivers of bacterial, fungal, and protistan community assembly and co-occurrence in agricultural rhizosphere environments.

## 2. Introduction

Climate change will have a substantial influence on agriculture as it will increase water demand, limit agricultural production, and exacerbate water scarcity. To be economically and agronomically viable, crop plants need to remain productive in water deficit (WD) environments, which are likely to become more frequent and intense in the future due to climate change (Cotter and Reyes, 2008). The ability of crops to face environmental stresses such as water scarcity can be partly mitigated by the microbiome inhabiting the soil, rhizosphere, roots, and other plants compartments (Naylor and Coleman-Derr, 2018; de Vries et al., 2020; Trivedi et al., 2020; Trivedi et al., 2022). Elucidating the dynamic relationships between soil microbes and plants during stress is essential for predicting and potentially managing plant–microbiome interactions to increase the resilience of crop production to abiotic stresses (Naylor and Coleman-Derr, 2018; de Vries et al., 2020; Trivedi et al., 2021; Trivedi et al., 2022). However, there is little information on how WD influences the complex microbial interactions supported by plant environments, and the degree to which such changes are conserved across different plant hosts.

WD-mediated changes in plant physiology and metabolism are reported to influence the structure and function of the plant microbiome with consequences on plant performance and health (Naylor and Coleman-Derr, 2018; Xu and Coleman-Derr, 2019; de Vries et al., 2020; Santos-Medellín et al., 2021; Trivedi et al., 2022). This is likely caused by a combination of top-down processes in the form of control exerted by the plant on its microbiome and bottom-up processes, namely the responses of the microbial community to the drought itself (Trivedi et al., 2022). The influence of the plant can be explained by the emerging ‘cry for help’ hypothesis that posits that plants intentionally recruit specific microbes that can alleviate plant stress (Rolfe et al., 2019; Rodriguez and Durán, 2020; Rizaludin et al., 2021). According to this hypothesis, upon perception of water stress, plants adjust their root exudation profiles, releasing exudates that can then serve as selective signals, chemo-attractants and/or nutritional sources to stimulate beneficial microbial communities to colonize and provide relief (de Vries et al., 2020). On the other hand, the microbial recruitment could be a general by-product mediated by the direct impact of WD on the microbial seed bank or the indirect impact of changes in the plant physiology (Trivedi et al., 2022). The complex ways these processes interact to shape the plant microbiome are highly circumstantial and currently not well understood.

Plant-associated microbial communities form highly complex ecological networks that include multiple associations between co-existing taxa. Climate extremes can reorganize networks of associations between co-existing soil microbial taxa (Zhou et al., 2011; de Vries et al., 2018; Bardgett and Caruso, 2020; Yuan et al., 2021) with essential feedback on plant resilience and performance in stress environments. For example, recent studies have shown that WD significantly influences microbial co-existence networks (de Vries et al., 2018; Zhang et al. 2021; Xie et al., 2021). These changes in the microbial co-existence network potentially impact the recovery of microbial communities and alter plant–microbe interactions under disturbances. In microbial co-existence networks, positive and negative associations represent aggregation and exclusion, respectively. Research on microbial co-existence networks and their topologies suggest that negative association patterns maximize robustness and stability under disturbances (Coyte et al., 2015; de Vries et al., 2018). While the impact of climate change stressors on the individual microbial networks for certain microbial groups have been studied (de Vries et al., 2018), there is limited information on how WD impacts the direction and strength of intra-kingdom associations across contrasting crops.

In plant environments, trophic interactions are governed by protists that act as top-down controllers of microbial communities and influence food webs by preying on a wide range of bacteria, fungi, and other eukaryotes (Geisen et al., 2018; Gao et al., 2019; Sun et al., 2021). Protists and their interactions with other microorganisms are also subject to change during environmental stress events (Geisen et al., 2018; Gao et al., 2019). Studies have shown that trophic cascades can be destabilized by strong changes to a few important interactions in a food web (de Vries et al., 2018; Bardgett and Caruso, 2020). Furthermore, few studies have shown that protists form central hubs in microbial co-existence networks, linking diverse bacterial and fungal groups (Xiong et al., 2018; Sun et al., 2021). Given their key position, protists can amplify or dampen the impact of environmental perturbations on the microbial co-existence networks in plant-associated environments. However, compared to bacteria and fungi, the impact of WD on rhizosphere protists has seldom been investigated.

Here, we explored the impact of WD on the diversity, community composition, and associations between multi-kingdom rhizosphere microbial groups (bacteria, fungi, and protists) of corn and sugar beet. We chose corn and sugar beet for their commercial relevance, ease of sampling, and physiological differences, as corn is a C4 monocot and sugar beet is a C3 dicot. We tested the following hypotheses. First, we hypothesized that WD will have variable influence on the rhizosphere microbial community composition of different microbial groups (bacteria, fungi, and protists). This hypothesis was based on past work showing that the response of microbes to WD can vary based on differences in molecular, cellular, physiological, and morphological traits (Naylor and Coleman-Derr, 2018; Xu and Coleman-Derr, 2019; de Vries et al., 2020; Trivedi et al., 2022). Second, we hypothesized that WD will decrease the connectedness of microbial co-existence networks by influencing inter-kingdom associations. This hypothesis was based on past work showing that the changes in the species interactions mediated by environmental disturbances promote destabilizing properties in microbial co-existence networks (de Vries et al., 2018; Bardgett and Caruso, 2020; Hernandez et al., 2021).

To test our hypothesis, we collected rhizosphere soil samples from eight sites across four mid-western states in the United States (Supplementary Table S1). We specifically selected for sites where corn and sugar beet were growing in adjacent fields, and the irrigation was maintained through center-pivot (also known as waterwheel and circle irrigation). Under these conditions a natural moisture gradient can be observed between the inner (>20 rows) and the outer (<15 rows) of planted crops. Thus, here, WD is established by a prolonged reduction in water availability compared with fully irrigated (IR) plants closer to the water source. We aimed to explore the taxonomic differences between the rhizosphere microbiomes of WD and IR plants using amplicon (for bacteria, fungi, and protists) sequencing. We also compared the inter-kingdom microbial networks of WD and irrigated plant microbiomes to offer insights into the connectedness and proportion of positive/negative interactions, which serves as a measure of network stability; the hubs within the network, which indicates prominent microbial groups; as well as highly correlated taxonomic groups, indicating microbes that tend to co-exist with one another.

Our results show that microbial co-existence networks differ significantly in key properties such as network connectivity and inter- and intra-kingdom interactions that might inform on their stability under water stress. We also show that a WD-induced shift in the microbial community composition varies with different microbial groups with bacteria and protists being more sensitive to water limitation compared to fungi. Disentangling the role of crucial microbial taxa in microbiome communities in WD conditions might provide suitable approaches to harness plant–microbiome interactions to alleviate water stress. Altogether, we provide novel evidence that WD significantly affects microbial community taxonomic composition and co-occurrence network structures in the rhizosphere, which have implications for the potential changes in their ecological functions under climate change.

## 3. Experimental Procedures

### 3.1 Site selection and sample collection

Eight sugar beet fields with directly adjacent corn fields were identified with help from the Western Sugar Cooperative and selected as sites (Supplementary Table S1.1). Each site contained two crop fields, one sugar beet and one corn. Each crop field contained two irrigation treatments, irrigated (IR), defined as within the reach of the irrigation machinery of the site, and non-irrigated or water deficit (WD) stress, defined as crops beyond the reach of the field's irrigation machinery. The yearly average rainfall from the sampling regions ranges from 431 to 533 mm. Under these conditions, farming for corn and sugar beet is not possible without supplemental irrigation. A lack of irrigation exerts a strong abiotic stress on plants in this region, which has a notable impact on plant and productivity that is equivalent to drought in other regions. Three plants from each crop type were collected from each treatment, totaling 12 plants per site. We collected plant samples from 30 to 40 and 8 to 12 rows inside the field representing IR and WD treatments, respectively. Sample collection was conducted in the summer of 2020 at the flowering time for both the plant species. Samples were shipped to the laboratory at Fort Collins on ice.

### 3.2 Sampling processing and DNA extraction

We used a detailed protocol from Simmons *et al*. (2018) to separate rhizosphere soil samples. Rhizosphere soils were defined as soil clinging tightly to the plant's roots. DNA was extracted from soils using the DNeasy Powersoil Kit (MO BIO Laboratories, Carlsbad, CA, USA) as per manufacturer's instruction. Extracted DNA was quality checked by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), quantity checked by Qubit Fluorometer (Thermo Fisher Scientific), and stored at −80 °C.

### 3.3 Measurement of soil physicochemical properties

Soil properties were determined following conventional methods. Soil gravimetric water content was determined by weighing 5 g of fresh, sieved composite sample, oven drying, and reweighing after no further mass loss. Soil organic carbon (OC) was determined by the combustion method on an element analyser using air-dried soils (Vario MAX C/N, Germany) pH was measured using a pH metre, in a 1:2.5 mass:volume suspension of soil and water.

### 3.4 Amplicon sequencing and bioinformatic analyses

The diversity and community structure of soil bacteria, fungi, protists, and invertebrates was determined by amplicon sequencing using an Illumina MiSeq platform. We used the primer sets 515F/806R (Caporaso et al., 2012), ITS1F/ITS2R (Caporaso et al., 2012) and Euk1391f/EukBr (Amaral-Zettler et al., 2009; Stoeck et al., 2010) to amplify a portion of the bacterial 16S rRNA gene, fungal ITS1 region, and the eukaryotic 18S rRNA gene, respectively. Bioinformatics processing was performed using a combination of USEARCH (Edgar, 2010) and UNOISE3 (Edgar, 2016). Amplicon sequence variant (ASV) tables based on 97% sequence similarity were generated using the USEARCH pipeline. Sequencing run quality was assessed using fastQC (Andrews, 2010). The raw sequences were discarded if they contained ambiguous nucleotides, had a low ($Q < 20$) quality score, or were short in length (<100 bp). Adapters and primers were removed using cutadapt (Martin, 2011). Then samples were demultiplexed. Paired-end reads were merged, and quality was assessed with an initial quality check test. The representative set database was created using the UCLUST and UPARSE algorithm (Edgar, 2013). Unique sequences were located and sorted into unique ASVs. ASVs were clustered using DADA2 and DeNoised using uNoise3 (Xiong et al., 2021a) as described (Xiong et al., 2021b). ASV tables were generated by mapping reads to the representative set database. ASVs were counted at the sample level. Protistan sequences based on the eukaryotic 18S rRNA gene data were taxonomically assigned against the Protist Ribosomal Reference (PR2) database (Guillou et al., 2012). Protists were defined as all eukaryotic taxa, except fungi, invertebrates (Metazoa) and vascular plants (Streptophyta) (Delgado-Baquerizo et al., 2020). Taxonomic identification of bacteria and fungi was obtained against the Silva (Pruesse et al., 2007) and UNITE database (Nilsson et al., 2019), respectively. Bacterial sequences that match host mitochondria and chloroplast were removed.

### 3.5 Statistical analysis

Samples were evaluated separately for bacterial (16S), fungal (ITS), and protistan (18S) communities. Samples were rarified to the lowest occupancy of 8000, 5000, and 3300 reads for 16S rRNA, ITS, and 18S rRNA, respectively. We used the R package ‘mctools’ to analyse microbial community structure (Leff, 2017). To examine beta diversity, Bray–Curtis dissimilarity distances were calculated then ordinated in multidimensional scaling using a constrained analysis of principal coordinates (CAPs) analysis to irrigation treatments.

Permutational multivariate analysis of variance (PERMANOVA) models were generated to determine significant beta-diversity differences correlating with niche compartment, species, site, and irrigation treatment. To examine alpha diversity, Shannon diversity indexes were calculated and evaluated through general linear models (GLMs). Tukey HSD tests were used to determine influence of the above variables on alpha-diversity. To investigate the indicator taxa involved in the differences between IR and WD community, a linear discriminate analysis (LDA) effect size (LEfSe) was conducted to explore the differential microbial populations at the phylum level for bacteria and family level for fungi and protists (Segata et al., 2011). A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study. All statistical analyses were completed using R v 4.0.5 (R Core Team, 2020).

Structure equation model (SEM) was used to analyse the relationships among soil water content, soil properties (pH, OC, moisture), microbial alpha and beta-diversity for both corn and sugar beet (Trivedi et al., 2016; Trivedi et al., 2017; Ochoa-Hueso et al., 2018). CAP1 and CAP2 were used to proxy the variance of microbial community composition. Shannon diversity indexes were used as a proxy for alpha-diversity. The a priori models included all possible pathways among these factors. The significance of each path-coefficient was analysed by calculating its critical ratio ($P < 0.05$). The overall model fit was evaluated with the Bentler comparative fit index, goodness-of-fit index and chi-square test (Trivedi et al., 2016; Trivedi et al., 2017; Ochoa-Hueso et al., 2018). The SEM was performed using Amos Graphics v22 (IBM Corp., Armonk, NY, USA).

### 3.6 Microbial correlation networks

Correlations among bacterial, fungal and protists ASVs were calculated to generate a co-existing network of the three groups. To minimize the influence of rare taxa, only ASVs with more than five reads and three observations were kept in the calculation. We controlled the false discovery rate by performing 1000 bootstraps on each correlation. We kept only the strong ($r > 0.60$) and robust ($P < 0.01$) correlations. The network calculation was performed using the SparCC-based (Friedman and Alm, 2012) algorithm Fastspar (Watts et al., 2019). The networks were displayed in the software Gephi (Bastian et al., 2009). Topological properties including nodes, edges numbers, degree, and Closeness centrality and between centrality were also calculated in Gephi. Scatter plots were generated using the ggplot2 package in R platform.

## 4. Results and discussion

### 4.1 Water deficit impacts the structure of rhizosphere microbiota

Our study shows that inter-kingdom microbial connections in plant rhizospheres are highly sensitive to WD stress in two contrasting crops; however, we also found that such impacts are soil taxa and site dependent. Our soil dataset showed significant (30%–63%) reductions in the moisture content in the WD compared to the irrigated (IR) treatments for both corn and sugar beet within each site (S1.1). The reduction in soil moisture in our study is similar to that in controlled greenhouse experiments designed to compare the impact of WD vs well-watered conditions on plant performance (Puértolas et al., 2017; Singh et al., 2021). Our analyses also showed mostly non-significant difference in pH and organic carbon (OC) in WD and IR treatments for corn and sugar beet within each site (S1.2; S1.3).

Using general linear models (GLMs), we found that the impact of WD on alpha-diversity was significant for bacteria ($P < 0.005$) and protists ($P < 0.05$) but not for fungi (Figure 1.1A).

The alpha diversity of bacteria decreased in WD as compared to IR treatments while the opposite trend was observed for protists (Figure 1.1A). Moreover, crop species were the most important driver of alpha diversity for fungi and protists ($P < 0.005$) but for bacteria, the influence of crop species was non-significant. The interaction between species:site:treatment was significant for bacteria ($P < 0.005$) and protists ($P < 0.05$) but non-significant for fungi (Table 1.1). Our results are in lines with those in de Vries et al. (2018) suggesting that fungal communities are usually resistant to water stress.

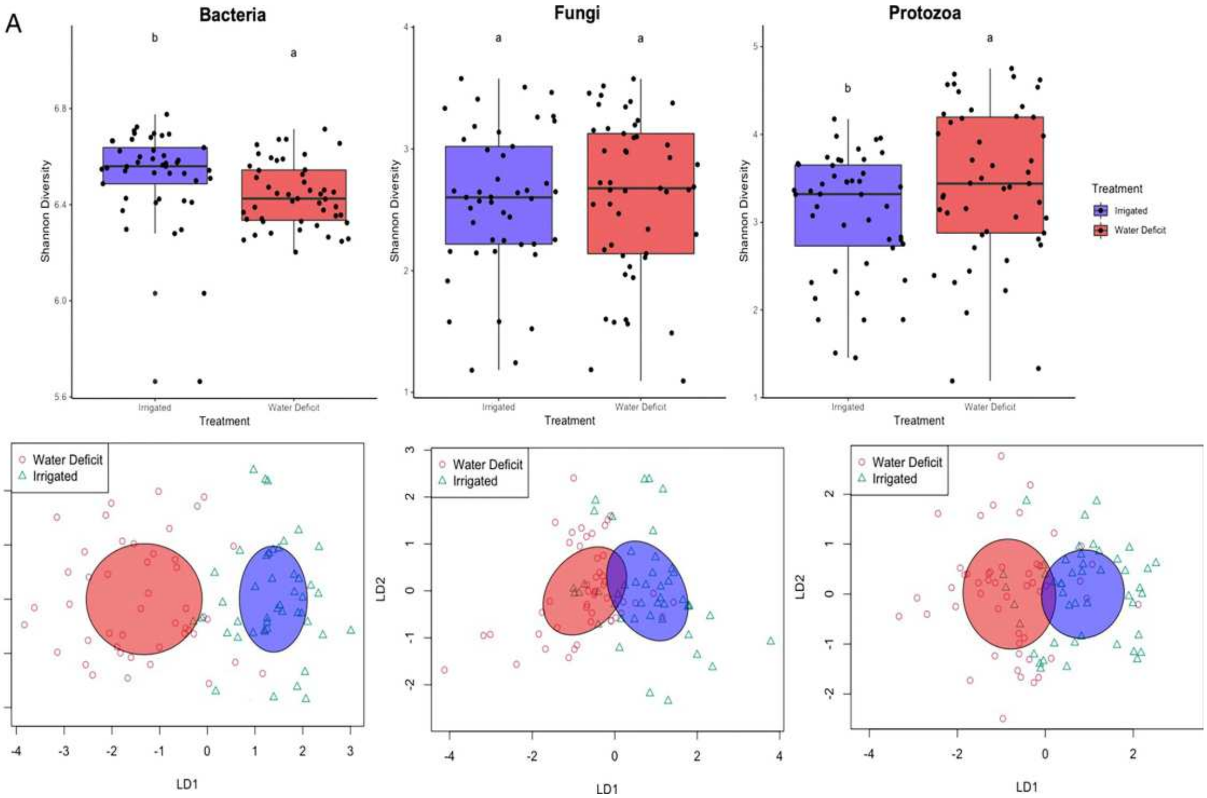

Figure 1.1. Impact of water deficit on the alpha (A) and beta (B) diversity of bacteria, fungi, and protists.

A. Differences between Shannon diversity of bacteria, fungi, and protists in treatments. The boxes show the average Shannon diversity of corn and sugar beet rhizosphere under irrigated and water deficit treatment for bacteria, fungi, and protists. Different letters indicate statistically significant differences ($P < 0.05$).

B. Ordination biplots for canonical analysis of principal coordinates (CAP) illustrating the impact of water deficit (red color circles) and irrigation (green colored triangles) on the combined rhizosphere bacteria, fungi, and protists community of corn and sugar beet. CAP analysis is based on the Bray–Curtis dissimilarity.

Table 1.1. The effects of plant species, site, treatment, and their interactions on the changes of alpha diversity of bacterial, fungal, and protistan communities based on linear mixed model (LMM).

| | **Bacteria** | | **Fungi** | | **Protist** | |
|---|---|---|---|---|---|---|
| **Drivers** | **F-value** | **P value** | **F-value** | **P value** | **F-value** | **P value** |
| Site | 5.187 | 0.0001165*** | 17.9972 | 0.001*** | 2.55 | 0.022* |
| Treatment | 9.645 | 0.0028979** | 0.2203 | 0.64 | 3.894 | 0.053. |
| Species | 0.3181 | 0.5748754 | 17.3878 | 0.001*** | 14.838 | 0.001*** |
| Site:Treatment | 0.729 | 0.6480487 | 1.0568 | 0.402 | 1.25 | 0.289 |
| Site:Species | 2.273 | 0.0402901* | 4.9451 | 0.0001*** | 1.075 | 0.39 |
| Treatment:Species | 3.7906 | 0.0562256 | 0.1262 | 0.724 | 10.124 | 0.002** |
| Site:Treatment:Species | 4.1175 | 0.0009398*** | 1.1797 | 0.327 | 2.164 | 0.049* |

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.001$.

Our results align with the findings of Schmitt and Glaser (2011) who reported that water limitation increased protistan diversity. Protists are primarily aquatic and therefore it is presumed that they will be sensitive to WD (Harder et al., 2016). However, protistan taxa have a variety of lifestyles and body sizes, exhibiting a range of tolerance to soil moisture conditions (Stefan et al., 2014; Fierer, 2017; Geisen et al., 2018). Our results also are in accordance with an earlier study that reported that protist community composition and dynamics are filtered by the influence of plants on their rhizosphere biological and physicochemical environment, resulting in similar patterns observed for rhizosphere bacterial communities (Ceja-Navarro et al., 2021).

We further assessed the relative contribution of multiple factors in terms of sampling sites, plant species, and WD in shaping the rhizosphere microbial communities. PERMANOVA analysis revealed that all examined drivers and their interactions have a significant impact ($P < 0.005$) on the structure of bacterial, fungal, and protistan communities (Table 1.2). As a result of differing environmental conditions (e.g. sites, soil moisture content), shifts in microbial community composition are driven by changes in relative abundance of microbial species, rather than complete disappearance, which explains the importance of all the studied factors in driving beta but not alpha-diversity (Naylor and Coleman-Derr, 2018). The greatest effect on the total microbiome was exerted by the sampling site ($R^2 = 0.47$ for bacteria; $R^2 = 0.53$ for fungi, and $R^2 = 0.39$ for protist; $P < 0.001$ for all three). Our sampling sites varied in soil properties including pH and organic C, both of which are reported to be the major drivers for microbial community composition (Trivedi et al., 2016; Fierer, 2017; Ochoa-Hueso et al., 2018). The sampling site effect represented the interaction effect of site-dependent environmental characteristics (e.g. climate and soil type) and has been shown as the major driver co-influencing the microbiome composition (Coleman-Derr et al., 2016; Santos-Medellín et al., 2017; Hamonts et al., 2018; Guo et al., 2021).

Table 1.2. PERMANOVA output showing importance of plant species, site, treatment, and their interactions as factors shaping the microbial community of bacteria, fungi, and protists.

| | **Bacteria** | | | **Fungi** | | | **Protist** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **F-value** | **R2** | **P value** | **F-value** | **R2** | **P value** | **F-value** | **R2** | **P value** |
| Species | 4.394 | 0.0185 | *** | 9.65 | 0.0281 | *** | 22.822 | 0.07965 | *** |
| Site | 15.805 | 0.46576 | *** | 25.135 | 0.51244 | *** | 16.133 | 0.39416 | *** |
| Treatment | 9.856 | 0.04149 | *** | 8.172 | 0.0238 | *** | 9.769 | 0.0341 | *** |
| Species:Site | 3.348 | 0.09867 | *** | 7.545 | 0.15383 | *** | 5.738 | 0.14019 | *** |
| Species:Treatment | 2.111 | 0.00889 | ** | 3.33 | 0.0097 | ** | 7.175 | 0.02504 | *** |
| Site:Treatment | 2.269 | 0.06686 | *** | 2.531 | 0.05159 | *** | 2.329 | 0.0569 | *** |
| Species:Site:Treatment | 1.603 | 0.04725 | *** | 1.96 | 0.03995 | ** | 1.907 | 0.04658 | *** |
| Residuals | | 0.2526 | | | 0.18058 | | | 0.22338 | |

- *** P < 0.001
- *** P < 0.01

WD was an important driver for all the three groups of microbial communities ($R^2 = 0.041$ for bacteria; $R^2 = 0.023$ for fungi, and $R^2 = 0.034$ for protists; $P < 0.001$ for all three) (Table 1, Figure 1.1B). Few studies have reported small or non-existent impacts of water limitation on soil or root fungal communities (Yuste et al., 2011; Bouasria et al., 2012; Fuchslueger et al., 2016). Our study observed that although the variation explained by WD on bacteria and protists was more significant than on fungi, the impact of WD on fungal communities was still significant. Fungal hyphae networks can allow remote access and redistribution of water that can improve host tolerance towards WD. It is reported that plant presence can modify the impact of water limitation on fungal communities, wherein significant variation was observed in rhizosphere and plant compartments but not in bulk soils (Veach et al., 2020).

Bacterial communities are reported to be more sensitive to WD than fungal communities (Naylor and Coleman-Derr 2018; de Vries et al., 2018). Bacteria and fungi differ in body size, diversity, metabolic activity, dispersal potential, and in their nature of interaction with host or other microbes, affecting species sorting and the community assembly process under environmental stresses. Interestingly, for bacterial communities, the variation explained by the WD was higher than crop species ($R^2 = 0.018$ and 0.041 for species and WD, respectively). Similar results were obtained by Santos-Medellín et al. (2021), wherein they reported that WD explains more bacterial community variation in race than host genotype. Our results thus suggest that WD weakens the correlation between the host phylogeny and bacterial community composition.

We then used structural equation models (SEMs) to explore the direct and indirect relationships among WD, soil properties – including organic carbon (OC), pH, moisture – and microbial community composition and alpha diversity (Figure 1.2). Our SEM showed that WD has a significant negative impact on soil moisture but does not affect OC and pH (Figure 1.2; S1.4). For bacteria, WD had a significant impact on the community composition for both corn and sugar beet. While WD directly impacts bacterial alpha-diversity in corn rhizospheres, the impact is indirectly mediated though moisture in sugar beet. For corn, we did not observe any impact of WD or moisture on the fungal communities. For sugar beet, our SEM showed a minor impact of both WD and moisture on the second CAP component. These results are in line with other studies suggesting that water stress has a more pronounced impact on bacteria than fungal communities (Naylor and Coleman-Derr, 2018; de Vries et al., 2018). For protists, our SEM analysis did not reveal a significant impact of WD and soil moisture on community composition or diversity in corn. For sugar beet, both WD and soil moisture impact the community composition of protists but not the diversity. Interestingly, while the impact of WD on the bacterial community composition was positive, SEM analysis revealed a significant negative impact of WD on fungal and protistan community.

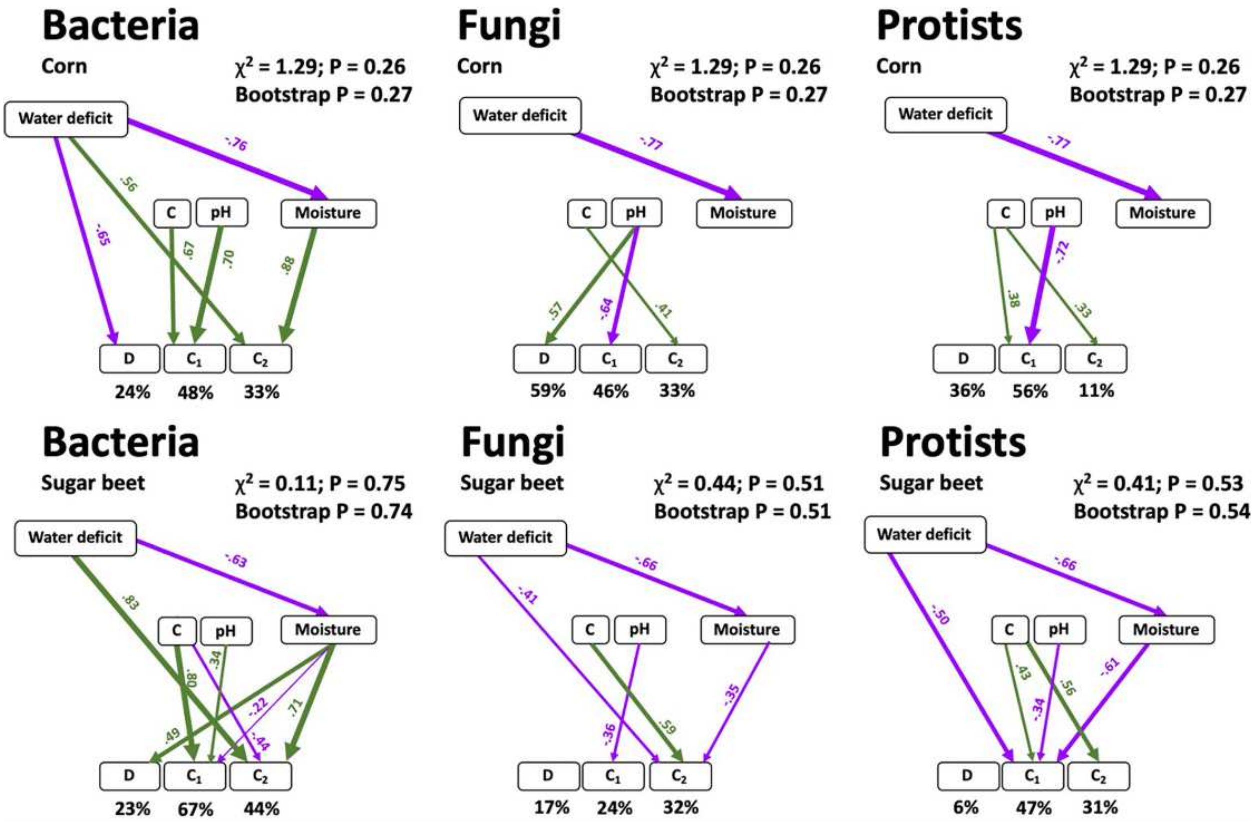

Figure 1.2. Structural equation models showing the effects of organic carbon (OC), pH, soil moisture and water deficit treatment on the community composition (C1 and C2) and Shannon diversity of bacteria, fungi, and protists in the rhizosphere of corn and sugar beet. Numbers adjacent to arrows are standardized path coefficients, analogous to partial regression weights and indicative of the effect size of the relationship. Arrow width is proportional to the strength of path coefficients. Green and purple arrows represent positive and negative effects, respectively. Model fitness details ($\chi 2$ and non-parametric Bootstrap parameters) are close to each figure.

### 4.2 Water deficit impacts the enrichment of selected microbial groups in the rhizosphere

We used linear discriminant analysis (LDA) effect size (LEfSe) to compare microbial communities and identify specific phylotypes of corn and sugar beet rhizosphere responding to WD (Figure 1.3). We observed that the microbial groups that responded to WD are similar for both the plant species. Generally, bacteria from the phyla Actinobacteria, Firmicutes, Chloroflexi, Deinococcus Thermus, Aramatimonadetes increased in WD while those belonging to the phyla Acidobacteria, Verrucomicrobia, Nitrospirae, Planctomycetes, Euryarchaeota, and class Gammaproteobacteria and Betaproteobacteria were depleted in relative abundance. Our results support the core response to water limitation at phylum level with a universal enrichment of monoderm (Gram-positive) bacteria and a depletion of most diderm (or Gram-negative) lineages (Naylor et al., 2017; Santos-Medellín et al., 2017, 2021; Fitzpatrick et al., 2018; Naylor and Coleman-Derr, 2018; Xu et al., 2018; Xu and Coleman-Derr, 2019). Resistance against water limitation involves deeply conserved traits such as osmolyte production, cell wall features or spore formation, which are present in similar groups within soil microbial communities, indicating that the context of a particular location does not affect the phylogenetic pattern of response.

Our results provide support for 'cry for help' hypothesis. First line of support comes from the selective enrichment of microbial groups particularly monoderm under water deficit conditions. The enrichment of the monoderm is driven in part by the interaction within the plant host and not just on the ability of monoderm to withstand water limitation (Fitzpatrick et al., 2018; Naylor and Coleman-Derr, 2018; Xu et al., 2018; Xu and Coleman-Derr, 2019; Santos-Medellín et al., 2021). For example, under drought stress plants secrete glyceraldehyde-3-phosphate, which can be efficiently transported and utilized by Actinobacteria (Xu et al., 2018;

Xu and Coleman-Derr, 2019). Furthermore, many monoderm strains are reported to provide drought resistance to several crop plants (Xu et al., 2018; Santos-Medellín et al., 2021).

For fungi, we observed only four groups at the family level that were indicators for the IR or WD treatments. This observation again suggests that fungi are more resistant to water limitation than bacteria. Our results showed an enrichment of members of the phyla Mortierellomycotaand depletion of members of Basidiobolomycota in WD compared to IR treatment. Members within both these groups are relatively less dominant and diverse with limited information on the traits that can be related to their drought response.

In comparison to fungi, there were more protist groups that responded to IR or WD. Soil moisture has been reported as the most influential edaphic factor differentially affecting different functional groups within protists (Fiore-Donno et al., 2019). Canarini et al. (2021) identified protists as the biomarker for drought along with the Gram-negative and Gram-positive bacteria. Members of WD indicator protists families, such as Acanthamoebidae and Flamella lineage, produce cysts that are very efficient in preserving protists for weeks and even years against environmental stresses such as drought (Geisen et al., 2018). Members of family Litostomatea, Acrasidae and Allapsidae were identified as WD indicators in our study and are reported to prefer dry environments (Oliverio et al., 2020). Overall, we have identified protistan families that are robust bioindicators for WD or IR treatments. Given the variety of functional trophic roles that protists play in shaping microbial dynamics (Bates et al., 2013; Gao et al., 2019; Sun et al., 2021) these bioindicators will be key to understand the trophic complexity in response to water stress.

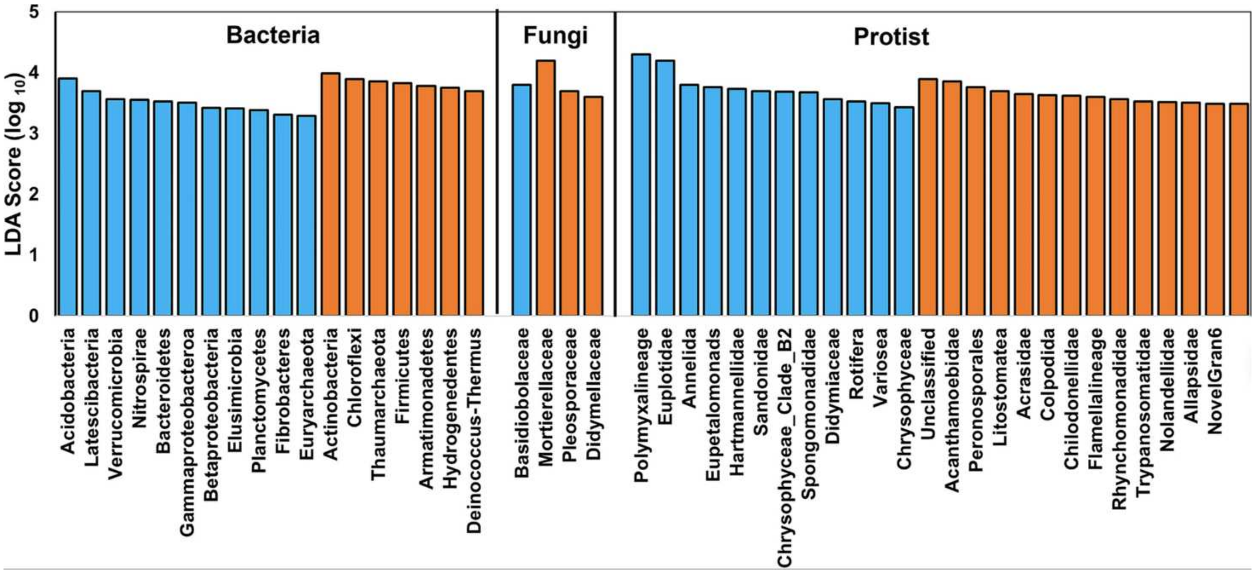

Figure 1.3. Linear discriminate analysis effect size (LEfSe) analysis of bacterial (phylum), fungal (family), protistan (family) groups that were indicators for irrigated (blue) and water deficit (brown) treatments (LDA score > 3).

### 4.3 Water deficit affects rhizosphere microbiome co-existence networks

Our results showed that microbial inter-kingdom network patterns shifted clearly in response to WD for both corn and sugar beet (Figure 1.4). We observed that the protist taxa separated distinctly and formed a separate cluster under WD conditions for both corn and sugar beet (Figure 1.4A–D). For corn, the hub microbial taxa in IR conditions were fungi and bacteria, whereas in WD conditions, the hubs were all protists (Figure 1.4I and J). For sugar beet, the hub microbial taxa in both IR and WD networks were solely protists (Figure 1.4K and L). In the IR networks for both corn and sugar beet, bacterial taxa have lower network connectivity (network degree) (5.36 and 6.01 for corn and sugar beet, respectively; $P < 0.005$) as compared to fungi (9.5 and 7.9 for corn and sugar beet, respectively) and protists (6.8 and 9.8 for corn and sugar beet, respectively) (no significant difference between fungi and protists) (S1.5). In the WD networks for both the plant species, the average number of degrees for protists (9.2 and 9.3 for corn and sugar beet, respectively) was significantly higher ($P < 0.005$) than bacteria (3.5 and 4.7 for corn and sugar beet, respectively) and fungi (5.7 and 3.7 for corn and sugar beet, respectively) (no significant difference between bacteria and fungi). For both the plant species, we observed a significant decrease ($P < 0.005$) in degree for bacteria (3.5 vs 5.3 and 4.7 vs 6.8 for corn and sugar beet, respectively) and fungi (5.8 vs 9.5 and 3.8 vs 7.8 for corn and sugar beet, respectively) in WD as compared to IR network. Our results thus suggest that in WD networks, protists are more central with a higher number of connections, and the relative centrality of bacteria and fungi is lower than in the IR networks.

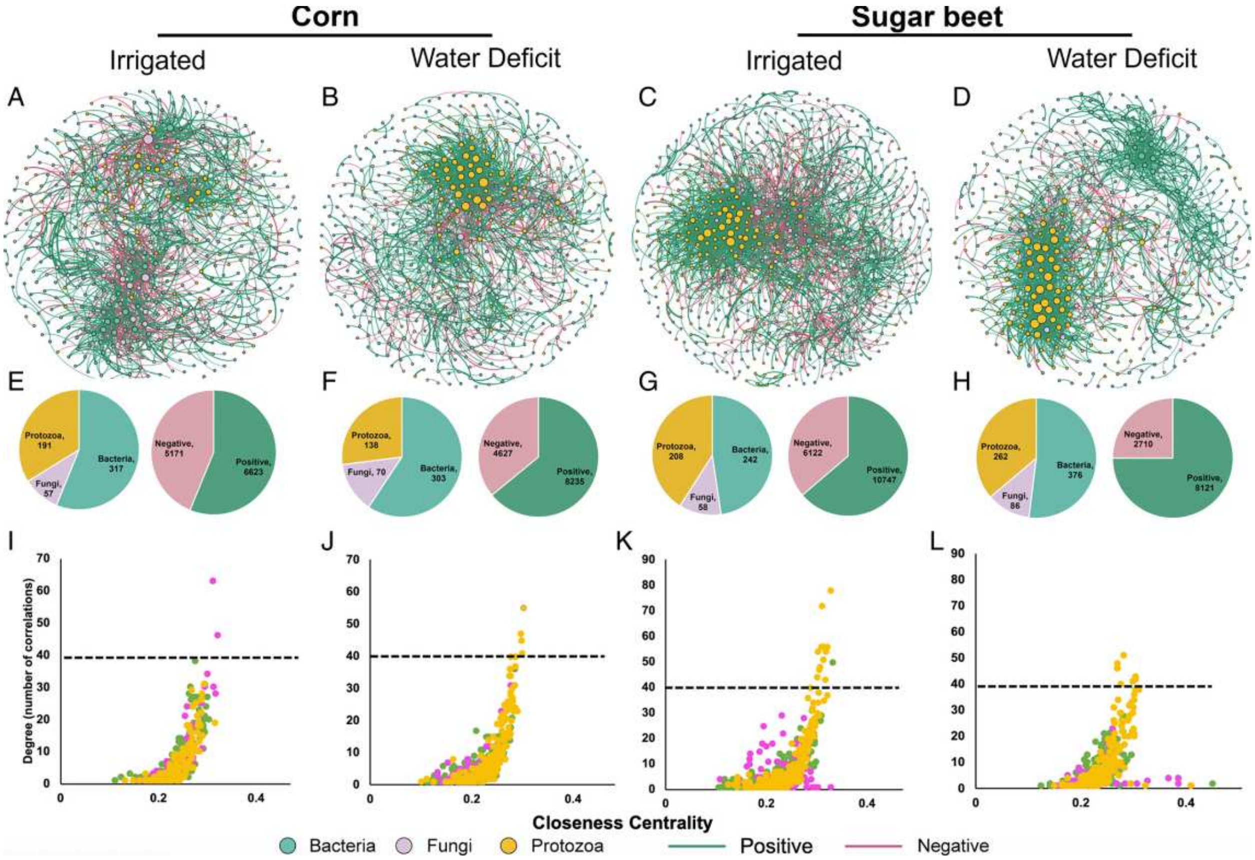

Figure 1.4. Co-existing networks of soil bacteria, fungi and protists under different treatments (A – Irrigated Corn; B – Water Deficit Corn; C – Irrigated Sugar Beet; d- Water Deficit Sugar Beet). Nodes indicate microbial ASVs (green – bacteria, purple – fungi, yellow – protists) and edges indicate strong ($r > 0.60$) and significant ($P < 0.01$) correlations among ASVs (green edges indicate positive correlation and red edges indicate negative correlation). Under each network, the left pie chart indicates the number of ASVs from each group in individual network, and the right pie chart indicates the number of correlations (positive vs. negative) in each network (E–H). The scatter plots below show the importance of nodes in each network (I–L). Higher betweenness centrality indicates a potential connector while higher closeness centrality indicates a potential module hub.

In microbiome studies, protists have received little attention despite their key role in controlling bacterial and fungal populations (Geisen et al., 2018; Guo et al., 2021). Protists are sensitive to environmental disturbances, occupy key position in inter-kingdom microbial networks, and are postulated to enhance microbial mediated functions (Xiong et al., 2018). We illustrate the importance of protists as possible top-down controllers of microbiome community interactions linked to plant stress response. We therefore propose that a holistic microbiome perspective, including bacteria, fungi, and protists, provides the optimal next step in predicting plant performance under water stress.

The percentage of positive correlations increased from 56% to 65% and 63% to 75% in WD as compared to IR network for corn and sugar beet, respectively (Figure 4E–H). A large proportion of positive links between the interacting members can cause instability in microbial networks. Conversely, higher positive interactions infer that the members respond similarly to environmental fluctuations resulting in positive feedback and co-oscillations (Coyte et al., 2015; de Vries et al., 2018). Our results thus suggest that water limitation will destabilize co-oscillation in communities and will weaken the stability of networks. Notably, inspection of network architecture indicates that in both the IR and WD networks, there were more positive intra-kingdom as compared to inter-kingdom correlations (S1.6). Interestingly, the percent increase of positive associations in WD was driven by an increase in the intra-kingdom associations while the inter-kingdom associations became more negative in WD as compared to IR networks (S1.6). For example, in sugar beet, the bacteria–bacteria positive associations increased from 63% to 94% in the WD as compared to IR networks. On the other hand, the negative associations between bacteria and protists increased from 59% to 80% in WD as compared to IR networks. The competition between microbial groups for the root exudates is postulated to contribute towards negative correlations between bacteria and eukaryotes (Durán et al., 2018). Taken together, these results suggest that the detected microbial inter-kingdom associations in the rhizosphere become more intense and competitive under WD. Further research involving reconstitution experiments to disentangle microbiome interactions will reveal mechanisms that govern microbiome assembly in WD. These insights on the complex plant–microbiome interactions will be crucial in the development of targeted and effective microbial amendments that can improve crop fitness and productivity under WD.

## 5. Conclusions

This study advances the understanding of ecological processes that occur in the rhizosphere of crops under water stress. Additionally, our study highlights the importance to consider protist and their associations with other microbes to evaluate the impact of environmental stresses on crop microbiome. We propose that a deeper understanding on how qualitative and quantitative changes in root exudation affect both competition for resources and cooperative relationships in the rhizosphere will illuminate the specific mechanisms underpinning these interactions, including how changes in microbial community interactions in response to shifting environmental regimes impact plant performance. Understanding the dynamics of inter-kingdom interactions under stressful conditions will provide a way forward to engineering complex crop microbiomes with predictable behavior and robust outcomes.

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## Authors contribution

P.T. conceived and supervised the study. P.T. and K.B. designed the experiments. K.B. performed the experiments. K.B., J.T., M.D.B., P.T., and B.K.S. analysed the data. P.T. and K.B. wrote the manuscript. All authors read and approved the final manuscript.

# CHAPTER 2: Water deficit stress alters the microbial community assembly, structure, and sources in corn and sugar beet

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## 1. Summary

Plant associated microbes have the potential to improve plant fitness under abiotic stress conditions like drought by providing stress relieving benefits to the host, however there is limited research on the complex ways in which microbial communities assemble in plants under varying environmental conditions. In a field study, we examined the bacterial, fungal, and protist communities of the rhizospheres, roots, and leaves of corn and sugar beet plants grown in the field under irrigated and water deficit conditions. We hypothesized that water deficit stress would alter community composition and structure of plant microbiomes by shifting the relative importance of community assembly processes and the patterns of movement from microbial sources to sinks. Using amplicon sequencing and community assembly and source tracking modeling approaches, we found that the water deficit treatment led to key differences in microbial community structure and that these changes were likely driven by differences in community assembly processes and microbial source communities. Altogether, these results indicate that plant microbiome communities are shaped by available microbial sources, host selection factors, microbial interactions, and stochastic forces, and that each of these factors are influenced by water deficits. These findings highlight the importance of applying ecological

concepts to plant microbiome research to elucidate the impacts of environmental factors on microbial community assembly.

## 2. Introduction

Drought poses significant threats to agricultural systems and food security and is increasing in frequency and severity in many regions due to climate change (Kuwayama et al. 2019; Bahadur et al. 2019; Dai 2013). Drought has caused significant crop losses in the last four decades (Lesk, Rowhani, and Ramankutty 2016) and is predicted to increase yield losses in the future (Leng and Hall 2019, Pradhan et al. 2022). For example, by the end of the 21st century, drought severity is predicted to increase the yield loss risk of corn, rice, and wheat by 9-19.4% (Leng and Hall 2019). Drought additionally increases the demand for irrigation, which accounts for 70% of global water consumption, with an increase of 10% expected by 2050 due to climate change (UN General Assembly, 2nd Committee) meaning that drought-induced crop loss cannot be mitigated by increased irrigation alone. Harnessing the plant microbiome to improve drought resiliency is increasingly considered a viable, sustainable future approach (Trivedi et al. 2022; De Vries et al. 2020; Naylor and Coleman-Derr 2018). However, optimizing the genomic potential of microbiomes as a new platform for enhanced crop production in a drier world requires a detailed understanding of the ecological processes that drive microbiome assembly and dynamics. In order to appropriately leverage the plant microbiome to improve crop resilience to drought stress, we must understand how drought affects the avenues by which microbes colonize the plant host, the dominant community assembly processes, and patterns of multi-kingdom microbial co-occurrence in various plant niches.

Plants sculpt the microbial communities of their various niches (Hacquard et al. 2017). The plant-associated microbiome is mainly derived from soils and gradually enriched and filtered at different plant compartments (Trivedi et al. 2020; Singh et al. 2023). The relative importance of the factors driving microbiome composition in plant niches varies for different microbial groups (Coleman-Derr et al. 2016; Hamonts et al. 2018; Xun et al. 2021). For example, plant associated bacterial communities are shaped more strongly by the influence of the plant, varying more strongly by niche compartment (bulk, rhizosphere, and rhizoplane), while fungal community is shaped more strongly by the sampling location and the preexisting soil community (Hamonts et al. 2018; Coleman-Derr et al. 2016). Drought can influence the structure and function of the plant-associated microbiome through various abiotic and biotic changes that include a reduction in water availability, changes in soil chemistry and physical properties, and altered plant physiology. Changes in soil properties under drought stress can alter the microbiome of bulk soils, the initial pool for microbial recruitment in plant habitats (Trivedi et al. 2021; Naylor and Coleman-Derr 2018). Similarly, changes in the root exudation patterns or immune response to drought can influence the host-mediated selection responsible for "filtering" effects that allow selective colonization of different microbial groups in plant microhabitats (Williams and de Vries 2020; Singh, Liu, and Trivedi 2020). While drought-mediated dysbiosis in the rhizosphere and root bacteria communities is well studied, little is known about the assemblies of different microbial groups along the soil–plant continuum, including root, rhizosphere, and phyllosphere for crops growing in fields under water stress.

Plant-associated microbial communities form highly intricate ecological networks that constitute numerous interactions between coexisting taxa, belonging to diverse groups in bacteria, fungi, and protists (van der Heijden and Hartmann 2016). Few studies have provided evidence that drought has a significant impact on key properties of microbial networks including network connectivity and inter- and intra-kingdom interactions (de Vries et al. 2018; Bazany et al. 2022; Vilonen et al. 2023; Lei et al. 2023; Gao et al. 2022). Drought has a stronger impact on the bacterial compared to fungal co-occurrence networks suggesting that soil bacterial communities are more vulnerable to drought than fungal communities (de Vries et al. 2018). Generally, drought responsive microbial taxa are highly central and connected within networks, suggesting that they are the major drivers of changes in network structure (de Vries et al. 2018; Bazany et al. 2022; Gao et al. 2022). Drought increases the overall positive links between the interacting members, potentially indicating instability in microbial networks (Bazany et al. 2022; Gao et al. 2022). Previous studies have reported the impact of drought on the individual microbial networks (de Vries et al. 2018; Peng et al. 2024) or multi-kingdom interactions in a single compartment (Bazany et al. 2022; Lei et al. 2023), however there is a limited information of the impact of drought on the direction and strength of multi-kingdom microbial associations for different compartments across contrasting crops.

Plant-associated microbial communities are shaped by complex ecological processes including selection, dispersal, diversification, and drift (Cordovez et al. 2019). Compared to larger organisms, microbes often possess greater capacity for dispersal due to their small size, dormancy capabilities which may be selected for in periods of abiotic stress like droughts, and the capability to rapidly evolve due to short lifespans and horizontal gene transfer (Wilkinson et al. 2012; Lennon and Jones 2011; Ochman, Lawrence, and Groisman 2000). While the reproducibility of microbial communities in controlled environments may indicate that plant microbiomes are largely defined by deterministic processes, like homogenous and heterogenous selection (Finkel et al. 2019), this has yet to be extensively verified in the field where more stochastic forces, like drift, dispersal limitation, and homogenizing dispersal are in affect (Zhou and Ning 2017). Climate change is postulated to alter the relative importance of ecological processes in controlling microbial community diversity and succession (Trivedi et al. 2022). For example, in grassland ecosystems, experimental warming decreased the relative importance of drift and increased homogeneous selection (Ning et al. 2020; Bei et al. 2023). Little is known about the assembly processes that determine the assembly processes of microbial communities in various plant niches under drought stress. Understanding the impact of drought on community assembly processes would elucidate how microbial communities form under drought. In this study we aimed to examine how water deficit (WD) influences the structure, assembly, and co-occurrence patterns of bacterial, fungal, and protistan communities in the rhizosphere, root, and phyllosphere of two most important crops of mid-Western US, corn and sugar beet. We hypothesize that: 1) At the community scale, environmental selection of drought-adapted microbial taxa will alter the community composition of plant-associated microbial communities; 2) The plant environments and host selection will drive drought-induced shifts in different plant compartments; 3) Drought will impact microbial community assembly patterns by reducing stochastic processes like dispersal and increasing the prevalence of selection. We believe that dispersal will be limited because many taxa require water for mobility, to move into and throughout the plant and that selection will increase both due to the direct influence of drought on the microbial communities; 4) Drought induced competitive inter and intra kingdom microbial interactions will influence the connectedness of microbial co-existence network in different plant compartments.

To test our hypotheses, we collected bulk soil, rhizosphere soil, root, and leaf samples from eight paired corn and sugar beet grown under irrigated and water deficit conditions in adjacent fields located across the mid-western United States (S2.1). We used amplicon sequencing to profile the bacteria, fungi, and protists associated with the rhizosphere, roots, and leaves of corn and sugar beet grown under regular irrigated conditions in adjacent fields. We then applied a suite of statistical and ecological modelling approaches to examine the impact of drought on community assembly, source-sink relationships, and multi-kingdom interactions in different plant compartments. This research addresses key knowledge gaps in the ways that bacterial, fungal, and protistan communities assemble in the plant rhizosphere, root, and phyllosphere and how these communities and the ecological processes driving their assembly are influenced by drought stress.

## 3. Methods

### 3.1 Sample Collection

We collected bulk soil cores and plants (leaves, roots, and rhizosphere soil) from adjacent corn and sugar beet fields in eight sites in Colorado, Montana, Nebraska, and Wyoming (S2.1). Samples were collected from both within the reach of the central pivot irrigation boom, called the irrigated treatment, and the planted area beyond the reach of the irrigation machinery, called the water deficit or the "drought" treatment. The average yearly rainfall in these sites ranged from 431 to 533 mm, which is insufficient for growing corn or sugar beet without additional irrigation, meaning these plants were grown under dry conditions and experienced significant osmotic stress. The soil in the non-irrigated treatment had a gravimetric water content of 30-63% less than the irrigated soils (S2.2). This is a similar water reduction to that of greenhouse studies designed to simulate drought (Singh et al. 2021; Puértolas et al. 2017). The irrigated plants were collected between 30-40 rows into the field and the water deficit plants were taken 8-12 rows into the field where irrigation did not occur. Three corn and three sugar beet plants were selected from each watering treatment resulting in three replicates for the plant associated compartments (leaves, roots, and rhizosphere soil) and a total of twelve plants per site. Two soil cores were collected outside the zone of planting of each corn and sugar beet field. These cores were subdivided into four technical replicates. All samples were collected in the summer of 2020 during the flowering stage of both species. Samples were placed on ice and shipped to Colorado State University for processing.

### 3.2 Sample Processing

Bulk soil samples were homogenized through a 2 mm sieve and frozen at -20 °C until further processing. Plant samples were divided into three niche compartments—rhizosphere soil, classified as any soil tightly bound to the plant roots after roots were thoroughly shaken; root compartment, consisting of the root plane and endosphere (Hamonts et al. 2018); and phyllosphere compartment, consisting of the leaf plane and endosphere. Rhizosphere soil was separated from plant roots using a protocol from Simmons et al. (2018) in which roots were shaken and the remaining soil clinging tightly to the roots was defined as the rhizosphere soil and scraped off. Roots were rinsed with DI water, placed in 50 mL tubes with 40 mL DI water, agitated on a shaker, then rinsed again. Leaves were rinsed to clear soil from the surface, though roots and leaves were not surface sterilized as root and leaf samples are intended as a composite of endosphere microbes and surface microbes (Hamonts et al. 2018). After the soil was removed, roots and leaves were frozen in liquid nitrogen, ground with mortar and pestle and kept frozen at -20 °C until further processing.

### 3.3 DNA Extraction and Amplicon Sequencing

DNA was extracted from the bulk and rhizosphere soil samples using the DNeasy PowerSoil Pro Kit from QIAGEN (QIAGEN Strasse, Hilden, Germany). From root and leaf samples, DNA was extracted using the DNeasy PowerPlant Pro Kit from QIAGEN (QIAGEN

Strasse, Hilden, Germany). DNA quality was assessed with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and DNA quantity was determined with a Qubit Fluorometer (Thermo Fisher Scientific).

Amplicon libraries of the 16S rRNA gene using the 515F/806R primer set (Caporaso et al. 2012), fungal ITS1 region using the ITS1/ITS2 primer set (Caporaso et al. 2012), and eukaryotic 18S regions using the Euk1391f/EukBr primer set (Amaral-Zettler et al. 2009; Stoek et al. 2010) were prepared using Earth Microbiome Project single barcoded primers and protocols (www.earthmicrobiome.org) to examine the bacterial, fungal, and protist communities respectively. The DNA was amplified in 30 cycles in 50 μL reactions with Invitrogen Platinum Hotstart PCR Master Mix 2x (Thermo Fisher Scientific). For the amplification of 16S rDNA, we used peptide nucleic acid (PNA) clamps, designed to specifically bind and block the amplification of plastid DNA (pPNA, 5′-GGCTCAACCCTGGACAG-3′) and mitochondrial DNA (mPNA, 5′-GGCAAGTGTTCTTCGGA-3′) (Lundberg et al. 2013). For 18S, we used PNA clamps designed to block the amplification of the plant host (PNA, 5’-CATTGGTCGGCTTGTCC-3’). All samples were pooled in equimolar concentrations and then sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) with a paired-end protocol at the Next Generation Sequencing Facilities at Colorado State University. More detailed descriptions on DNA amplification are available in Supplementary Materials and Methods.

### 3.4 Bioinformatics Analysis

USEARCH v11 (Edgar 2010) and UNOISE3 (Edgar 2016) were used to process reads into operational taxon unit (OTU) tables. FastQC was used to determine the quality of each sequencing run (Andrews 2010). Adapters and primers were removed with cutadapt (Martin

2011), and demultiplexed by a single barcode sequence. Once demultiplexed, the sequencing runs were combined with the cat command in Linux. Reads were merged with fastq_mergepairs with a minimum overlap of 200 basepairs (bp) and a maximum mismatch of 20 bps between forward and reverse reads for 16S rRNA, minimum overlap of 100 and maximum mismatch of 20 for ITS, and minimum overlap of 50 and maximum mismatch of 10 for 18S rRNA. Merged sequence qualities were evaluated and reads with greater than 1 error were removed from the analysis with the fastq_filter command in USEARCH (Robert C. Edgar 2010). The representative datasets were created with UCLUST and UPARSE algorithms (Robert C Edgar 2013). Amplicon Sequence Variants (ASVs) were clustered and denoised with DADA2 and with UNOISE3. Sequences with >97% similarity were clustered into one OTU (Robert C. Edgar 2016). The De Novo OTU tables were built by aligning the clustered OTUs to the representative datasets with the otutab command in USEARCH. The 16S and 18S ASV tables were assigned taxonomies by aligning sequences to the SILVA database (Pruesse et al. 2007). Protists were defined as all eukaryotic taxa, except fungi and invertebrates (Metazoa) (Delgado-Baquerizo et al., 2020). Bacterial sequences that match host mitochondria and chloroplast and 18S sequences that matched plant or fungal DNA were removed. The ITS OTU table was aligned to the UNITE database (Nilsson et al. 2019).

### 3.5 Statistical Analysis

Unless otherwise stated, all statistical analysis steps were performed in R (v4.2.1). To assess the influence of water deficit, site, host genotype, and host niche compartment on alpha diversity, the Shannon index of each sample was determined using the vegan package in R (Oksanen et al. 2022). An Analysis of Covariance (ANOVA) test was performed using the Anova() function in the car package on General Linear Models that were built using the glm()

function to determine the relative importance of each variable on the Shannon index (Fox 2012). Tukey HSD tests were performed to assess alpha diversity differences by treatment. Shannon diversity was then visualized in boxplots using ggplot2 (Wickham 2011). We ran permutational multivariate analysis of variance (PERMANOVA) using the host compartment, sampling location, and water deficit treatment to establish which one of them is the strongest in shaping the bacterial, fungal, and protistan communities. In cases where irrigation treatment was significant, Canonical Analysis of Principle Coordinates (CAP) analysis was performed using the vegan package and visualized in ggplot2 to determine the influence of water deficit treatment for each microbial kingdom within each plant-associated habitat. To determine the indicator taxa enriched or depleted under water deficit conditions, we generated volcano plots in DESeq2 (Love, Huber, and Anders 2014).

Phylogenetic trees were generated with MAFFT alignment in QIIME2 with the q2-phylogeny plugin align_to_tree_mafft_fasttree (Katoh and Standley 2013). OTUs with greater than 20 reads were included in the tree. The .qza files were converted to .nwk with the QIIME2 export plugin. Significantly enriched and depleted taxa were defined as taxa with an FDR < 0.25 determined with MaAsLin2 (Mallick et al. 2021). Trees were visualized in iTOL (Ciccarelli et al. 2006). Source tracker analyses were performed using Fast Expectation Microbial Source Tracking (FEAST) (Shenhav et al. 2019) to determine the proportion of the different potential sources of microbes of each given sink environment. We used the iCAMP package (community assembly mechanisms by phylogenetic bin-based null model analysis), developed by (Ning et al. 2020) to determine the relative importance of different of community assembly processes in forming the bacterial, fungal, and protistan communities in the rhizosphere, roots, and leaves. Multi-kingdom microbial co-existence networks were generated by calculating correlations among bacteria, fungal, and protists OTUs. To minimize the influence of rare taxa, only OTUs with more than twenty reads were kept in the calculation in the data subset. We controlled the false discovery rate by performing 1000 bootstraps on each correlation. The network calculation was performed using SparCC correlations in Python 3 (Friedman and Alm 2012). Different plant compartments have significant differences in both the microbial diversity and community structure. As our aim was to evaluate the impact of drought on the microbial co-existence networks between two similar compartments, we used different SparCC correlation cutoffs for each plant compartment. A correlation strength of $|r| > 0.7$ was used for the rhizosphere, $|r| > 0.65$ was used for the roots, and $|r| > 0.5$ was used for the leaves with a cutoff of $p < 0.01$ for all niche compartments. Networks were visualized in Gephi (Bastian, Heymann, and Jacomy 2009). Topological properties including nodes, edges numbers, degree, and closeness centrality and betweenness centrality were also calculated in Gephi. Further details on the methods used for these statistical tests can be found in the supplemental methods section.

## 4. Results and Discussion

### 4.1 Sugar beet 18S sequencing was dominated by host reads in the root and leaf compartments

Overall, paired-end sequencing resulted in 13,108,603; 8,639,870; 17,649,253 high-quality reads for bacteria, fungi, and protists, respectively. The reads could be assembled into 33,448; 8,445; 10,548 OTUs for bacteria, fungi, and protists, respectively. Samples were singly rarefied to 5000, 5000, and 1000 reads for 16S, ITS, and 18S respectively using the MCToolsR package in R (https://github.com/leffj/mctoolsr/). Samples with fewer total reads than the number rarefied to were removed from the analysis. This included 14 leaf samples from 16S, 11 samples from ITS of mixed hosts and sample types, and 112 samples from 18S. We observed a dominance of host reads in the 18S sequencing runs for Sugar Beet roots and leaves, likely because the PNA used in library prep was designed for monocots and proved less effective in dicots. We removed the majority of the Sugar Beet root and leaf samples from the dataset. As a result, Corn and Sugar Beet samples were considered and reported separately and we opted to report on the Corn subset in the main figures. Sugar Beet analyses is included in the supplementary figures.

### 4.2 Water deficits impact the microbial community structure in all plant compartments

For both plant host species, we observed a significant ($p < 0.001$) decrease in the microbial diversity between soil (bulk soil and rhizosphere) and plant associated (root and phyllosphere) environments (Figure 2.1a; Table 2.1; S2.3a; S2.4). Habitat filtering, induced mainly by physical barriers and the plant defense responses, facilitates selective microbial colonization in the plant compartments, with plants exerting the least selection on the rhizosphere and the most in the phyllosphere (Trivedi et al. 2022; Singh et al. 2023; Xiong, Zhu, et al. 2021). While all microbial kingdoms had a significantly reduced alpha-diversity in the endosphere compartments, protists had a much larger reduction (Figure 2.1a; S2.3a), likely because protists are larger organisms and may face greater obstacles in colonizing the endosphere compartments. We observed a reduction in the Shannon diversity of bacterial communities of soil, roots, and leaves under water deficit while no significant changes in any niche compartments for fungi or protists (Figure 2.1a; S2.3a) were attributed to the watering treatment. Bacteria communities are typically more sensitive to drought than fungi and protists, experiencing reductions in diversity and microbial biomass (Preece et al. 2019; Bazany et al. 2022). Differences in the body size, nutrient acquisition potential, cellular metabolism, cell wall structure and constituents, and foraging strategies can lead to the differences in the drought tolerance between bacteria, fungi, and protists (Chen et al. 2022; Meisner et al. 2018; Stefan et al. 2014).

Plant niche, site (growing region), watering treatment and their interactions explained significant portions of the variation observed in plant-associated bacterial, fungal, and protistan beta-diversity (Table 2.2; S2.5). Our results demonstrate that while site characteristics can result in differences in the initial microbial pool, compartment filtering is a dominant determinant of overall microbial community assemblage, in line with previous studies (Deng et al. 2021; Xiong, Zhu, et al. 2021; Singh et al. 2023; Durán et al. 2018). Our results further revealed that water deficit treatment significantly influences the community structure of all three microbial groups in each niche compartment ($P < 0.001$) (Figure 2.1b; S2.3b; S2.5). In contrast with the significant impact of water deficit on the community composition, non-significant changes in the alpha-diversity for fungi and protists (Figure 2.1a; Table 2.1; S2.3a; S2.4) indicate that water deficit drives changes in relative abundance rather than outright abolition of drought-susceptible taxa. Both bacteria and fungi experience plant compartment specific drought-induced community changes (Santos-Medellín et al. 2017), though bacterial communities are generally observed to be more influenced by drought than fungal communities (Bouasria et al. 2012). Water deficit induces a range of molecular, physiological, architectural, and developmental responses in plants that can indirectly restructure the microbial community assemblages in plant compartments (Karlowsky et al. 2018; Xu et al. 2021; Williams and Vries 2020). Water deficit can also directly impact microbial communities by selecting for drought-tolerant species in different plant compartments and in the bulk soil which serves as a reservoir of organisms from which plants select members of their microbiome (Yandigeri et al. 2012; Naylor and Coleman-Derr 2018; Ullah et al. 2019).

The response of bacterial communities to water deficit is taxonomically consistent across sites. The members of Actinobacteria and Firmicutes were enriched while bacteria belonging to Proteobacteria and Chloroflexi were the depleted for water deficit as compared to irrigated treatments (Figure 2.2). Our results are in line with previous studies that reported the core impact of water deficit on plant associated bacterial communities is driven by the enrichment of a few specific linages (Naylor and Coleman-Derr 2018; Barnard, Osborne, and Firestone 2013; De Vries et al. 2020; Yandigeri et al. 2012; Williams and Vries 2020). Monoderms with thick cell walls like Actinobacteria and Chloroflexi are generally drought tolerant or even opportunistic, while more permeable diaderms like Acidobacteria and Proteobacteria experience reductions in drought conditions (Xu et al. 2018; Sun et al. 2020). Many bacteria can form spores to survive extended periods in drought conditions (Filippidou et al. 2016; Schimel 2018). The enrichment of the members of phylum Actinobacteria is not just due to their cellular makeup to tolerate drought conditions but it is postulated that plants intentionally alter their root exudation profile to actively recruit different Actinobacterial groups—an example of the "cry for help" hypothesis (Rolfe, Griffiths, and Ton 2019; López-Ráez, Pozo, and García-Garrido 2011; Ait-El-Mokhtar, Meddich, and Baslam 2023; Wang and Song 2022; Xu et al. 2018; Liu, Li, and Singh 2024). The recruited members of Actinobacteria phylum can help the plants to tolerate drought stress through the production of osmolytes (Rangseekaew et al. 2022; Niu et al. 2022; Santos-Medellín et al. 2021), 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce stress ethylene, indole-3-acetic acid (IAA), an important plant growth hormone (Jog et al. 2014; Zhang et al. 2021; Tamreihao et al. 2016; Chukwuneme et al. 2020; Qin et al. 2017).

For fungi and protists, there were no clear phylum trends with treatment (S2.6). In line with earlier studies, our study shows that fungi and protists are generally more drought-resistant than bacteria (Barnard, Osborne, and Firestone 2013; Bazany et al. 2022; de Vries et al. 2018). As a whole, fungi and protists are able to persist in drier conditions than bacteria, as they frequently have larger bodies and have mechanisms for desiccation tolerance (Sun et al. 2020; Evans and Wallenstein 2012). Fungi leverage their hyphal networks to obtain water from small soil pores, thereby maintaining nutrient and water uptake over longer distances under water deficit conditions (Guhr et al. 2015; Evans and Wallenstein 2012; Six et al. 2004; Duan et al. 1996; Augé 2004). Because protists generally depend on water for movement through soils, and different types of protists have different drought tolerances, soil protistan taxa are differentially impacted by drought (Stefan et al. 2014). Most soil protists can tolerate the water-deficit conditions by undergoing to resting stage as cysts (Lennon and Jones 2011; Geisen et al. 2018). While there was a strong overlap in the water deficit responsive taxa between different compartments at higher phylogenetic levels, several drought-responsive taxa were compartment specific. These patterns can arise due to the differences in the bacterial community composition between compartments as well as the differences in the influence of water deficit on the plant-mediated processes affecting individual compartments. Further research is needed to elucidate taxonomic trends of enrichment for fungi and protists under drought conditions.

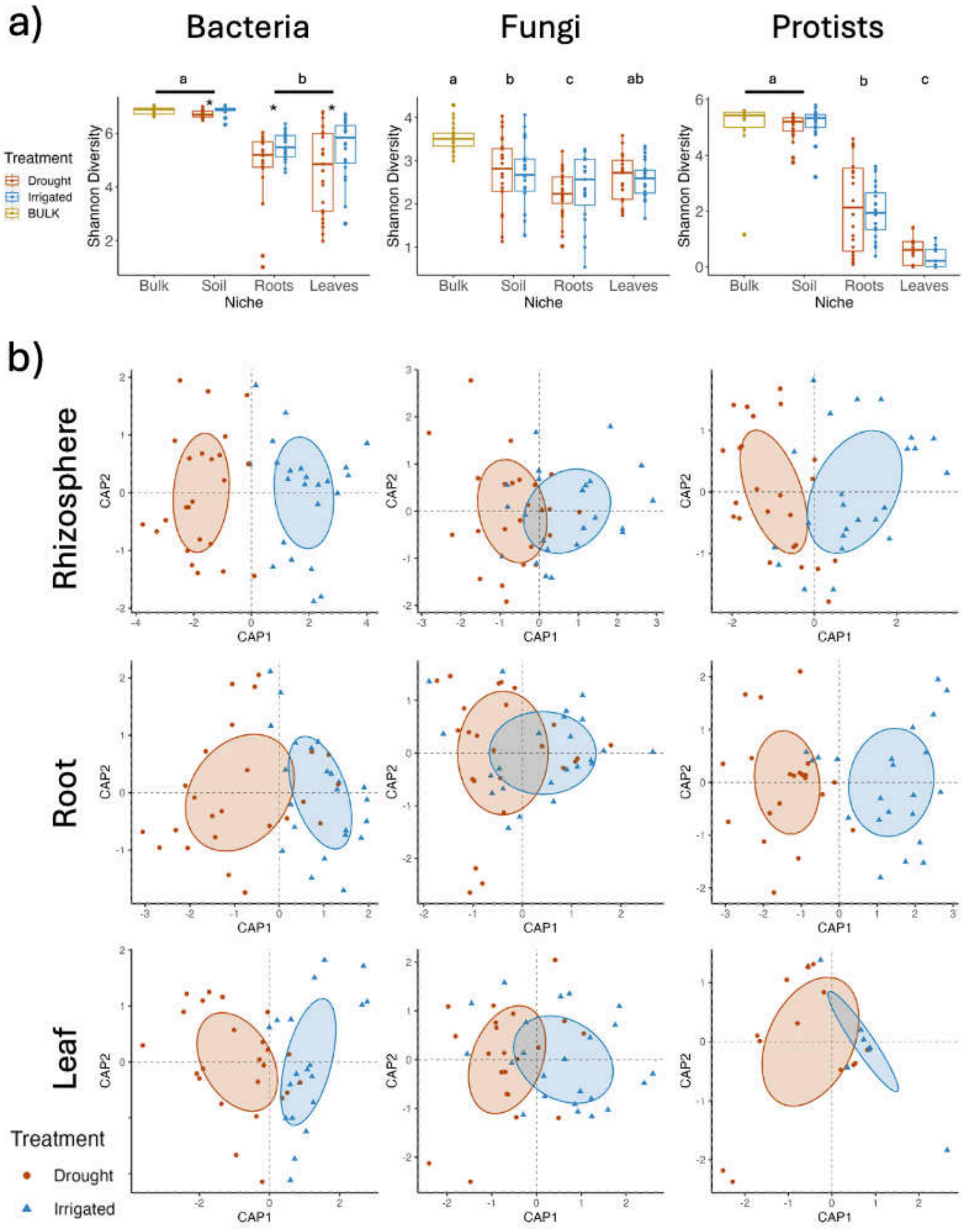


Figure 2.1. Alpha and beta-diversity of Corn. (a) The alpha diversity (Shannon-Index) of the different niche compartments—bulk (bulk soil), soil (rhizosphere soil), roots, and leaves—and the different irrigation treatments (drought and irrigated) for bacteria, fungi, and protists. Asterix indicated significance ($p < 0.05$) by Tukey HSD test between the irrigation treatments. Letters indicate the significance among the various niche compartments. (b) CAPs ordinations showing the beta-diversity of the drought and irrigated treatments for bacteria, fungi, and protists (left to right) within the rhizosphere, root, and leaf compartments (top to bottom).

Table 2.1. Analysis of Variance (ANOVA) performed on general Linear Models (GLMs) of the Shannon Indexes for Corn for bacteria, fungi, and protists.

| | Bacteria | | Fungi | | Protist | |
|---|---|---|---|---|---|---|
| Effect | F-statistic | p-value | F-statistic | p-value | F-statistic | p-value |
| Site | 3.806 | 0.001 ** | 8.995 | < 0.001 *** | 3.409 | 0.003 ** |
| Watering | 15.737 | < 0.001 *** | 0.0398 | 0.842 | 0.288 | 0.593 |
| Niche | 62.008 | < 0.001 *** | 11.5613 | < 0.001 *** | 420.330 | < 0.001 *** |
| Site:Watering | 1.177 | 0.322 | 1.128 | 0.351 | 4.357 | < 0.001 *** |
| Site:Niche | 1.075 | 0.389 | 7.3910 | < 0.001 *** | 4.482 | < 0.001 *** |
| Watering:Niche | 2.178 | 0.118 | 0.674 | 0.512 | 1.348 | 0.266 |
| Site:Watering:Niche | 1.296 | 0.222 | 1.716 | 0.063 | 1.382 | 0.204 |

Table 2.2. Permutational Multivariate Analysis of Variance (PERMANOVA) on Corn for bacteria, fungi, and protists.

| | Bacteria | | Fungi | | Protist | |
|---|---|---|---|---|---|---|
| Effect | $R^2$ | p-value | $R^2$ | p-value | $R^2$ | p-value |
| Site | 0.15459165 | < 0.001 *** | 0.18754221 | < 0.001 *** | 0.1558085 | < 0.001 *** |
| Watering | 0.01338723 | < 0.001 *** | 0.00928524 | < 0.001 *** | 0.01273833 | < 0.001 *** |
| Niche | 0.20706322 | < 0.001 *** | 0.30635186 | < 0.001 *** | 0.23102539 | < 0.001 *** |
| Site:Watering | 0.04517112 | < 0.001 *** | 0.0459203 | < 0.001 *** | 0.06334373 | < 0.001 *** |
| Site:Niche | 0.14843698 | < 0.001 *** | 0.17614598 | < 0.001 *** | 0.22353861 | < 0.001 *** |
| Watering:Niche | 0.0180138 | < 0.001 *** | 0.01337471 | 0.002 ** | 0.01364684 | 0.002 ** |
| Site:Watering:Niche | 0.07835945 | < 0.001 *** | 0.06462002 | < 0.001 *** | 0.05798762 | < 0.001 *** |
| Residual | 0.33497655 | | 0.19675968 | | 0.24191097 | |

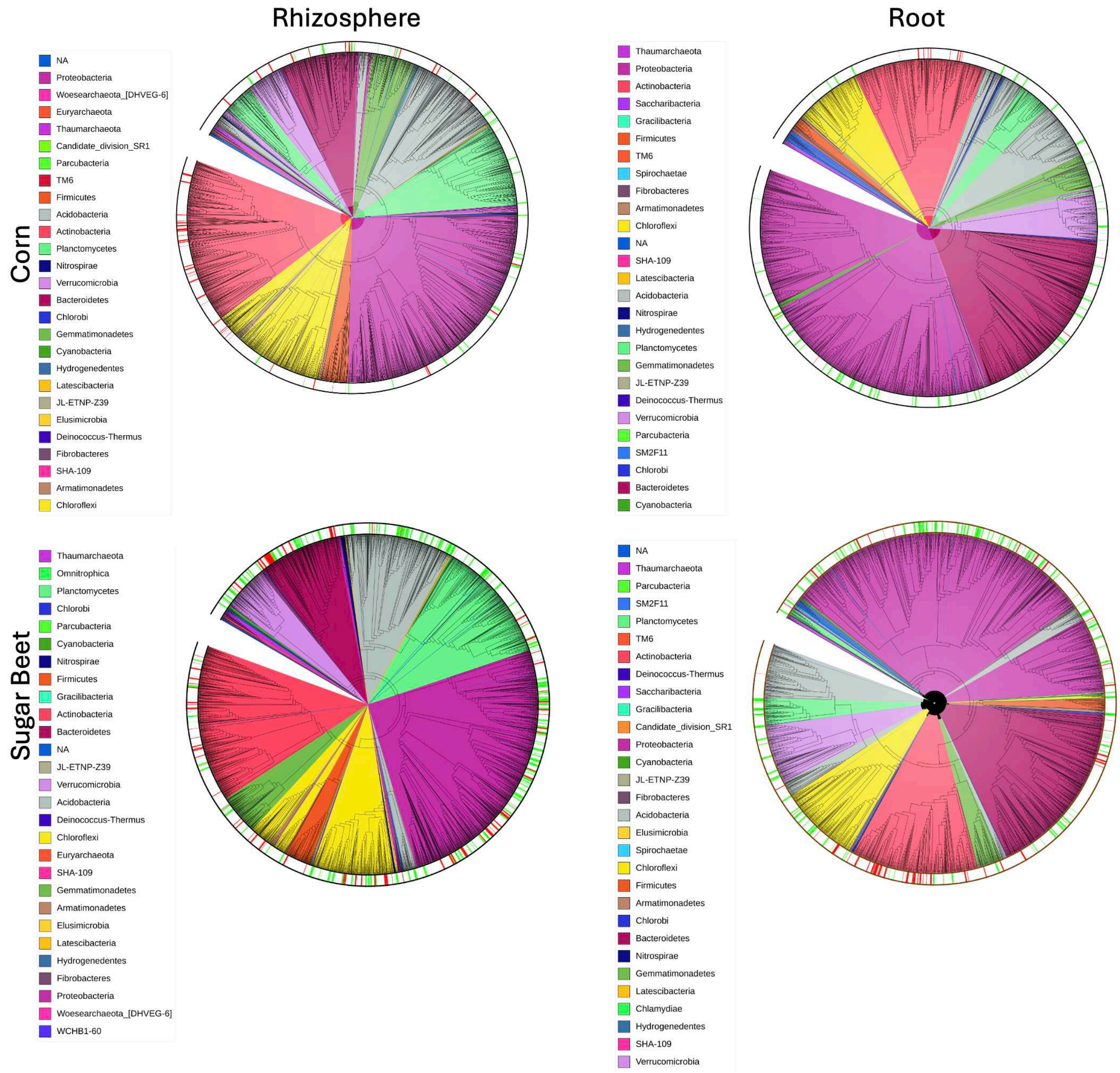


Figure 2.2. Phylogenetic trees of the bacterial communities in Corn (top) and Sugar Beet (bottom) rhizospheres (left) and roots (right). Trees are color coded by phylum. Outer ring shows relative enrichment (red) or depletion (green) in drought compared to the irrigated control. Trees were aligned with MAFFT and visualized in iTOL. Significance was determined with MaAsLin2. OTUs with an FDR < 0.25 were considered significantly enriched or depleted with irrigation treatment.

### 4.3 Water deficit influences the microbial sources of the plant microbiome

FEAST based source-tracking analysis (Shenhav et al. 2019) was conducted to identify the impact of water deficit on the sources of observed bacterial, fungal, and protistan communities in plant environments. For both Corn and Sugar Beet, we observed that plant associated microbial communities were mainly derived from bulk soil communities and filtered by host compartment (Figure 2.3; S2.7). The nearest species pool was generally major contributor to a particular sink. For example, the primary source for the rhizosphere is the bulk soil, the primary source for the root is the rhizosphere and so on. In general, between 30 and 90% of a given plant associated sink was derived from the nearest source, with the known source values greater than 30% in most cases (Figure 2.2). Our study revealed that water deficit influenced multiple source-sink relationships of different microbial groups between plant compartments. Within the nearby species source-sink pools, there were no significant differences in the recruitment of bulk soil microbes to the rhizosphere for bacteria and fungi communities (76 vs. 76% for bacteria and 92 vs. 94% for fungi for irrigated and drought respectively), whereas for protists, there was slightly greater recruitment in the drought treatment (55 vs. 70% for irrigated and drought respectively), though the percentage of root species sourced from the rhizosphere was not affected by watering (30 vs. 30%). There was a higher percentage of root bacteria sourced from the rhizosphere under irrigated conditions (53 vs. 48% for irrigated vs. drought), but the opposite was true for fungi (34 vs. 39% for irrigated vs. drought).

Drought reduced the percentage of leaf microbes sourced from the roots for all the three microbial groups (31 vs. 13% for bacteria; 32 vs. 23% for fungi; 71 vs. 49% for protists for irrigated vs. drought respectively). Drought stress impairs the vascular system of plants, reducing the flow of water in the xylem, the primary transport mechanism of microbes between plant endosphere compartments (Sevanto 2014). Increased contribution of leaf bacteria and fungi from bulk soil and rhizosphere under water deficit indicates direct transfer from the soil sources rather than internal transport from roots. We also observed that the water deficit increased the contribution of unknown sources to the leaf microbial communities, particularly for fungi (19 vs. 44%, irrigated vs. drought) and protists (9 vs. 34%, irrigated vs. drought). The microbial communities on the leaves of fake plants varied with seasonal and environmental conditions, suggesting that the air microbiome is an important source of phylloplane microbes (Xiong, Singh, et al. 2021). Other sources like the native seed endosphere and insects and other pollinators are also likely important sources, as the aerobiome is strongly influenced by the soil microbiome (Zhou et al. 2021). Drought likely increases the capabilities of bacterial and fungal spores to disperse via air due to increased movement of particulate matter (Borlina and Rennó 2017), indicating the impact of weather and climate conditions on microbial dispersal. As many microbes rely on water to move, it stands to reason that the colonization of the various plant compartments by microbes is impacted by drought. Flagellate and ciliate bacteria and protists rely on water for movement around soil particles and may experience affects in the soil far before aboveground evidence of drought (Stefan et al. 2014). These changes in the relative contributions of microbial sources to various plant-associated habitats indicate that drought changes the path by which microbes colonize plant hosts and that these paths of colonization vary by microbial kingdom.

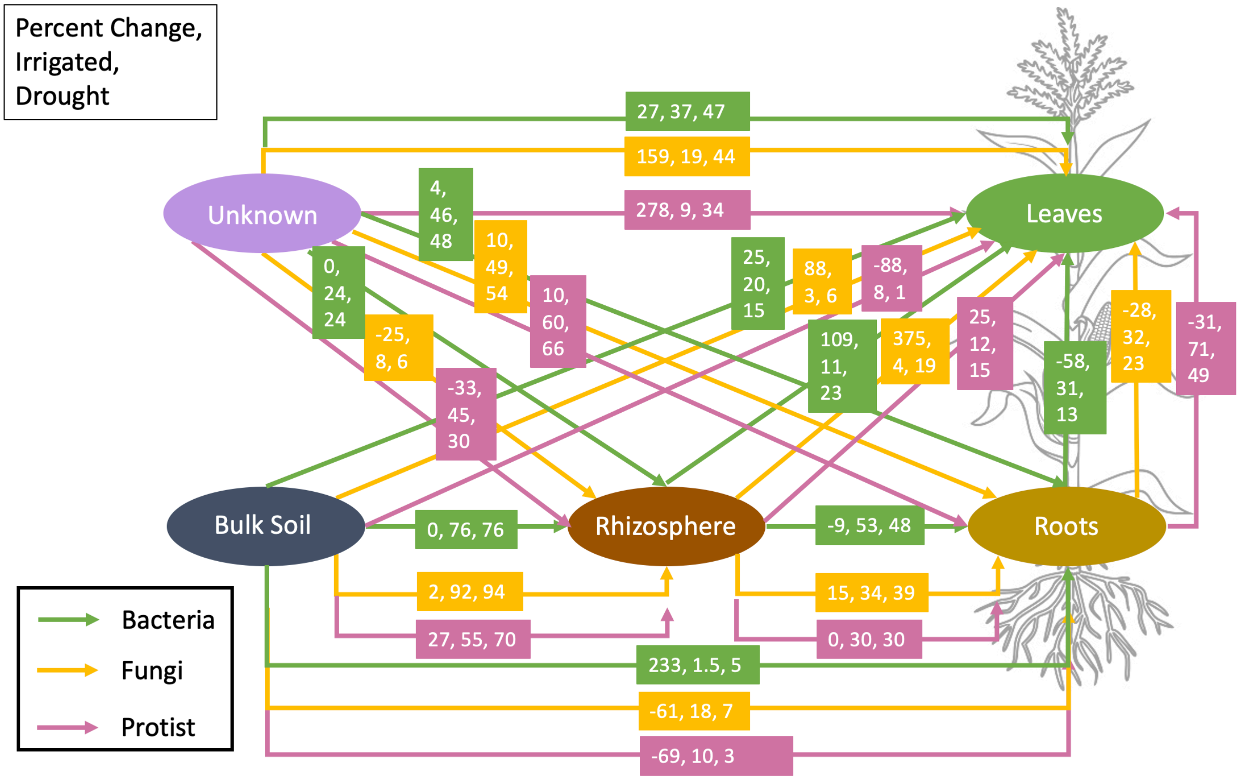


Figure 2.3. FEAST source tracker analysis showing the path of microbial assembly for bacteria, fungi, and protists in Corn. Arrows indicated the proposed direction of microbial colonization from a source to a sink. The numbers indicate the percent change between treatments [(percent drought – percent irrigated) / percent irrigated], followed by the percent sourced in the irrigated treatment, followed by the percent sourced in the drought treatment.

### 4.4 Microbial community assembly processes are influenced by drought

From the metacommunity perspective, microbial community assembly is a comprehensive result of deterministic (selection) and stochastic (random) processes, including selection, dispersal limitation (working in concert with drift), homogenizing dispersal, and drift (acting alone) (Leibold et al. 2004; Stegen et al. 2012; Vellend 2010). To determine the impact of water deficit on the ecological drivers controlling the microbial assembly in different plant compartments, we performed iCAMP analysis (Ning et al. 2020). Our results revealed that stochastic processes (drift, dispersal limitation, and homogenizing dispersal) were dominant over deterministic processes (homogenous and heterogeneous selection) in controlling the assembly of microbial communities in rhizosphere, roots, and leaf compartments. The overall importance of stochastic process was surprising given the importance of host selection processes in determining microbial communities of their various niche compartments (Xiong, Zhu, et al. 2021; Wagner et al. 2016). However, the relative importance of host selection varies with the plant compartment (Singh et al. 2023), time, (Wagner et al. 2016) and plant growth stage (Xiong, Singh, et al. 2021; de Souza et al. 2016). For example, deterministic processes (homogenous and heterogenous selection) are the primary drivers of microbial community assembly in the early growth stages while stochastic processes (homogenizing dispersal, dispersal limitation, and drift) are dominant drivers at later stages of plant development (Xiong, Singh, et al. 2021). As we have collected the plant before the reproductive stage there is a high likelihood that stochastic dominate deterministic assembly processes in our study. The importance of stochastic processes varies with the microbial group and plant compartment (Figure 2.4; S2.8; S2.9). In the corn rhizosphere, the relative importance of drift and unknown processes was highest for bacteria (69.4% in irrigated corn) followed by fungi (50.7%) and protists (21.1%), while an opposite trend was noticed for dispersal limitation (18.5%, 34.2%, and 68.1%, in irrigated corn in bacteria, fungi, and protists respectively). In the roots and leaves, drift was more important than dispersal limitation in controlling bacterial communities (33.8% in roots, 19.2% in leaves of irrigated corn), however the opposite trend occurred for fungal communities (55.1% in roots, 36.4% in leaves of irrigated corn). Interestingly, the protist communities in the root and leaves were overwhelmingly controlled by drift and unknown processes (64.3% in roots, 88.3% in leaves of irrigated corn) with a negligible impact from dispersal limitation (0.2% in roots, 0% in leaves of irrigated corn). The differences in the importance of different stochastic process in controlling the assembly of microbial groups in plant compartments can be linked to differences in the metabolic potential and propagule size within microbial groups. For example, fungi are generally larger and more sedentary organisms than bacteria, therefore they are more likely to have limited dispersal (Fan et al. 2024). Order of arrival has been shown to have a major impact in shaping fungal microbiomes (Leopold and Busby 2020). Bacteria, due to their small size, fecundity, and comparatively high mobility are much more likely to be impacted by drift and other stochastic processes than dispersal (Hamonts et al. 2018; Liu et al. 2023; Chen et al. 2023).

Drought is postulated to have differential impacts on community assembly processes. Drift and other stochastic processes decreased in the water deficit treatment for both bacteria and fungi in each niche compartment for corn and sugar beet, suggesting that drought will reduce the random recruitment of fungi and bacteria from the regional species pool (Figure 2.4; S2.8; S2.9). Selection processes, here heterogeneous and homogenous selection, were higher in the drought treatment for the rhizosphere (7.6 vs. 8.9% for bacteria, 12 vs. 14% for fungi, 10.8 vs. 13.3% for protists in corn in irrigated vs. drought) and root compartments (6.1 vs. 9.7% for bacteria, 4.7 vs. 9% for fungi, 1.4 vs. 4.9% for protists in corn) and lower in the leaves (8.1 vs. 6.6% for bacteria, 8.5 vs. 7.7% for fungi, 4.7 vs. 2.9% for protists in corn) for bacteria, fungi, and protists. The drought-mediated decrease in the stochastic community assembly patterns in the rhizosphere and roots is likely the result of an increased importance of competitive interactions and host selection in shaping the community composition of rhizosphere soil and root microbiomes under drought stress (Chave 2004; Nemergut et al. 2013). This is likely due to the selective pressure of drought itself on microbial communities, resulting in the elimination or limitation of drought sensitive taxa and the proliferation of taxa with a competitive advantage in drought, and to plants selectively recruiting certain taxa under drought stress in their roots and rhizospheres. For example, monoderm bacteria are frequently enriched in the rhizospheres and especially in the roots of drought stressed plants (Naylor et al. 2017; Xu et al. 2018). The reduction of selection processes observed in the leaves under drought conditions aligns with the increased percentage of unknown microbial sources found in the source tracker analysis. In drought conditions, soil-derived taxa are more likely to be directly deposited on the leaf surface, likely mediated by air, allowing for a more stochastically formed community of the leaves during drought (Peñuelas et al. 2012).

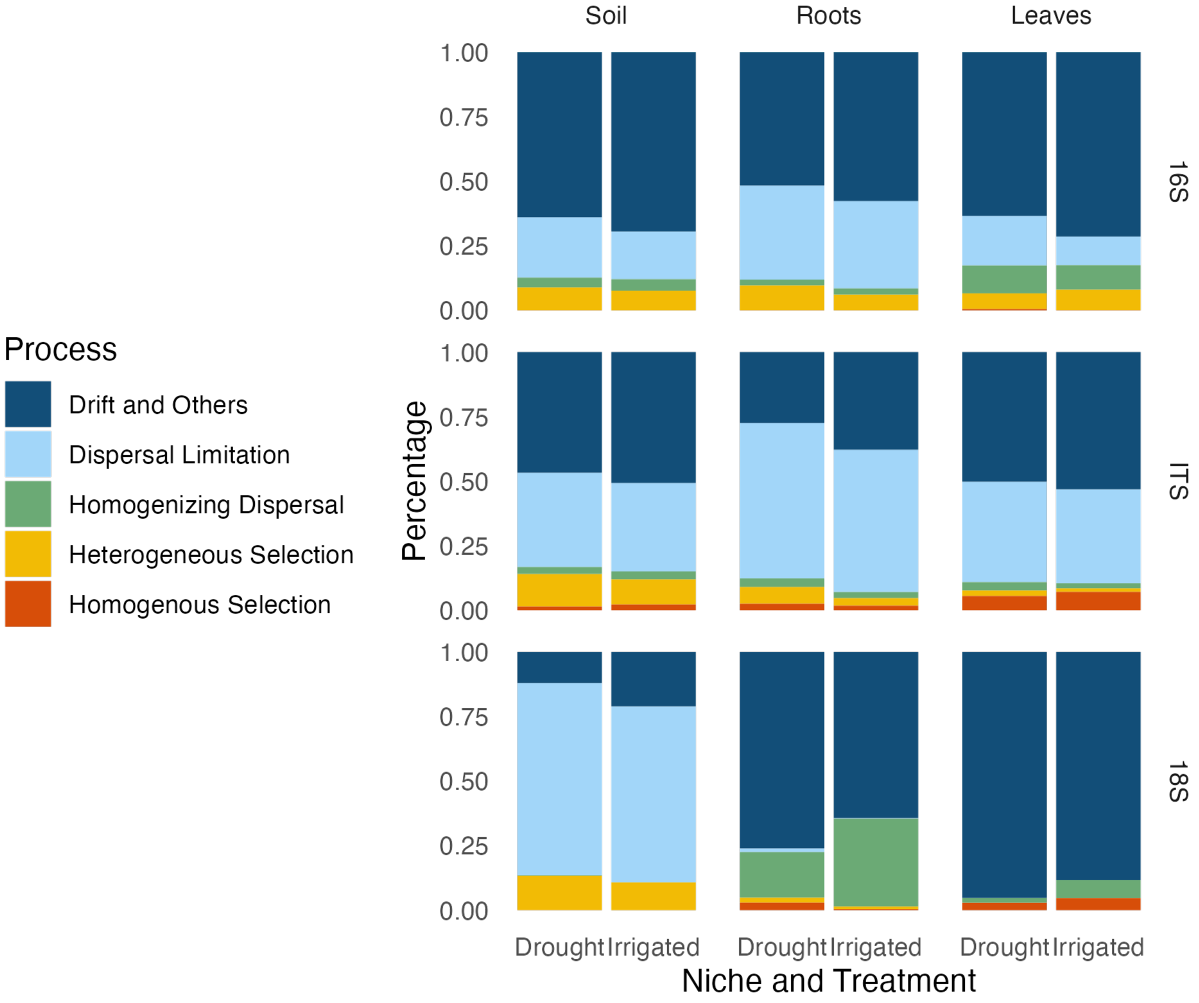


Figure 2.4. Stacked bar charts representing the results of the iCAMP analysis in Corn to determine the prevalence of different microbial community assembly processes in the soil (rhizosphere soil), roots, and leaves (left to right), for the different microbial kingdoms 16S (bacteria), ITS (fungi), and 18S (protists), in the drought and irrigated treatments. The processes tested include stochastic processes (drift and others, dispersal limitation, and homogenizing dispersal) and deterministic processes (heterogeneous selection and homogenous selection).

### 4.5 Water Deficit Shapes Microbial Co-Occurrence Networks

The stability of microbial communities relies on not only the composition, but also on associations that may occur among co-existing members (de Vries et al. 2018; Gao et al. 2022). Here we used the metacommunity co-occurrence network based on correlation relationships to examine how drought impacts the co-occurrence of bacteria, fungi, and protists and plant rhizospheres, roots, and leaves. As expected, the network complexity was highest in the rhizosphere, with root networks being less complex, and leaf networks being far simpler overall. In order to emphasize comparisons between the irrigation treatments as opposed to comparisons between niche compartments, different SparCC correlation cutoffs were used for each taxa, where |r| > 0.7 was used for the rhizosphere, |r| > 0.65 was used for the roots, and |r| > 0.5 was used for the leaves (Figure 2.5; S2.10; S2.11). Interestingly, we found that the response of microbial networks to water deficit differed for different plant compartments, though network complexity and modularity generally decreased with drought. In corn leaves and roots, there was a greater percentage of positive connections in the drought treatment than in the irrigated treatment (77.4 vs. 64.8% in leaves and 85.1 vs. 61.9% in roots). The increases were due largely, to correlations between fungi in the leaves and between bacteria in the roots during water deficit, where the relative frequency of positive correlations was increased, and the frequencies of negative correlations were decreased. Increases in the relative frequencies of positive correlations due to drought can weaken the stability of microbial networks (de Vries et al. 2018; Coyte, Schluter, and Foster 2015).

In the rhizosphere co-occurrence networks, we observed that the water deficit treatment has a greater percentage of negative connections compared to the irrigated treatment (32.2 and 18.9% in water deficit and irrigated, respectively). Compared to the leaf and root, the co-occurrence networks of rhizospheres had greater contributions from protists in both the drought (3.4, 13.7, 37.1% in leaf, root, and rhizosphere, respectively) and in irrigated treatments (2.8, 7.7, 39% in leaf, root, and rhizosphere, respectively). In the rhizosphere, the water deficit treatments increased the proportion of connections between protists while decreasing connections between bacteria. For interactions between fungi and other microbial kingdoms in the rhizosphere, there was a greater increase in the number of positive connections in the water deficit (245) than in the irrigated treatment (69). Both bacteria (Barnard, Osborne, and Firestone 2013; Gao et al. 2022) and protists (Stefan et al. 2014) are reported to be more responsive to water deficit while the fungi are resistant (Guhr et al. 2015; Barnard, Osborne, and Firestone 2013; Gao et al. 2022). Our results indicate that under water deficit, stronger positive interactions with the resistant groups can allow benefits for sensitive groups towards stress tolerance, though this could also indicate a general reduction in network stability given the stress gradient hypothesis (Gao et al. 2022). While water deficit increased the overall number of connections between bacteria and protists in the rhizosphere (both positive and negative), there was a significant increase in the negative bacteria-protist connections in the drought treatment compared to irrigated treatment (577 and 359 in drought and irrigated, respectively). The negative interactions between the bacteria and protists can be the result of the competition for similar resources (Hassani, Durán, and Hacquard 2018) or predation (Gao et al. 2019), as protists can mediate a decrease in plant stress via grazing induced shifts in the rhizosphere microbial communities (Guo et al. 2021; Kuppardt et al. 2018). These findings begin to illustrate the complex and varied influences of drought on plant microbiomes, demonstrating that drought can impact co-occurrence network architecture by mediating changes in multi-kingdom interactions.

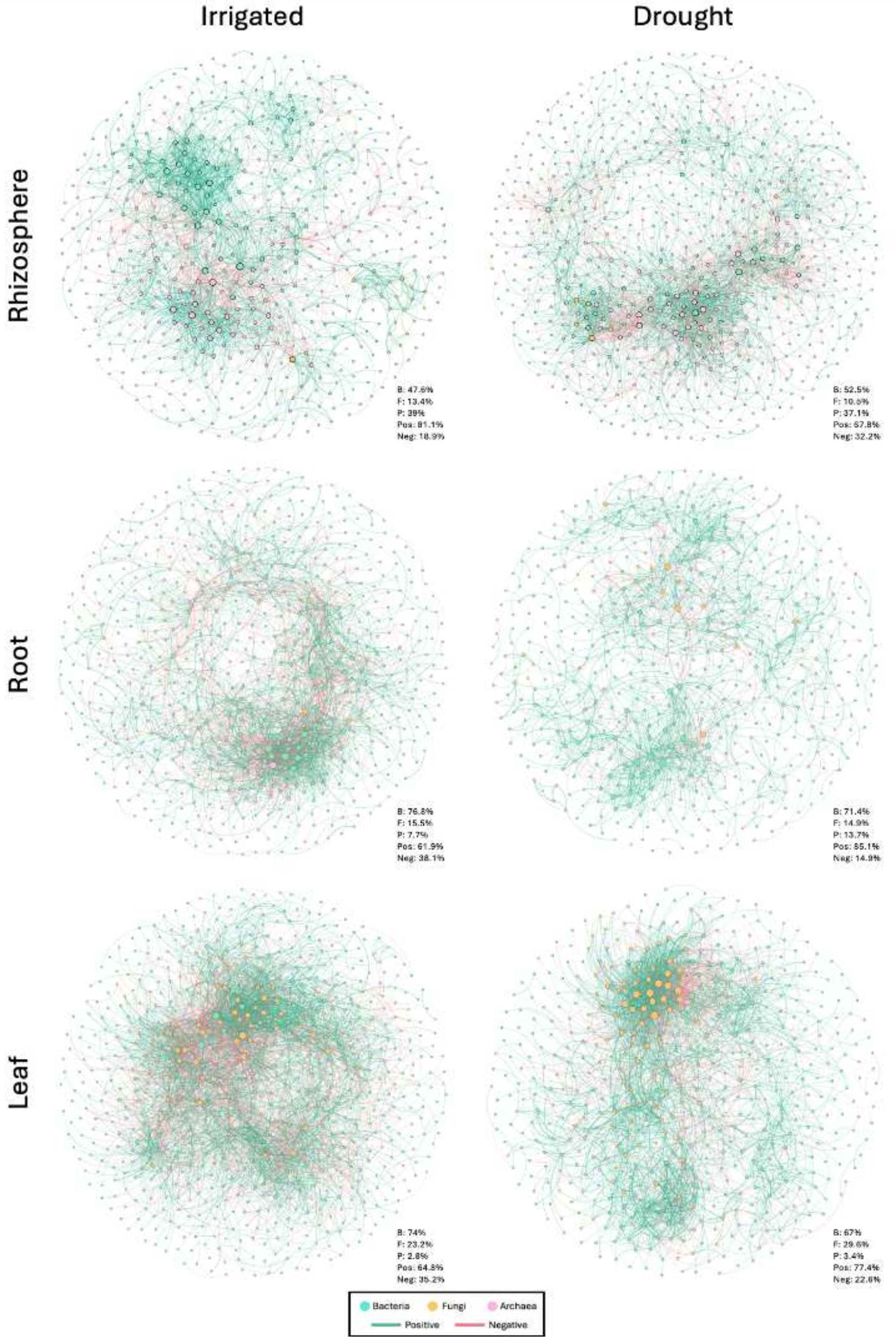


Figure 2.5. Co-occurrence networks of the bacterial, fungal, and protistan communities of corn in the irrigated and drought conditions (left to right) for the rhizosphere, root, and leaf

compartments (top to bottom). Green points represent bacterial nodes, yellow points represent fungal nodes, and pink points represent fungal nodes. Green lines represent positive correlations between nodes and pink lines represent negative correlations between nodes. The percents at the bottom right of each network show the percent of bacterial (B), fungal (F), and protistan (P) nodes in each network and the percent of positive (pos) and negative (neg) edges. Correlations were performed in SparCC on OTUs with at least 150 reads in the total dataset. Correlation cutoffs were adjusted based on niche compartment to allow for clearer comparison between irrigation treatments. For the rhizosphere networks we kept correlations where $|\text{SparCC}| > 0.7$, for the roots $|\text{SparCC}| > 0.65$, and the leaves $|\text{SparCC}| > 0.5$.

## 5. Conclusion

We examined the impact of drought and host species on the bacterial, fungal, and protistan communities and community assembly processes of corn and sugar beet rhizospheres, roots, and leaves. We found that water deficit stress impacted not only these communities, but the relative importance of different source communities and the ecological processes by which these communities assembled. We recommend that researchers consider the influence of host and environmental factors like abiotic stress on the recruitment of certain microbes. Future research should consider the ecology of microbial communities and their plant hosts as well as the mechanisms behind microbial recruitment to allow for more predictable results on the impacts of employing different management strategies and microbial amendments under varying environmental conditions for different crop species.

# CHAPTER 3: Arbuscular mycorrhizal fungi inoculation, drought treatment, and soil influence the rhizosphere and root microbiomes and fitness of wheat

## 1. Summary

Arbuscular mycorrhizal fungi (AMF) form associations with most agricultural plants, performing beneficial functions for plant and soil health that can help to ameliorate drought stress for the plant. However, we don’t fully understand the factors driving the formation of plant-AMF associations, nor how these associations may impact plant fitness and the plant microbiome in different plant hosts, under varying environmental circumstances, and native microbial communities. In a large greenhouse experiment, we tested the impact of soil, drought, host genotype, and AMF inoculation on plant fitness, the soil, and the rhizosphere and root microbiomes of wheat. Using amplicon sequencing, we found that AMF inoculation reduced bacterial and fungal diversity in the rhizosphere while increasing the bacterial and fungal diversity in the roots for most soil types and drought treatments. Bacterial and fungal beta-diversity of both the rhizosphere and root compartment varied significantly with soil type, AMF inoculation, and drought. Cross-kingdom microbial co-occurrence networks revealed that the AMF inoculation simplified rhizosphere soil communities, especially in initially low diversity soil. AMF inoculation also increased soil organic carbon and soil moisture while reducing available phosphorus compared to non-inoculated treatments. These findings begin to demonstrate the complexity behind the formation and impacts of AMF associations. Further elucidation of these complex interactions will allow for future engineering of tailored microbial solutions to plant stress that consider host genotype, environmental factors, and interactions with the native microbial community.

## 2. Introduction

Arbuscular mycorrhizae fungi (AMF) associate with the roots of over 90% of plant families including major agricultural crops (i.e. rice, wheat, corn, barley). The fungi increase the absorptive area of the plant rhizosphere in addition to providing additional metabolic capabilities in resource acquisition and stress tolerance to several types of stress including salinity, drought, heat, limiting nutrients, and pathogen and herbivore attack (Rillig and Mummey 2006; Thirkell et al. 2017; Chen et al. 2018). For this reason, the interaction between AMF and host plants has been exploited in forestry management, in bio-energy production and in high-value timber plantations as well as in agriculture (Berruti et al. 2016; Rillig and Mummey 2006; Thirkell et al. 2017). A great deal of effort has gone into determining how to foster these beneficial relationships through different land management techniques and using various commercially available inoculants (Li et al. 2021; Ryan and Angus 2003; Frew, Heuck, and Aguilar-Trigueros 2023). However, these results are often difficult to replicate due to complex interactions among the plant and fungal genotypes as well as the local environment (Meier and Hunter 2018; Berdeni et al. 2018; Ryan and Graham 2018). The context dependence of these interactions is a major factor limiting exploitation of AM fungi in agriculture and remediation, while unpredictable plant benefits hinder large scale application of these fungi to increase microbiome mediated benefits.

Current crop yield increases will not meet the global food needs under prevalent farming practices, highlighting the need to develop new technologies and better apply long-known technologies, such as growth-promoting microbial symbionts of plants, in a more efficacious manner (Rodriguez and Sanders 2015; Pathania et al. 2020). The effects of AMF on plant growth and fitness are not always beneficial (Corrêa et al. 2014; Rodriguez and Sanders 2015; Ryan and Graham 2018). This inconsistency is exacerbated by the poor quality of many commercial products (Ryan and Graham 2018; Salomon et al. 2022) and management recommendations that are poorly suited to existing agronomic practices. Recent research has demonstrated that in addition to abiotic factors, the local microbiome plays a very important role in the establishment of beneficial-host AM relationships (Svenningsen et al. 2018; Zhang et al. 2018). These findings clearly suggest that if fungal inoculants are not matched with an appropriate plant host and suitable microbiome, the maximal benefits from these relationships will not be achieved as host growth promotion is significantly correlated to the percentage of roots colonized by a mutualistic fungus.

Plants are associated with a diverse microbiome that greatly affects plant productivity, and interactions among the members of microbiome can even allow persistence of less beneficial microbes (Leach et al. 2017; Compant et al. 2019). Root traits themselves select for a particular microbiome that is related to the patterns of root exudation and root architecture (Herms et al. 2022; Escudero-Martinez and Bulgarelli 2019). Therefore, expecting that the introduction of AMF to a well-established plant-associated microbiome will lead to consistent increments in crop yield is too simplistic (Rodriguez and Sanders 2015). Field soils have huge amount of variability with regards to the root colonization ability of the introduced AFM (Lutz et al. 2023). In fact, field soils can suppress the extra-radical mycelium production, significantly reducing AMF activity (Svenningsen et al. 2018a). The “conductivity” or “suppression” of AMF colonization and activity has been attributed to the local soil microbiome (Svenningsen et al. 2018a). AMFs themselves can recruit plant growth promoting bacteria and mycorrhiza helper bacteria that promote AMF activity and development (Jiang et al. 2021; Branco et al. 2022; Battini et al. 2016; Turrini et al. 2018). A better understanding of the ecology and population biology of the plant-associated microbiome, and how introduced the AMF interact with existing microbial communities, is essential to predict which AMF taxa will form a given assemblages in a given locality.

The effect of AMF on plant growth and fitness (termed “mycorrhizal growth response” or MGR) is diverse and varies dramatically along a continuum from strong to weak mutualism or even parasitism (Kaur, Campbell, and Suseela 2022; Van Der Heijden et al. 2006). Variation in host response to inoculation may be partly explained by the identity of the host and the fungus. Plants with hindered ability to access soil nutrients, for example plants with short root hairs are more likely to benefit from AMF associations (de Vries et al. 2021; Wang et al. 2020). Context is important as in some cases the carbon cost of maintaining the AMF association may not be worth the nutrient acquisition benefits. In addition, the effects of AMF on plant performance are likely to differ among AMF species or strains (Al-Karaki and Williams 2021). The pairwise interactions between AMF and other symbionts may depend upon the presence or identity of a third partner (Larimer, Clay, and Bever 2014). The strength of interactions between a native AMF community and other members of the soil microbiome also changes significantly in response to biotic/abiotic stresses. Biotic context, including the presence of other microbes, can also drive contextual variation in plant responses to AMF (Hoeksema et al. 2010; Wang et al. 2023; Kaur, Campbell, and Suseela 2022; Nasslahsen et al. 2022). Overall, the context dependency of the AMF-host interactions makes it difficult to predict the effects of AMF on plant fitness in a given host and environment.

It is not fully understood what factors drive AMF to form associations to host plants, but there is evidence that plant stress, either from nutrient limitation or water limitation, signals the release of root exudates which facilitate AMF colonization by providing a desirable photosynthetically derived carbon source (Chialva et al. 2024; Hoang et al. 2022). In low stress conditions, for example high available phosphorus, plants demonstrate the ability to reject AMF partners, not only by reducing carbon exudation, but also by producing phenolic compounds, which serve as defensive chemicals against microbes (Frew, Heuck, and Aguilar-Trigueros 2023). Additionally, there is evidence that plants reduce their immune response in the face of abiotic stress, which allows for colonization by AMF partners though this also makes plants more vulnerable to pathogen attacks (Carbone et al. 2021). For example, strigolactones—important plant signaling molecules involved in AMF colonization—are produced in greater quantities by the plant when under stress and recruit beneficial AMF while also increasing the plant's vulnerability to pathogens (López-Ráez, Pozo, and García-Garrido 2011). Contrarily, there is also evidence that environmental stress like drought or heat stress can reduce AMF growth and reproduction (Branco et al. 2022) which could indicate that intermediate stress levels could result in greater AMF assimilations than high stress or no stress conditions.

The extent to which AMF associations will impact plant fitness under drought stress is not clear. AMF can ameliorate drought symptoms in the host through a variety of mechanisms (Tang et al. 2022), including aiding in nutrient acquisition that is hindered by soil drying, altering plant phenotype including root morphology and exudation patterns (Hoang et al. 2022; Ganugi et al. 2022) and enriching other beneficial microorganisms, though many of these responses vary with host genotype (Ganugi et al. 2022). While AMF can help plants acquire vital nutrients under drought conditions, the exchange of nutrients facilitated by AMF is not always straightforward with some nutrient exchanges being easier to facilitate than others (Ma et al. 2022; Ryan and Angus 2003).

Our goal for the study was to examine the impact of pre-existing soil microbiome, AMF inoculation (*Rhizophagus irregularis*), drought stress, and plant genotype on the plant fitness, the soil, and the structure, function, and co-occurrence networks of the plant-associated microbiome. We hypothesized: (a) AMF colonization would be higher in the AMF-inoculated treatments and in the drought treatments compared to the controls, (b) plant fitness would be greater in the AMF-inoculated drought treatments than in the non-inoculated drought treatments, (c) AMF-inoculation would alter the structure and co-occurrence networks of the plant-associated microbiome, and (d) the impact of the AMF-inoculation on AMF colonization, plant fitness, and the plant microbiome would vary by soil type.

We conducted a full factorial greenhouse experiment with three soil treatments, two AMF inoculation treatments (*Rhizophagus irregularis* inoculated, and a non-inoculated control), three watering treatments, and three wheat genotypes, each with different root morphologies. We found that AMF inoculation generally reduced plant biomass and resulted in a reduction in bacterial and fungal diversity in the rhizosphere soil, but increased diversity in the roots. This is likely an indication that AMF inoculation and the resulting changes in the plant microbiome led to a compromised plant biomass in most of the treatments tested.

## 3. Methods

### 3.1 Soil sampling

Soil was collected from three different agricultural fields where winter wheat was grown in the previous season. The first site (ARDEC), located at the Agricultural Research, Development and Education Center (ARDEC), Fort Collins, Colorado (40.6525; -105.0000) had comparatively high nutrient status and comparatively low initial soil microbial diversity. The second site (Fruita), located at the Western Colorado Research Center, Fruita, Colorado (39.1803; -108.6990) was of intermediate soil diversity and nutrient status. The final site (WYO) located at The Sustainable Agriculture Research and Extension Center, Lingle, Wyoming (42.159367; -104.364377) had the highest soil diversity and lowest soil nutrient status of the three. Soil diversity was determined with 16S and ITS amplicon sequencing and soil chemistry and nutrient status was determined with standard biogeochemistry methods performed at WARD Laboratories Inc. (https://www.wardlab.com/). Altogether, our sampling sites varied considerably in their soil properties and microbial community structure and composition (S1) and thus provide an excellent framework to test our hypothesis.

### 3.2 Greenhouse Experiment

Each soil was passed through a 2 mm sieve and combined 1:1 with an autoclaved potting medium. For the AMF inoculation treatment, a powder formulation containing *Rhizophagus irregularis* was purchased from Sustainable Village (https://sustainablevillage.com/) and mixed thoroughly into the soil before planting at a rate of 2,000 CFU per L of soil which amounted to 5 grams of inoculum or 1500 CFU per plant. Plants were grown in 0.75 L pots.

We selected three different winter wheat genotypes based on root architecture and drought tolerance (Awad et al. 2018; Becker et al. 2016). Byrd, a relatively drought-tolerant commercial variety with complex root architecture, TAM107, an experimental variety that is highly drought tolerant with highly branched and high biomass roots, and Snowmass, a commercial variety that is drought sensitive with more simple, lower biomass roots. Three seeds were planted in each .75 L pot and thinned to a single plant after germination.

Plants were watered with Hoagland solution at a rate of 100 mL every other day for the first 14 days after planting. After 14 days, watering treatments were initiated. For the Control watering treatment, plants were watered with tap water at a rate of 100 mL every other day. For the Press watering treatment, plants were watered at the same every other day interval but were only partially saturated with 50 mL of water. For the Pulse treatment, water was withheld for 5 days, followed by a watering of 100 mL on the 6th day. Both drought treatments resulted in significant osmotic stress and reductions in soil moisture which led to an approximately 50% reduction in plant biomass and soil moisture reductions similar to that of an environmental drought. This design resulted in a total of 54 combined treatments, each with 9 replicates, for a total of 486 plants.

### 3.3 Sample collection and processing

Plants were harvested by gently shaking the soil from the roots. Leaves and roots were rinsed, patted dry, and air-dried for several weeks then weighed for biomass analysis (3 replicates). The roots from the other 6 replicates were gently separated from the pot, placed in a bag and thoroughly shaken to dislodge the soil that clings tightly to the roots. We considered this soil the rhizosphere soil and set it aside for DNA extraction (6 replicates). The roots were then flash frozen in liquid nitrogen and stored at -80 C for DNA extraction (6 replicates).

### 3.4 Soil moisture

We measured soil moisture in both the field and the lab. We used a hand-held soil moisture probe to measure in-situ soil moisture to a depth of 15 cm at the time of soil sampling. We additionally dried field-collected soil (the same soil used to measure nutrients) for 48 hours at 60°C to calculate moisture and soil wet soil/dry conversion factors.

### 3.5 Plant phenotype

One day before harvesting the experiment (day 41) plant tillers were counted and plant heights were measured from the base of the stem to the tallest leaf. On the day of the harvest (day 42) plant chlorophyll was calculated with a ChL fluorescence meter (atLEAF CHL STD Handheld Chlorophyll Meter). Three leaves were measured from each plant and averaged. Soil moisture was taken as percent volumetric water content (VWC) with a soil moisture probe. These observations were performed on all 9 replicates.

### 3.6 Enzymatic Activity

We examined the enzymatic activity of several enzymes produced by microbes using methods from Bell et al. (2013) and Trivedi et al. (2016). We examined the carbon-cleaving enzymes a-Glucosidase (AG), b-Glucosidase (BG), b-D-cellulosidase (CB), and b-Xylosidase (XYL), the nitrogen-cleaving enzymes N-acetyl glucosaminidase (NAG) and leucyl aminopeptidase (LAP), and the phosphorus-cleaving enzyme phosphatase (PHOS). Substrates for each enzyme were attached to a highly fluorescent cleaving product. 4-methylumbelliferyl (MUB) was used for every enzyme except for LAP, which was attached to 7-amino-4-methylcoumarin (MUC). A Tris buffer solution was added to the bulk soil samples of three replicates from each treatment, and to three replicates from each of the original field soils used in the experiment. The soil slurry was shaken for 40 minutes at room temperature. Samples were pipetted into deep-well 96-well plates. MUB and MUC standard curves were created for each sample. To simulate soil conditions, plates were incubated in the dark for 3 hours at 25 C. After incubation, fluorescence was measured with a Tecon Infinite M200 plate reader (Switzerland) with 365-nm excitation and 460-nm emission filters.

### 3.7 Soil physiochemical properties

Soil physiochemical properties were measured with standard methods. Total soil carbon and nitrogen were determined on dry soil using a combustion elemental analyzer (Vario MAX

C/N, Germany). pH was determined by suspending soil in a 1:2.5 soil:water ratio and measuring with a pH meter. Soil chemistry and nutrient status was determined with standard biogeochemistry methods performed at WARD Laboratories Inc. (https://www.wardlab.com/).

### 3.8 DNA extraction and amplicon sequencing (16S, ITS, SSU)

Plants were harvested on day 42 of the experiment. Plants were removed from the pot and shaken until only the soil clinging tightly to the roots remained. Roots were placed in bags and shaken additionally to collect the soil that remained clinging tightly to the roots, called the rhizosphere soil. Leaves were cut away and roots and rhizosphere soil were collected and stored at -20 C until further analysis. DNA was extracted from the rhizosphere soil using the PowerSoil Pro Kit and from the roots using the PowerPlant Pro Kit. Root samples were flash frozen in liquid nitrogen and ground before extracting.

16S and ITS libraries were prepared using primers and protocols from the earth microbiome project (https://earthmicrobiome.org/) to examine the bacterial and general fungal diversity of the samples. The primer sets 515F/806R were used to amplify part of the 16S rRNA gene, while ITS1F/ITS2R primers were used to amplify part of the fungal ITS1 region (Caporaso et al. 2012).

The DNA was amplified in 50 µL reactions with Invitrogen Platinum Hotstart PCR Master Mix 2x (Thermo Fisher Scientific). For the amplification of 16S rDNA, we used peptide nucleic acid (PNA) clamps, designed to specifically bind and block the amplification of plastid DNA (pPNA, 5′-GGCTCAACCCTGGACAG-3′) and mitochondrial DNA (mPNA, 5′-GGCAAGTGTTCTTCGGA-3′) (Lundberg et al. 2013).

Small subunit (SSU) libraries were prepared using WANDAF/AML2 reverse primers for a more refined look at AMF. PCR was performed in 50 uL reactions with 25 uL of Platinum Hotstart 2x, 14 uL of nuclease-free water, 6 uL of extracted root DNA, and 5 uL of forward and reverse barcoded primers mixed at equal volumes. Samples were amplified for 30 cycles with a 95 C denaturation for 3 minutes, followed by 30 cycles of 95 C for 15 seconds, 54 C for 15 seconds, and 72 C for 15 seconds. After the last cycle, the samples were given an additional minute at 72C followed by an infinite hold at 12 C.

After PCR, the 16S, ITS, and SSU libraries were tested and pooled with the same methods. Samples were validated with gel electrophoresis to ensure a bright band of the right approximate size (~250, ~150 and ~530 bps for 16S, ITS, and SSU respectively) and the DNA of each sample was quantified with PICO green.

Samples for each library were pooled in equimolar concentrations. 16S and ITS libraries were sequenced on the Illumina MiSeq2000 platform (Illumina Inc., San Diego, California) with a paired-end protocol and a 2x250 kit at the Next Generation Sequencing Facilities at Colorado State University. The SSU library was sequenced on a NextSeq2000 at the Dartmouth University Sequencing Center with a 2x300 kit.

Each library required a custom index design. Information on the custom library design for the 16S and ITS libraries can be found on the Earth Microbiome Project website (https://earthmicrobiome.org/). The SSU library is a new custom design developed by the Bala Chaudhary Lab (See Supplementary Materials and Methods).

### 3.9 AMF Quantification

We used qPCR following the methods of Bodenhausen et al. (2021). In short, we used the AMG1F and AM1 primers to amplify a highly conserved sequence in AMF on the small subunit (SSU). Additionally, we used the RiEFa primer set (Olson et al. 2022) which detects the *R. intraradices* (former name for *R. irregularis*) elongation factor gene and the 197198 primer set specifically designed for the detection and quantification of *R. irregularis* (Boussageon et al. 2023). Additionally, we performed microscopy on a subset of the samples using the intersect method developed by (McGonigle et al. 1990). Roots were bleached in 10% KOH and stained with a 1:9 black ink:vinegar solution. We examined 100 intersects per plant and noted the presence of arbuscules or hyphae at each intersect to determine the percent of colonization.

### 3.10 Bioinformatics

We performed amplicon sequencing to examine the bacterial (16S rRNA), general fungal (ITS), and AMF (SSU) communities of the rhizosphere and roots. The 16S rRNA and ITS libraries were sequenced on an Illumina MiSeq, whereas the SSU library was sequenced on an Illumina NextSeq. USEARCH (Edgar 2010) and UNOISE3 (Edgar 2016) were used to determine operational taxon units (OTUs) which we defined as sequences with >97% similarity. We used the following bioinformatics pipeline: first, the quality of the raw sequences was determined using fastQC (Andrews 2010) and sequences with ambiguous nucleotides, low quality score ($Q < 20$), or short lengths (<100 base pairs) were discarded from the analysis. Adapters and primers were cut from the sequences using cutadapt (Martin 2011). Next, the sequences were demultiplexed and merged and the quality of the merged sequences was assessed with fastq once more (Andrews 2010) and filtered based on quality by the same metrics as above. Remaining sequences were dereplicated and then made into OTUs with UNOISE3 (Edgar 2016). The reads were then mapped to the de novo database to build the OTU table and then taxonomies were added. For 16S, taxonomies were assigned by blasting the reads to the Silva database (Pruesse et al. 2007), the ITS taxonomies were assigned with the UNITE database (Nilsson et al. 2019), and the SSU taxonomies were assigned with the MaarjAM database (Öpik et al. 2010).

### 3.11 Statistical analysis

Statistical analyses were performed in R v4.2.2 and python3. For initial tests on alpha-diversity and beta-diversity, bacterial, general fungal, and AM fungal communities were evaluated separately. OTUs present in <10% of samples and OTUs belonging to the plant host (chloroplasts or mitochondria) were removed from the analysis. For the rhizosphere soil, 16S and ITS samples were uniformly rarified to 5000 and 3000 reads respectively. For the roots, 16S, ITS, and SSU samples were uniformly rarified to 9000, 10000, and 10000 reads respectively. Rarefication was performed in the R package MCToolsR (Leff 2017). Alpha diversity was examined using general linear regression models and analysis of variance (ANOVA) models. Significant differences between individual treatments were defined with Tukey's honestly significant differences (HSD) tests. To examine the effects of inoculation, drought, soil type, and wheat genotype on microbial beta-diversity, permutational multivariate analysis of variance (PERMANOVA) models were generated. Bray-Curtis dissimilarity distances were calculated and ordinated in non-metric multidimensional scaling models (NMDS). To identify differentially enriched taxa, the logarithm of the fold change of the relative abundance of each ASV between treatments and the p-value of these fold changes were calculated in DESeq (Love, Huber, and Anders 2014) and visualized in volcano plots.

### 3.12 Microbial co-occurrence networks

To generate co-occurrence networks, OTU tables were subset by treatment. As wheat genotype was not a significant factor in determining microbial alpha or beta-diversity in the rhizosphere, the genotypes were pooled for the network analysis. Rare taxa with less than 30 total reads were removed from the dataset. Correlations among bacterial and fungal OTUs were determined in SparCC3 (Friedman and Alm 2012). 100 iterations were run to reduce the false discovery rate. Strong ($r > 0.70$) and robust ($p > 0.01$) correlations were kept. Networks were visualized in Gephi (Bastian, Heymann, and Jacomy 2009).

## 4. Results

### 4.1 Impact of AMF inoculation and drought on plant growth parameters

Overall, the plant fitness metrics varied significantly with the experimental treatments (Figure 3.1, Table 3.1). Wheat genotype, while significant for every plant fitness metric, explained less of the variation in biomass and tillers than any other treatment, though genotype strongly influenced chlorophyll content (F-stat = 34.46). All plant fitness metrics varied significantly with soil type except for root biomass which was strongly influenced by the drought treatment (F-stat = 69.83) and the inoculation treatment (F-stat = 48).

Above and belowground biomass and tiller counts were most strongly influenced by drought and the inoculation treatments. Unsurprisingly, drought led to reductions in plant above and belowground biomass, chlorophyll, and tiller number (Figure 3.1). However, the AMF inoculation treatment also led to reductions in biomass and tiller number (Figure 3.1) and explained more of the variation in tiller number (inoculation F-stat = 327.87; drought F-stat = 104.51) and above ground biomass (inoculation F-stat = 87.28; drought F-stat = 78.52) than drought. Belowground biomass was influenced more by drought than inoculum (inoculation F-stat = 48.00, drought F-stat = 69.83). We observed general reductions in tiller number in the AMF inoculated treatment that were significant in each of the watering treatments for ARDEC soil and in the Pulse drought treatment for Fruita soil, and in both the Pulse and Press treatments for WYO soil (Figure 3.1).

Biomass reductions with inoculation treatments were more apparent in the low diversity, high nutrient soil ARDEC than in either Fruita (intermediate diversity and nutrient status) or WYO (high diversity and low nutrients). The above and below ground biodiversity reduction in the ARDEC soil and Control watering treatment were significant, while there were visible but non-significant reductions in the Press and Pulse drought treatments (Figure 3.1). The combinations of the inoculation crossed with the soil treatment and drought crossed with the soil treatment resulted in significant variation in all of the plant phenotype metrics (Table 3.1).

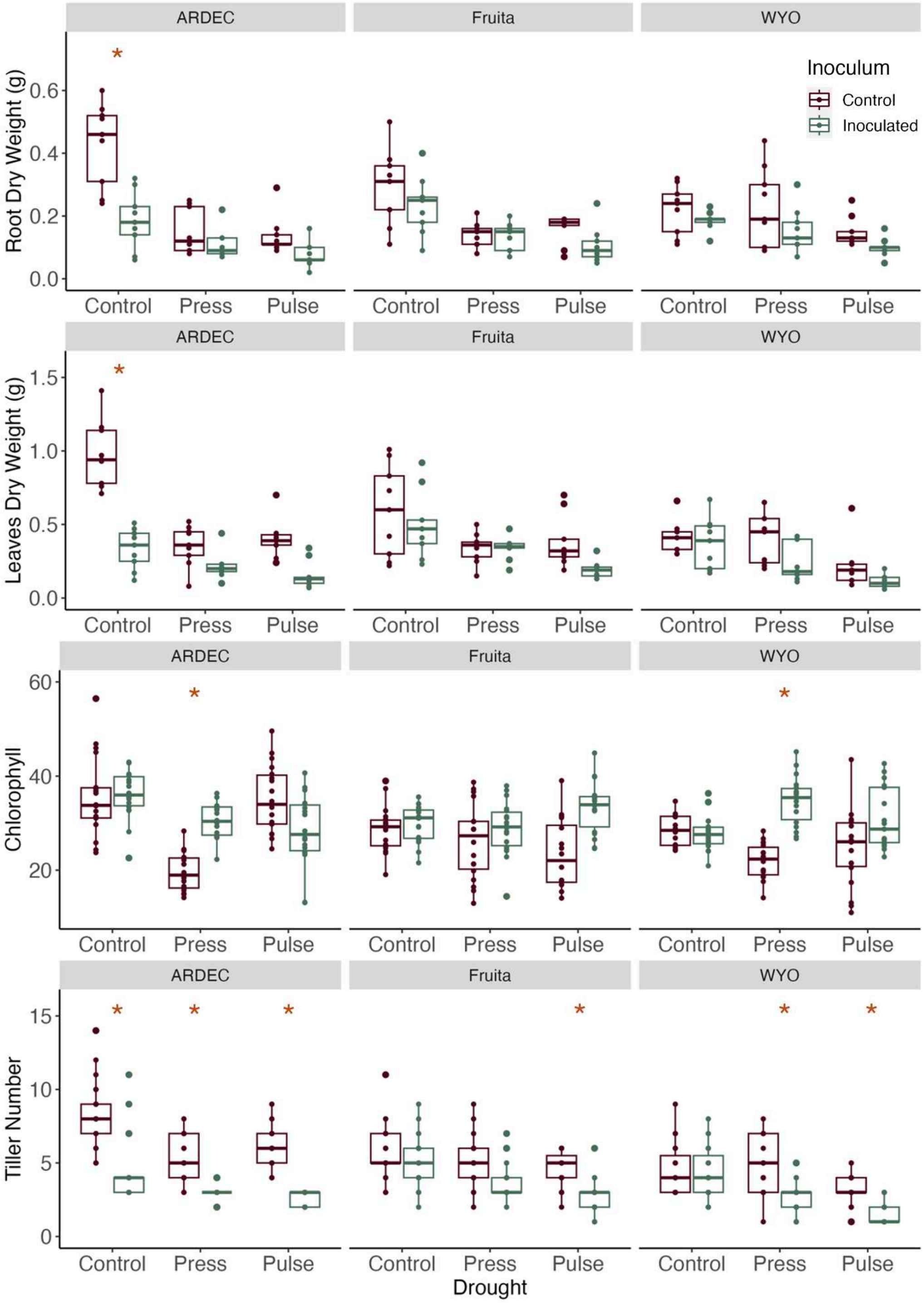


Figure 3.1. The biomass of the roots (a) and leaves (b) of airdried plants, and the number of tillers (c) in the ARDEC, Fruita, and WYO soils for the control, Press, and Pulse watering treatments, in the non-inoculated control plants and the inoculated control. * indicate significance between inoculation treatments based on Tukey HSD tests.

Table 3.1. Analysis of Variance (ANOVA) performed on General Linear Models (GLM) on the root dry weight (left), leaf dry weight (middle), and tiller counts (right).

| | | **Root Weight** | | **Leaf Weight** | | **Chlorophyll** | | **Tiller Number** | |
|---|---|---|---|---|---|---|---|---|---|
| | Df | F value | *p* | F value | *p* | F value | *p* | F value | *p* |
| Inoculum | 1 | 48 | *** | 87.28 | *** | 68.95 | *** | 327.87 | *** |
| Drought | 2 | 69.83 | *** | 78.52 | *** | 23.03 | *** | 104.51 | *** |
| Soil | 2 | 0.44 | | 13.07 | *** | 10.03 | *** | 66.54 | *** |
| Genotype | 2 | 4.15 | * | 4.37 | * | 34.46 | *** | 56.61 | *** |
| Inoculum × Drought | 2 | 5.02 | ** | 5.45 | ** | 20.85 | *** | 2.96 | |
| Inoculum × Soil | 2 | 5.3 | ** | 17.27 | *** | 6.7 | ** | 38.3 | *** |
| Inoculum × Genotype | 2 | 1.43 | | 2.43 | | 1.57 | | 0.27 | |
| Drought × Soil | 4 | 8.69 | *** | 7.11 | *** | 13.37 | *** | 7.63 | *** |
| Drought × Genotype | 4 | 1.17 | | 1.29 | | 9.36 | *** | 6.42 | *** |
| Soil × Genotype | 4 | 3.66 | ** | 7.78 | *** | 2.98 | * | 8.92 | *** |
| Inoculum × Drought × Soil | 4 | 5.77 | *** | 9.53 | *** | 20.93 | *** | 9.48 | *** |
| Inoculum × Drought × Genotype | 4 | 3.27 | * | 0.77 | | 9.09 | *** | 2.73 | * |
| Inoculum × Soil × Genotype | 4 | 2.71 | * | 2.86 | * | 6.01 | *** | 1.58 | |
| Drought × Soil × Genotype | 8 | 3.38 | ** | 4.89 | *** | 2.83 | ** | 6.49 | *** |
| Inoculum × Drought × Soil × Genotype | 7 | 2.41 | * | 2.62 | * | 5.55 | *** | 1.98 | * |
| Residuals | 262 | | | | | | | | |

* p < .05, ** p < .01, *** p < .001

## 4.2 Impact of AMF inoculation and drought on soil chemistry and enzymatic activity

The experimental treatments resulted in changes to the soil chemistry and enzymatic activity. The soil treatments were particularly influential as they as they had very different chemistry and microbial communities at the start of the experiment. Of all the treatments, the soil treatment had the greatest impact on the organic matter (F-stat = 138.06), Olsen phosphorus (F-stat = 354.42), and nitrate content (F-stat = 9.09) (Table 3.2). Soil also had the strongest impact on carbon cycling enzymes, BG + CB + XYL (F-stat = 58.14) and PHOS (F-stat = 21.12), but interestingly soil did not significantly impact LAP (Table 3.3).

Genotype did not influence nitrate (F-stat = 1.23), carbon cycling (F-stat = 0.26), or PHOS activity (F-stat = 0.37), and had less influence than other treatments on organic matter (F-stat = 4.98) and Olsen P (F-stat = 7.8). Interestingly, genotype had a greater influence than on LAP activity (F-stat = 10.43) than soil (F-stat = 2.25). The drought treatments resulted in slight reductions in organic matter (F-stat = 7.49) and Olsen P (F-stat = 12.96) (Figure 3.2) but did not influence carbon cycling (F-stat = 1.26) or PHOS (F-stat = 0.83) (Figure 3.3). Drought did not influence soil nitrate content (F-stat = 0.79) but did significantly influence LAP (F-stat = 18.06).

Inoculation with the *R. irregularis* culture led to significant changes to both the soil chemistry (Figure 3.2; Table 3.2) and enzymatic activity (Figure 3.3; Table 3.3). Inoculation increased organic matter (F-stat = 8.12) and reduced Olsen P (F-stat = 171.17). Organic matter increases with inoculation varied within soil and drought treatments, while Olsen P reductions were visible in all soil and drought treatments and significant in the ARDEC and Fruita drought treatments (Figure 3.2). Nitrate was fairly stable across the experiment and not affected by inoculation (F-stat = 0.95), though LAP activity was significantly impacted by inoculation (F-stat = 34.45) and increased significantly in the ARDEC and WYO inoculated controls (Figure 3.3).

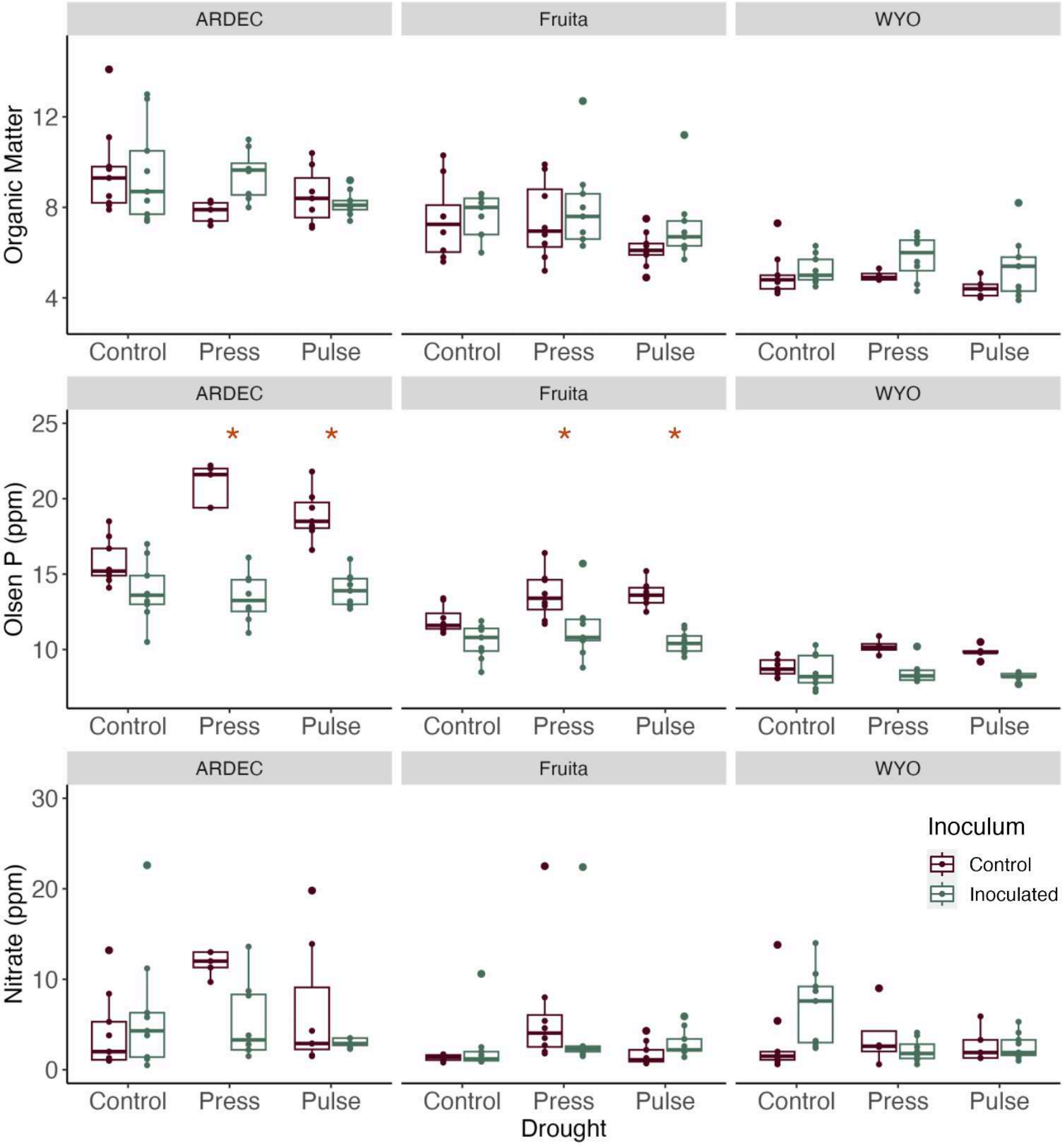
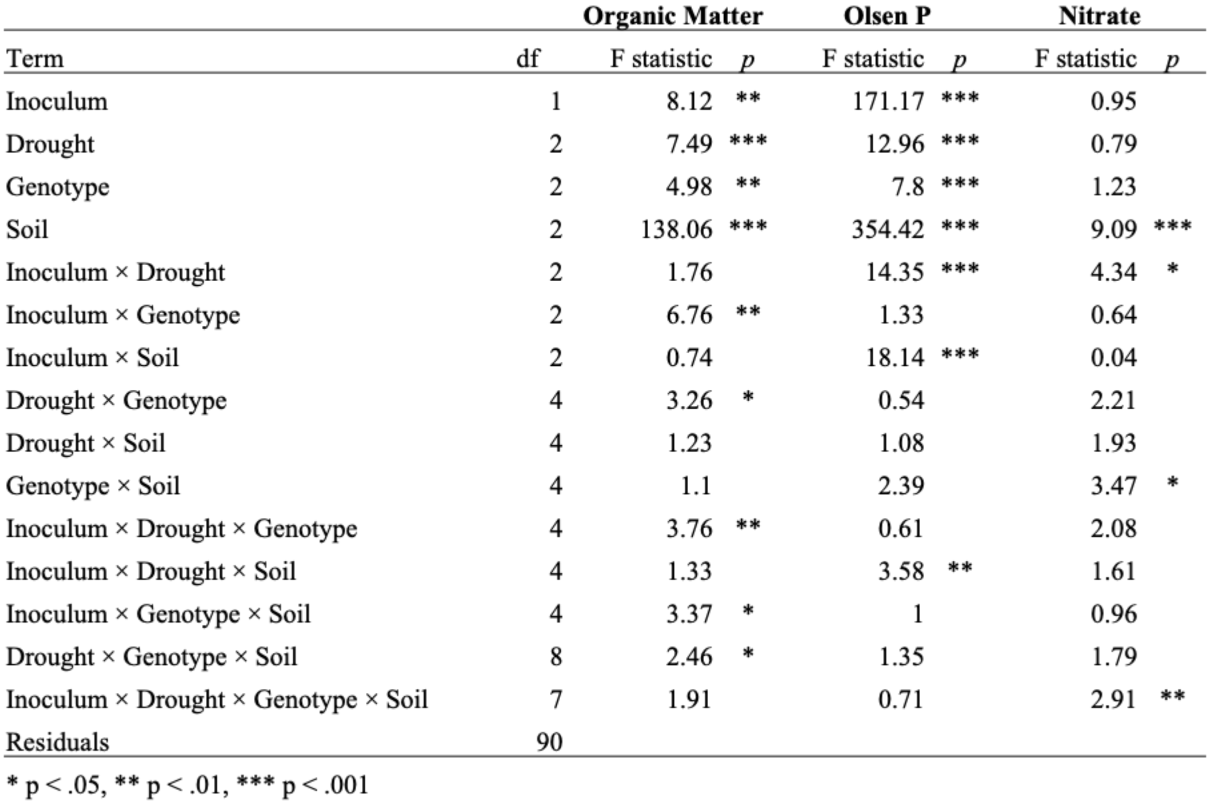


Figure 3.2. Soil chemistry analysis to determine the organic matter, Olsen phosphorus, and nitrate of the rhizosphere soil samples. * indicate significance between the inoculation treatments as determined by Tukey HSD tests.

Table 3.2. ANOVAs performed on GLMs of the soil analyses of the rhizosphere samples: organic matter, Olsen phosphorus, and nitrate.



| | | **Organic Matter** | | **Olsen P** | | **Nitrate** | |
|---|---|---|---|---|---|---|---|
| Term | df | F statistic | *p* | F statistic | *p* | F statistic | *p* |
| Inoculum | 1 | 8.12 | ** | 171.17 | *** | 0.95 | |
| Drought | 2 | 7.49 | *** | 12.96 | *** | 0.79 | |
| Genotype | 2 | 4.98 | ** | 7.8 | *** | 1.23 | |
| Soil | 2 | 138.06 | *** | 354.42 | *** | 9.09 | *** |
| Inoculum × Drought | 2 | 1.76 | | 14.35 | *** | 4.34 | * |
| Inoculum × Genotype | 2 | 6.76 | ** | 1.33 | | 0.64 | |
| Inoculum × Soil | 2 | 0.74 | | 18.14 | *** | 0.04 | |
| Drought × Genotype | 4 | 3.26 | * | 0.54 | | 2.21 | |
| Drought × Soil | 4 | 1.23 | | 1.08 | | 1.93 | |
| Genotype × Soil | 4 | 1.1 | | 2.39 | | 3.47 | * |
| Inoculum × Drought × Genotype | 4 | 3.76 | ** | 0.61 | | 2.08 | |
| Inoculum × Drought × Soil | 4 | 1.33 | | 3.58 | ** | 1.61 | |
| Inoculum × Genotype × Soil | 4 | 3.37 | * | 1 | | 0.96 | |
| Drought × Genotype × Soil | 8 | 2.46 | * | 1.35 | | 1.79 | |
| Inoculum × Drought × Genotype × Soil | 7 | 1.91 | | 0.71 | | 2.91 | ** |
| Residuals | 90 | | | | | | |

* p < .05, ** p < .01, *** p < .001

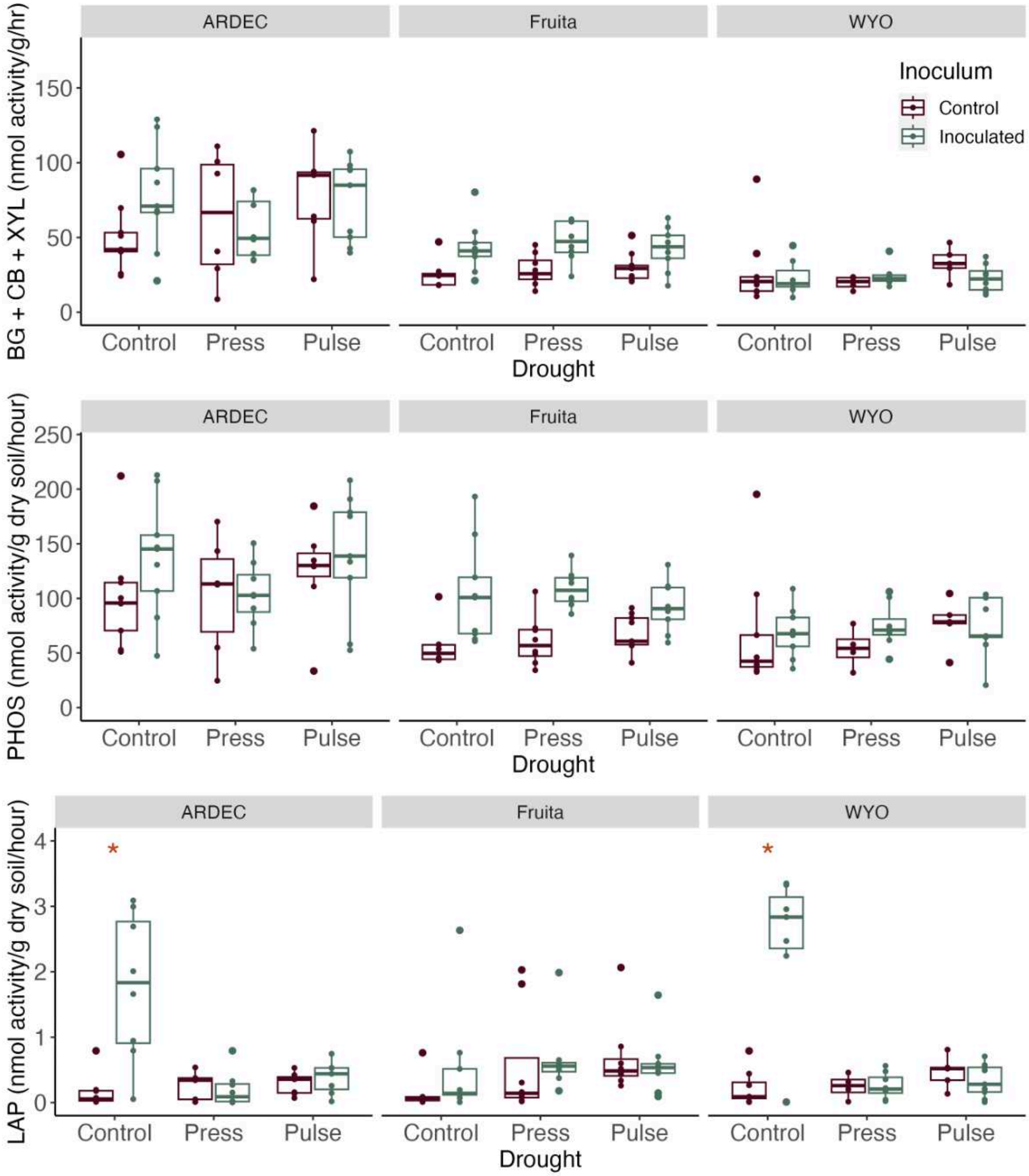


Figure 3.3. Enzyme analysis to determine the nanomols of activity per gram of dry soil per hour. The enzymatic activity examined are a) phosphotase (PHOS), b) leucyl aminopeptidase (LAP), c) N-acetylglutamate synthase (NAG), d) beta-glucosidase (BG), e) alpha-glucosidase (AG), f) xylanase (XYL), and g) cellobiohydrolase (CB).

Table 3.3. ANOVAs performed on GLMs of the enzymatic activity of the rhizosphere samples. The sum of three enzymes controlling carbon cycling—beta-glucosidase (BG), cellobiohydrolase (CB), and xylanase (XYL); phosphotase (PHOS), involved in phosphorus cycling; and leucyl aminopeptidase (LAP), involved in nitrogen cycling.

| | | **BG + CB + XYL** | | **PHOS** | | **LAP** | |
|---|---|---|---|---|---|---|---|
| Term | df | F statistic | *p* | F statistic | *p* | F statistic | *p* |
| Inoculum | 1 | 5.34 | * | 12.16 | *** | 34.45 | *** |
| Drought | 2 | 1.26 | | 0.83 | | 18.06 | *** |
| Genotype | 2 | 0.26 | | 0.37 | | 10.43 | *** |
| Soil | 2 | 58.14 | *** | 21.12 | *** | 2.25 | |
| Inoculum × Drought | 2 | 1.77 | | 0.58 | | 23.69 | *** |
| Inoculum × Genotype | 2 | 3 | | 0.06 | | 0.07 | |
| Inoculum × Soil | 2 | 3.67 | * | 2.41 | | 3.46 | * |
| Drought × Genotype | 4 | 3.5 | * | 0.81 | | 0.3 | |
| Drought × Soil | 4 | 0.99 | | 0.85 | | 12.16 | *** |
| Genotype × Soil | 4 | 1.28 | | 1.41 | | 2.59 | * |
| Inoculum × Drought × Genotype | 4 | 1.07 | | 0.25 | | 0.03 | |
| Inoculum × Drought × Soil | 4 | 1.98 | | 0.67 | | 4.95 | *** |
| Inoculum × Genotype × Soil | 4 | 6.54 | *** | 2.69 | * | 5.13 | *** |
| Drought × Genotype × Soil | 8 | 1.9 | | 1.11 | | 4.19 | *** |
| Inoculum × Drought × Genotype × Soil | 7 | 0.98 | | 0.52 | | 3.81 | *** |
| Residuals | 79 | | | | | | |

* p < .05, ** p < .01, *** p < .001

### 4.3 Alpha-diversity of bacterial and fungal communities in the rhizosphere and roots

The bacterial and fungal alpha-diversity responded in similar ways to the inoculation and drought treatment (Figure 3.4; Table 3.4). Genotype did not affect bacterial or fungal alpha-diversity in the roots or the rhizosphere (Table 3.4). Unsurprisingly given the initial diversity differences of the soils (S3.1), alpha-diversity for bacteria and fungi did vary with soil in both the rhizosphere and root compartments, with some interesting trends. For the alpha-diversity of the rhizosphere bacterial community, soil (F-stat = 11.25) had less of an impact than the inoculation (F-stat = 665.57), whereas for the roots the effect of the soil (F-stat = 23.03) was greater than the inoculation (F-stat = 20.35). However, for the fungal alpha-diversity, soil had a greater impact than inoculation in the rhizosphere (Soil F-stat = 71.94; Inoculum F-stat = 36.73), but a lesser impact in the roots (Soil F-stat = 15.68; Inoculum F-stat = 142.09). The drought treatments did not impact the alpha-diversity in the rhizosphere for either bacteria (F-stat = 0.34) or fungi (F-stat= 1.37) but was the strongest driver of alpha-diversity changes in the roots for bacteria (F-stat = 36.85) and second only to inoculation in the roots for fungi (F-stat = 27.78).

In the rhizosphere, inoculation led to reductions in bacterial and fungal alpha-diversity (Figure 3.4). This reduction was observed in all soil types and was significant in all soils, aside from the fungal diversity of the WYO treatment, where the reduction was not significant. In the roots the opposite trend was observed. Inoculation resulted in increases in the bacterial alpha-diversity in the ARDEC soil and non-significant increases were observed in Fruita and WYO (Figure 3.4). For the fungal communities of the roots, alpha-diversity increased significantly with inoculation in all soils (Figure 3.4). Of all the treatments, inoculation had the greatest impact on the bacterial alpha-diversity of the rhizosphere (F-stat = 665.57) and on the fungal alpha-diversity of the roots (F-stat = 142.09). For bacterial root alpha-diversity, inoculation was significant (F-stat = 20.35) though less so than drought (F-stat = 36.85) and soil (F-stat = 23.03). For fungal rhizosphere alpha-diversity, inoculation was significant (F-stat = 36.73) though less influential than soil (F-stat = 71.94).

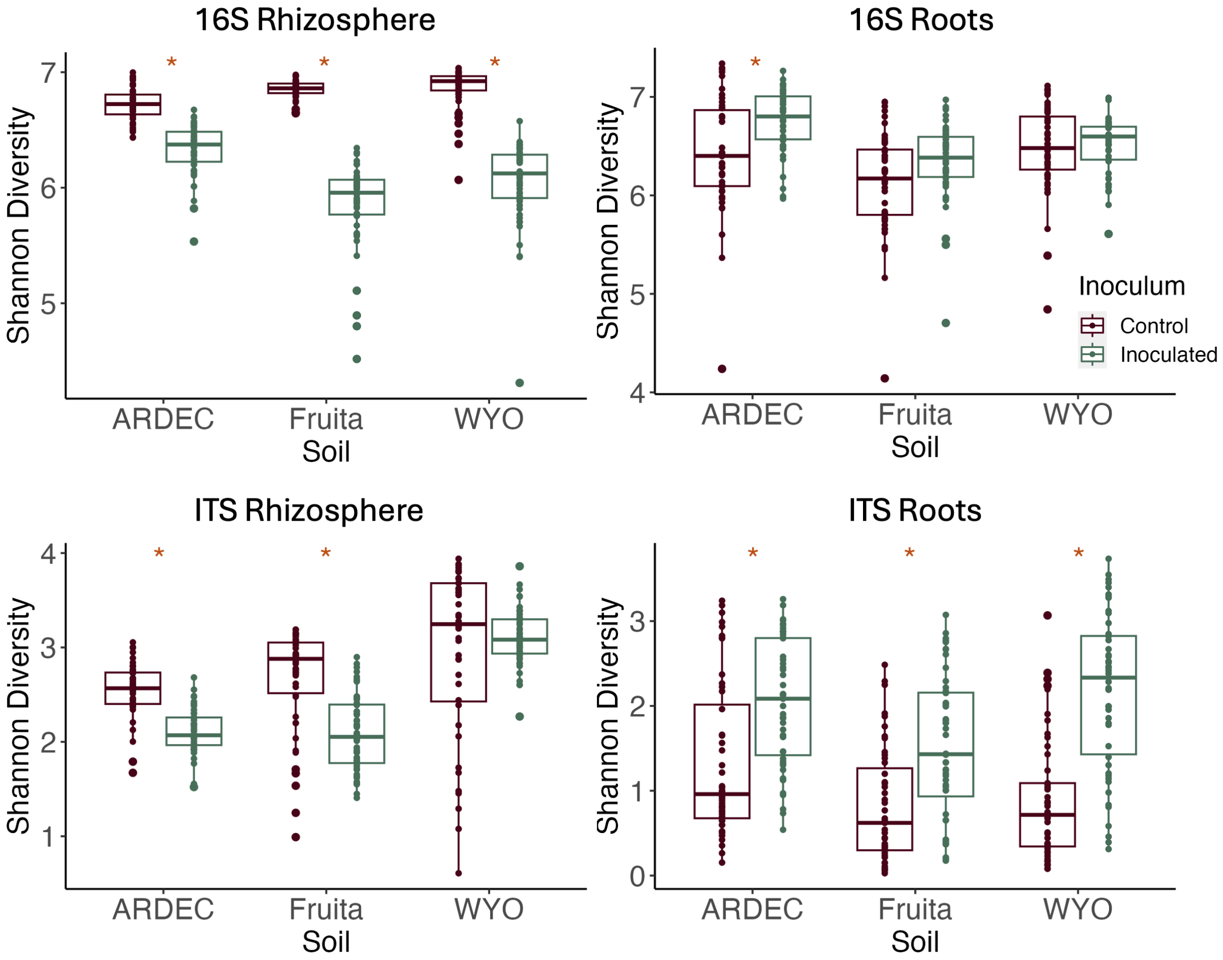


Figure 3.4. The Shannon diversity for the 16S bacterial amplicon library (top) and the ITS fungal library (bottom) for the rhizosphere soil post-harvest (left), and the roots (right). Red * indicate significance between the inoculation treatments as determined by Tukey HSD tests.

Table 3.4. Analysis of Variance (ANOVA) performed on General Linear Models (GLM) on the Shannon diversity of the bacterial (16S) and fungal (ITS) communities of the rhizosphere and roots.

| | | **16S Rhizo** | | **16S Roots** | | **ITS Rhizo** | | **ITS Roots** | |
|---|---|---|---|---|---|---|---|---|---|
| Term | df | F statistic | *p* | F statistic | *p* | F statistic | *p* | F statistic | *p* |
| Inoculum | 1 | 665.57 | *** | 20.35 | *** | 36.73 | *** | 142.09 | *** |
| Drought | 2 | 0.34 | | 36.85 | *** | 1.37 | | 27.78 | *** |
| Genotype | 2 | 1.46 | | 1.06 | | 1.92 | | 1.01 | |
| Soil | 2 | 11.25 | *** | 23.03 | *** | 71.94 | *** | 15.68 | *** |
| Inoculum × Drought | 2 | 0.6 | | 11.82 | *** | 4.32 | * | 0.55 | |
| Inoculum × Genotype | 2 | 0.29 | | 0.85 | | 0.69 | | 12.17 | *** |
| Inoculum × Soil | 2 | 37.87 | *** | 5.45 | ** | 12.2 | *** | 7.63 | *** |
| Drought × Genotype | 4 | 1.45 | | 2.18 | | 1.19 | | 6.07 | *** |
| Drought × Soil | 4 | 2.91 | * | 0.59 | | 4.91 | *** | 4.25 | ** |
| Genotype × Soil | 4 | 0.82 | | 1.57 | | 2.33 | | 1.23 | |
| Inoculum × Drought × Genotype | 4 | 2.97 | * | 3.63 | ** | 1.69 | | 1.68 | |
| Inoculum × Drought × Soil | 4 | 1 | | 4.19 | ** | 1.48 | | 5.02 | *** |
| Drought × Genotype × Soil | 8 | 1.41 | | 0.64 | | 0.64 | | 1.7 | |
| Inoculum × Drought × Genotype × Soil | 12 | 0.93 | | 1.21 | | 1.05 | | 3.43 | *** |
| Residuals | 245 | | | | | | | | |

* p < .05, ** p < .01, *** p < .001

### 4.4 Influence of AMF inoculation, drought, soil, and host genotype on bacterial and fungal beta-diversity

Bacterial and fungal beta diversity of the rhizosphere and root compartments were affected by the soil type, inoculation, drought treatment and in some cases, the plant genotype (Figure 3.5; Table 3.5). Soil type had the greatest impact on the microbial beta-diversity of the rhizosphere bacteria (R2 = .32), the root bacteria (R2 = .23), the rhizosphere fungi (R2 = .46), and the root fungi (R2 = .12). The visual differences between soil treatments were distinct for the rhizosphere, diminished slightly for root bacteria, and diminished considerably for root fungi (Figure 3.5). Drought influenced the beta-diversity of bacteria and fungi in both compartments

(Table 3.5). Similar to alpha-diversity, the impact of drought was greater in the root compartment for bacteria (R2 = 0.06) and fungi (R2 = 0.05) than in for bacteria (R2 = 0.01) and fungi (R2 = 0.01) in the rhizosphere. Genotype influenced the beta-diversity of the bacteria in the rhizosphere and roots, and the fungi in the rhizosphere but not the roots, however the beta-diversity changes driven by genotype were generally less than other factors (Table 3.5).

The inoculation treatment was a significant driver of bacterial beta-diversity in the rhizosphere (R2 = 0.14) and roots (R2 = 0.07) and of fungal beta-diversity in the rhizosphere (R2 = 0.13) and roots (R2 = 0.09). Though soil had a greater influence on beta-diversity in all compartments, there was visible separation between the inoculation treatments, though this varied with microbial kingdom, plant niche compartment, and soil type (Figure 3.5). For both microbial kingdoms, differentiation by inoculation treatment was greater in the rhizosphere than in the roots (Figure 3.5). Bacterial beta-diversity was more strongly influenced by inoculation than fungal beta-diversity. For rhizosphere bacteria, there was clearer differentiation between inoculation treatments in the higher diversity soils, Fruita and WYO, than in the low diversity soil ARDEC (Figure 3.5).

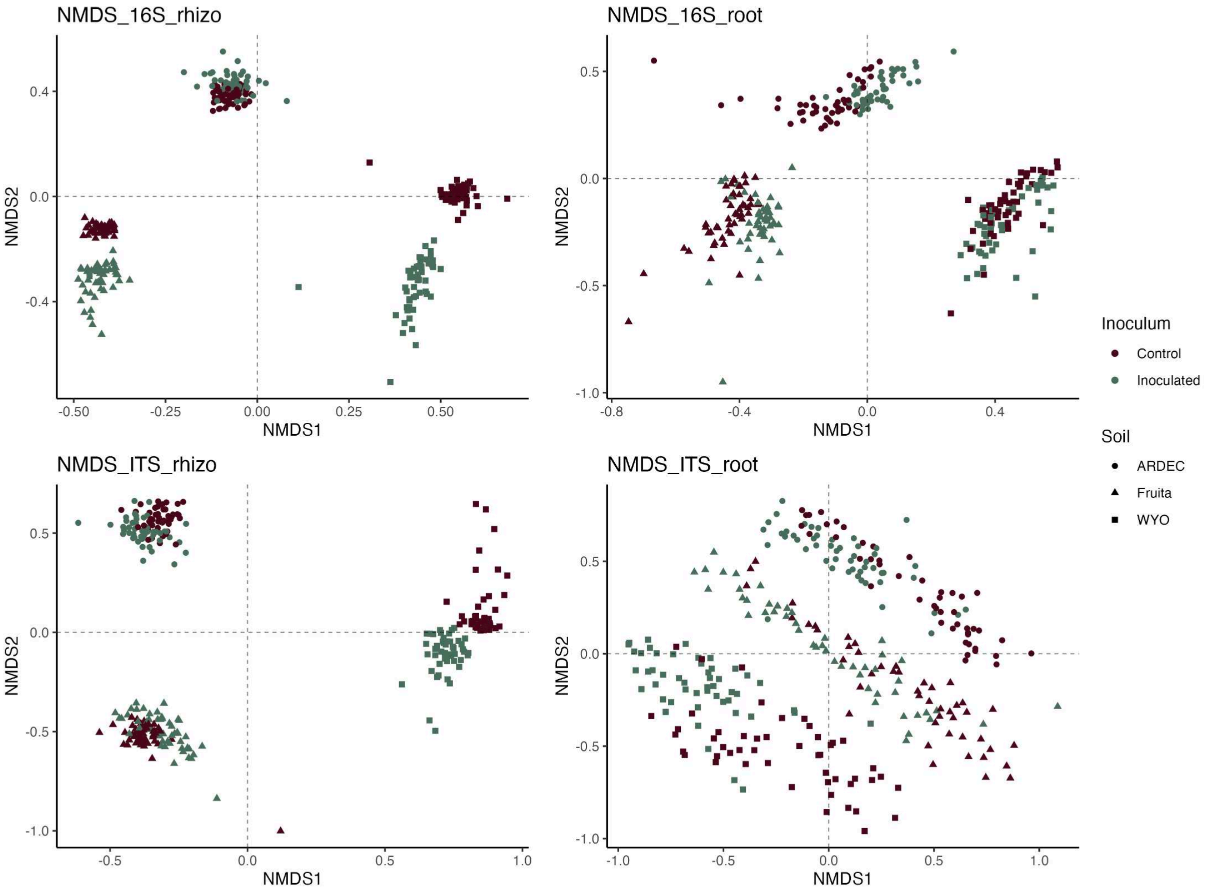


Figure 3.5. Non-Metric Multi-Dimensional Scaling (NMDS) ordinations for the 16S bacterial amplicon library (top) and the ITS fungal library (bottom) for the rhizosphere soil post-harvest (left), and the roots (right). Shapes indicate soil types and colors represent the inoculation treatment.

Table 3.5. Permutational Multivariate Analysis of Covariance (PERMANOVA) performed on the 16S bacterial sequencing and ITS fungal sequencing for the rhizosphere and roots**.**

| | | **16S Rhizo** | | **16S Roots** | | **ITS Rhizo** | | **ITS Roots** | |
|---|---|---|---|---|---|---|---|---|---|
| Term | *df* | $R^2$ | *p* | $R^2$ | *p* | $R^2$ | *p* | $R^2$ | *p* |
| Inoculum | 1 | 0.14 | *** | 0.07 | *** | 0.13 | *** | 0.09 | *** |
| Drought | 2 | 0.01 | *** | 0.06 | *** | 0.01 | *** | 0.05 | *** |
| Genotype | 2 | 0.01 | ** | 0.01 | *** | 0.01 | ** | 0.01 | |
| Soil | 2 | 0.32 | *** | 0.23 | *** | 0.46 | *** | 0.12 | *** |
| Inoculum × Drought | 2 | 0.01 | * | 0.02 | *** | 0 | * | 0.01 | ** |
| Inoculum × Genotype | 2 | 0 | * | 0.01 | *** | 0.01 | ** | 0.02 | *** |
| Inoculum × Soil | 2 | 0.06 | *** | 0.03 | *** | 0.06 | *** | 0.02 | *** |
| Drought × Genotype | 4 | 0.01 | | 0.01 | *** | 0.01 | | 0.02 | ** |
| Drought × Soil | 4 | 0.01 | ** | 0.03 | *** | 0.01 | ** | 0.05 | *** |
| Genotype × Soil | 4 | 0.01 | * | 0.02 | *** | 0.01 | ** | 0.02 | ** |
| Inoculum × Drought × Genotype | 4 | 0.01 | * | 0.02 | *** | 0.01 | | 0.02 | * |
| Inoculum × Drought × Soil | 4 | 0.01 | | 0.02 | *** | 0.01 | ** | 0.02 | *** |
| Inoculum × Genotype × Soil | 4 | 0.01 | * | 0.02 | *** | 0.01 | * | 0.02 | *** |
| Drought × Genotype × Soil | 8 | 0.01 | | 0.03 | *** | 0.01 | * | 0.03 | * |
| Inoculum × Drought × Genotype × Soil | 8 | 0.01 | | 0.03 | *** | 0.01 | * | 0.04 | *** |
| Residual | 245 | 0.37 | | 0.42 | | 0.25 | | 0.47 | |
| Total | 298 | 1 | | 1 | | 1 | | 1 | |

Note. * p < .05, ** p < .01, *** p < ***

## 4.5 Impact of drought and AMF inoculation on the enrichment and depletion of bacteria and fungi in the rhizosphere and roots

The treatments, especially inoculation and drought led to the relative enrichment and depletion of particular taxa in the rhizosphere and roots. In the rhizosphere, inoculation led to the enrichment of members of the phyla Firmicutes, Proteobacteria, and Verrucomicrobia while depleting members of Actinobacteria, Acidobacteria, and Chloreflexi. Most fungal phyla were reduced in the rhizosphere under inoculation, except for Basiciomycota which were selectively enriched (S3.2). Interestingly, the patterns of enrichment were vastly different in the roots where the abundance of several bacterial and fungal phyla were enriched in the inoculation treatment including members of Acidobacteria, Bacteroidetes, Proteobacteria, Plantomycetes, Firmicutes, Verrucomicrobia from bacteria and Ascomycota, Basidiomycota, Glomeromycota from fungi, while there were no noticeable depletions for either bacteria or fungi (S3.3).

Drought treatments resulted in the enrichment of Actinobacteria and the depletion of Acidobacteria, Bacteroidetes, Proteobacteria, Chloreflexi, Firmicutes, Gemmatimonodales, Plantomycetes, Verrucomicrobia in the rhizosphere (S3.4). In the roots, Acidobacteria, Actinobacteria, Plantomycetes, and Verrucomicrobia were enriched and Proteobacteria and Bacteroidetes were reduced (S3.5). For fungi, Ascomycota and Glomeromycota were reduced in the rhizosphere while all fungal phyla were reduced in the roots (S3.4; S3.5).

When functional modes were assigned to the ITS taxonomies using FUNGUILD, AMF inoculation reduced the abundance of saprotroph and saprotroph-symbiotroph fungal functional modes in the rhizosphere. In the roots, many fungal functional modes were enriched, particularly symbiotrophs, saprotrophs, and pathotroph-saprotroph-symbiotrophs (S3.6).

### 4.6 Impact of AMF inoculation and drought on patterns of bacterial and fungal co-occurrence in the rhizosphere

The rhizosphere co-occurrence networks varied with soil type, drought treatment, and AMF-inoculation (Figure 3.6; S3.7). In the drought and inoculation controls, ARDEC had the simplest network architecture overall with a lower number of nodes and edges than Fruita and WYO. Surprisingly, the network for the intermediate diversity soil, Fruita, was more complex and modular than the WYO network, and had a greater proportion of negative edges (37%) than ARDEC (25.4%) and WYO (28.2%). WYO had a higher proportion of fungal nodes (48.8%) than ARDEC (32.7%) and Fruita (23%). Fruita had a higher proportion of bacterial nodes

(72.8%) than ARDEC (59.1%) and WYO (46.5%). Fruita and to a lesser extent ARDEC had some modularity by microbial kingdom with a positively correlated module for bacteria and another for fungi, whereas the WYO network primarily had a primarily fungal module (Figure 3.6). Each of the networks had a greater proportion of positive correlations within kingdoms and a greater proportion of negative correlations across kingdoms, a trend which was most pronounced in WYO soil (S3.7).

Interestingly, the effect of the drought treatments on microbial co-occurrence varied with soil type. In the ARDEC and Fruita soil, drought increased the prevalence of fungal nodes (ARDEC: control = 32.7%; Press = 42.7%; Pulse = 38.3%; Fruita: control = 23%; Press = 32%; Pulse = 29.5%) whereas in WYO the prevalence of fungal nodes was reduced in drought (control = 48.8%; Press = 29.8%; Pulse = 31%). Interestingly the network complexity and proportion of negative correlations—generally considered to be indicators of network stability—were not consistently reduced in drought. The ARDEC Press network had a greater proportion of negative correlations (44.2%) than the control (25.4%) and both WYO drought treatments had a higher proportion of negative correlations (Press = 44%; Pulse = 37.2%) than the control (28.2%). The WYO drought networks were more highly correlated than the WYO control network (S3.7). For Fruita and WYO, the drought treatments resulted in a decrease in kingdom-specific modularity, with an increased number of negative correlations between kingdoms, especially between bacteria and fungi (Figure 3.6; S3.7). In the WYO soil the control treatment had considerably fewer negative bacteria-fungi correlations (75) than in the Press (263) or the Pulse (191) drought treatments. This trend was also observed when comparing Fruita Pulse (209) to the watering control (131), and when comparing the ARDEC Press (181) to the watering control (36).

In general, the AMF inoculation treatment increased the prevalence of fungal nodes in the network compared to the non-inoculated control, however this varied with soil type and drought treatment. In Fruita, the proportion of fungal nodes was higher in the inoculated network for every watering treatment (Figure 3.6). In ARDEC soil, the proportion of fungal nodes increased with inoculation in the watering control (control = 32.7%; inoculated = 36.1%), but not in either the Press drought (control = 42.7%; inoculated = 41%), or the Pulse drought (control = 38.3%; inoculated = 32.9%). In the WYO soil the trend was the opposite, with the inoculation reducing the proportion of fungal nodes in the watering control (control = 48.8%; inoculated = 36.2%), while increasing them in the Press (control = 29.8%; inoculated = 44.8%), and Pulse drought treatment (control = 31%; inoculated = 41.9%).

In every case, the AMF inoculation reduced the proportion of negative correlations (Figure 3.6) which is an indication of reduced network stability. The overall complexity of the AMF inoculated networks was generally lower than in the non-inoculated control, however this varied considerably with soil type and drought. In ARDEC soil, AMF inoculation resulted in significantly simpler networks with fewer overall nodes and edges (Figure 3.6; S3.7). This trend was observed in Fruita and WYO soil to a lesser degree and varied with watering treatment. For example, in the inoculated Fruita and WYO networks, we observed a module of highly positively correlated, unknown fungi. The impact of this new fungal hub varied with watering and soil treatment. In the watering control Fruita network, the main fungal and bacterial modules present in the control network are still present in the inoculated network, along with a new module of highly correlated fungi, however the original bacterial and fungal modules have fewer nodes and a greater prevalence of positive correlations (Figure 3.6). However, in the WYO watering control, the predominantly fungal module in the non-inoculated network is replaced by a primarily bacterial module in the inoculation treatment and the fungal nodes in the network are more dispersed and less central (Figure 3.6). Additionally, the new module of unknown fungi has slightly more correlations with the rest of the network, whereas the unknown fungal module in the Fruita watering control is highly positively correlated within the nodule but has very few correlations with other nodes in the network (Figure 3.6).

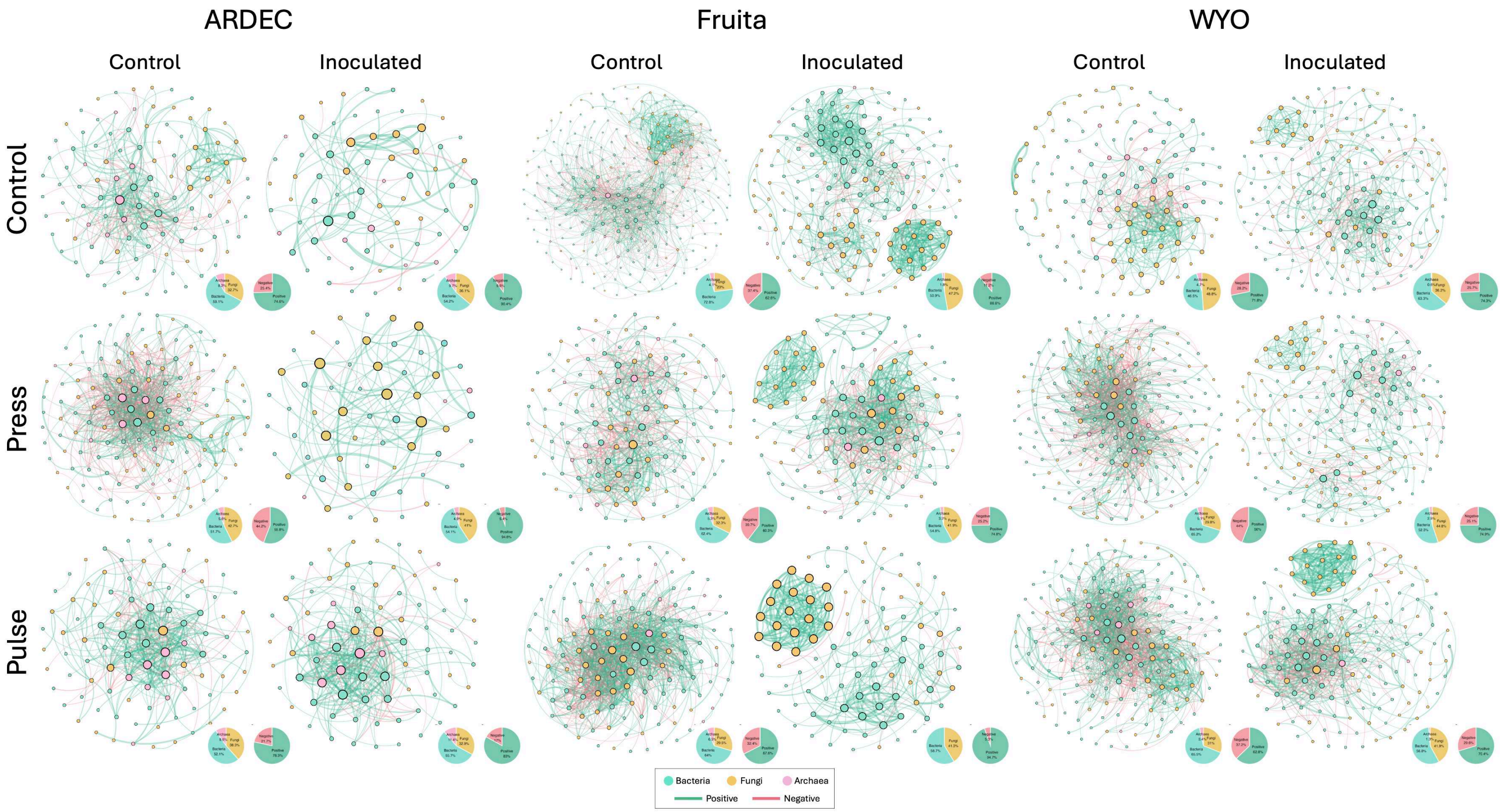


Figure 3.6. Rhizosphere co-occurrence networks based on SparCC correlations, subset by soil type, drought treatment, and inoculation treatment. Nodes represent bacterial and archaeal OTUs from the 16S sequencing library and fungal OTUs from the ITS sequencing library. Edges represent strong (|SparCC| > 0.7) and significant ($p\text{-val} < 0.05$) correlations. Pie-charts at the bottom-right corner of each network show the percentage of bacterial (green), fungal (yellow), and archaeal (pink) nodes and the percent of positive (green) and negative (pink) edges in each network.

## 4.7 Impact of AMF inoculation and drought on patterns of bacterial, AM fungal, and general fungal co-occurrence in the roots

In general, the root networks were more stable across soil, drought, and AMF-inoculation treatments than the rhizosphere networks. The root networks were for the most part densely populated with approximately even ratios of positive and negative edges (Figure 3.7; S3.8). While the soil type did not alter the root networks as much as the rhizosphere networks, there were a few trends. The WYO and Fruita soil treatments generally had more complex network architecture than the ARDEC soil with a higher number of nodes and edges in the network (Figure 3.7; S3.8). The WYO control network had a higher proportion of AMF nodes (37.2%) compared to the ARDEC (22.1%) and Fruita (23.4%) networks. Interactions between AMF nodes and between AMF and bacterial nodes were far more prominent in the WYO and Fruita soil treatments than in ARDEC (S3.8).

Compared to the watering control both drought treatments increased the prevalence of AMF nodes in the network regardless of soil type (Figure 3.7). Unlike in the rhizosphere networks, drought led to reductions in network complexity and connectedness. This was especially pronounced in the Press drought treatments. Drought led to slight reductions in the proportion of negative correlations in the ARDEC and Fruita treatments and slight increases in the proportion of negative edges in the WYO treatment, however these trends were slight as all of the root networks had a roughly even ratio of positive and negative correlations (Figure 3.7).

Interestingly the effects of AMF inoculation on the root networks were highly variable with soil and drought treatment. In Fruita soil, the AMF inoculation led to a greater prevalence of AMF nodes in the network in the control watering treatment (control = 23.4%; inoculated = 27%), the Press drought (control = 31.4%; inoculated = 39.4%), and in the Pulse drought (control = 29.3%; inoculated = 39.3%). For the other soil types, the effect of AMF inoculation on the percent of AMF nodes varied with watering treatment. In ARDEC soil, the proportion of AMF in the root network increased with AMF inoculation in the watering control (control = 22.1%; inoculated = 28.4%), and in the Pulse drought (control = 35%; inoculated = 40.6%) but was reduced in the Press drought (control = 55.9%; inoculated = 33.2%). In the WYO soil, the AMF inoculation reduced the prevalence of AMF nodes in the root networks considerably in the control (control = 37.2%; inoculated = 26.7%) and in the Press drought (control = 51.4%; inoculated = 33%), but not in the Pulse drought (control = 52%; inoculated = 52.2%). Regardless of treatment, AMF nodes were highly connected and centrally located in the root networks compared to bacteria and other fungi (S3.8).

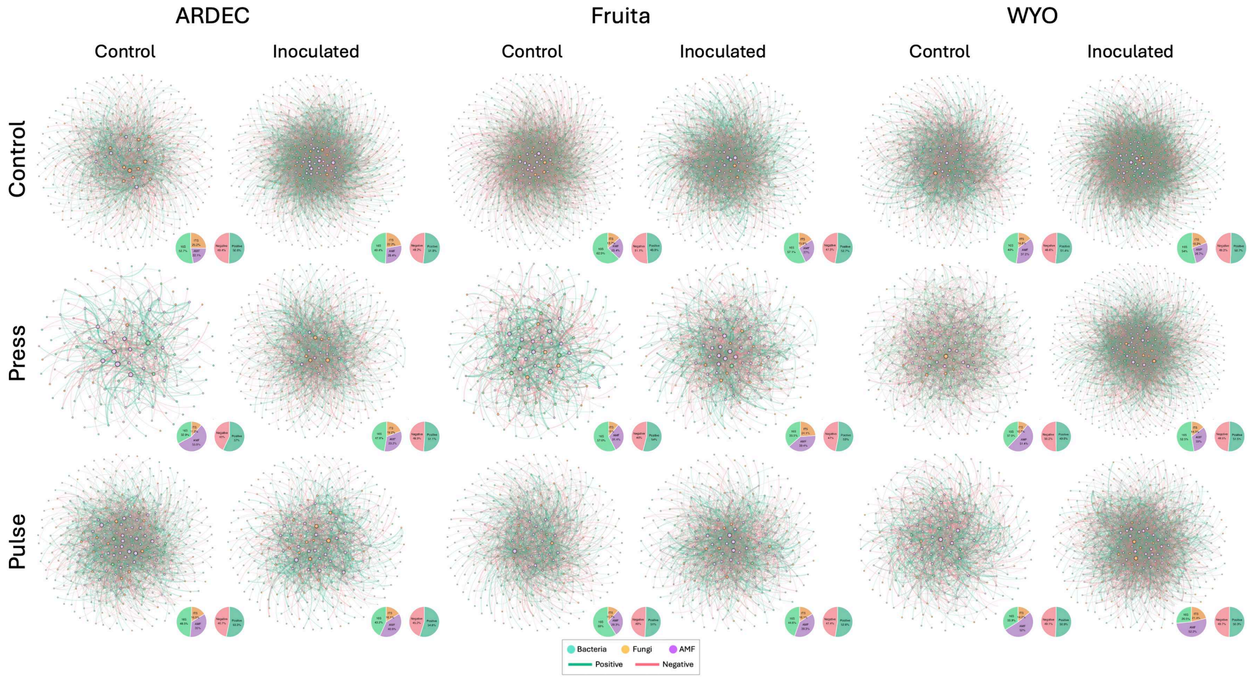


Figure 3.7. Root co-occurrence networks based on SparCC correlations, subset by soil type, drought treatment, and inoculation treatment. Nodes represent bacterial and archaeal OTUs from the 16S sequencing library and fungal OTUs from the ITS sequencing library. Edges represent strong (|SparCC| > 0.7) and significant (p-val < 0.05) correlations. Pie-charts at the bottom-right corner of each network show the percentage of bacterial (green), fungal (yellow), and archaeal (pink) nodes and the percent of positive (green) and negative (pink) edges in each network.

### 4.8 AMF inoculation resulted in no consistent increase in AMF colonization of the roots

Plants treated with the AMF inoculant had no notable increase in either the percent of the root colonized as determined with microscopy nor with the total gene copies per gram of sample (Figure 3.8). The AMF colonization percentage as determined by microscopy was 65.7% and 68% for inoculation and non-inoculated control, respectively. This was true for AM primers, a general gene present in the vast majority of AMF species, meant to represent the total AMF population, and for RiEF-a, *R. irregularis* elongation factor, and for the 197198 primer set which is also specific to *R. irregularis*, the advertised inoculation species (Figure 3.8). For example, the AM primers there were 5.15 x 105 gene copies per gram of root material in the non-inoculated treatment and 3.77 x 105 gene copies per gram of root material in the inoculated treatment. AMF library sequencing revealed no enrichment in the Glomeraceae family, the family to which *R. irregularis* belongs. Instead, we observed an enrichment in the family Claroideoglomeraceae (Figure 3.8).

## 5. Discussion

Our study revealed complex interactions between genotypes, soils, AMF inoculation, and drought, influencing plant growth, soil properties, and plant-associated microbiota diversity and community composition. Although genotypes significantly influenced plant growth parameters and soil properties (Figures 3.1-3.2; Tables 3.1-3.2), they had little impact on the diversity and community composition of plant-associated microbiota (Figures 3.3-3.4; Tables 3.1-3.4). AMF inoculation, soil, and drought had more pronounced effects on this study's soil, plant, and microbiota variables, therefore, we will focus our discussion on how AMF-inoculation impacted the soil, the plant, and the plant-associated microbiome under different watering conditions with unique endemic soil microbiomes.

### 5.1 AMF inoculation led to soil physical, chemical, and functional shifts

We did not observe a typical growth promotion that AMF often cause in planting trials (Figure 3.1; Table 3.1). The absence of the plant growth promotion by AMF inoculation is not uncommon (Püschel et al. 2023; Bitterlich et al. 2024). A recent study has reported that the growth response of AMF inoculation was highly variable, ranging from −12% to +40% (Lutz et al. 2023). Several factors including reduced pot size, associated high root densities and the moderate light intensities can influence AMF mediated plant growth benefits (Koide 1991; Poorter et al. 2012; Bitterlich et al. 2024). Overall, the AMF addition was not able to rescue the plants against the negative impact of drought on the plant growth parameters (Figure 3.1; Table 3.1). This can be due to the breakdown of the AMF-plant symbiosis where the lack of photosynthates from the host curtails the AMF exert their role “for free” resulting in lower biomass (Johnson 2010; Pons and Müller 2022). The ability to increase the plant growth under drought stress is a direct function of the ability of the AMF to colonize the plant’s roots (Augé 2004; 2001). Insufficient colonization of AMF as observed in our study (S3.2) can also result in the non-significant impact on plant growth parameters in response to AMF inoculation.

In general, we observed changes in the soil organic carbon (increased in response to AMF inoculation) and soil phosphorus (decreased in response to AMF inoculation) in the AMF inoculation treatment both in control and drought treatments (Figure 3.1; Table 3.1). These trends could be mediated by the impact of AMF inoculation on the activities of enzymes involved in C and P cycling. We observed a general increase in the activity of C cycling enzymes that regulate soil C turnover (Figure 3.2; Table 3.2). The increased activity of the phosphatase activity can result in increased availability of soluble P that can be taken up by the plants resulting in lower P concentrations in the soil. AMF associations generally increase the enzymatic activity of the soil (M. Qin et al. 2020). The reduction of available phosphorus in the soil in AMF inoculated treatments has been observed in other experiments along with increases in foliar phosphorus content (S. Qin et al. 2017; Wu, Zou, and He 2011). A functional root association with AMF would nevertheless contribute to plant P nutrition regardless of a net effect of plant growth since plant P uptake is a result of the interaction between the direct root P uptake pathway compared to the (indirect) P uptake pathway via AMF (Smith, Smith, and Jakobsen 2004). The increase in the soil enzyme activities may be either a function of the AMF itself, or it may be due to the impact of AMF inoculation on the plant and soil associated microbial communities (Jiang et al. 2021; L. Wang et al. 2023). AMF also increases processes related to chitin formation and degradation (M. Qin et al. 2020) which we observed in the form of marginal increases in NAG activity which is related to chitin synthesis.

### 5.2 Drought and AMF inoculation altered the rhizosphere and root microbiome diversity and community composition

Our results showed that drought significantly ($p < 0.001$) reduced the bacterial and fungal alpha diversity but had no significant impact on the rhizosphere (Table 3.1). Although drought significantly ($p < 0.001$) impacted the bacterial and fungal community composition in both the root and rhizosphere, the impact was more pronounced in root compared to the rhizosphere

(Table 3.2). Our results align with previous studies suggesting the compartment-specific restructuring of the plant-associated microbiota, with root communities displaying a more pronounced response than rhizosphere communities (Santos-Medellín et al. 2017; 2021). Under drought conditions, plants exert greater selective pressure on their endosphere compartments than their rhizospheres, leading to greater changes in the microbial communities of the root compartment.

AMF significantly impacted alpha diversity and community composition of bacteria and fungi in both the root and rhizosphere (Figures 3.1-3.2; Tables 3.1-3.2). AMF can influence the plant-associated microbiota directly through competition with native microbiota or by enriching a unique hyphae-associated microbiome (G. Wang et al. 2023; L. Wang et al. 2023). Indirect influence can be due to changes in plant physiology, exudation patterns, or plant defense responses. Although the mechanisms enabling members of AMF-associated bacterial communities to establish in plant environments and become endophytic are still unknown, recent data suggest that AMF and their associated bacteria could be an important factor affecting bacterial recruitment (Lastovetsky et al. 2022; 2024). When AMF hyphae are separated from plant roots, it is shown that the microbial community that forms around the hyphae is distinct from the rhizosphere community (L. Wang et al. 2023; G. Wang et al. 2023; Emmett, Lévesque-Tremblay, and Harrison 2021). When hyphae and plant roots are not separated, the hyphosphere and rhizosphere should overlap to form a new community. The overall bacterial and fungal alpha-diversity of the rhizosphere may be reduced due to the selective enrichment of certain taxa by the AMF itself and by AMF-induced changes to the soil chemistry and physical properties (Fall et al. 2022). While the increased bacterial and fungal alpha-diversity in the roots is surprising, it could be that the physical penetration of the cell walls of the plant roots creates an avenue through which both bacteria and fungi can colonize the plant roots. There may be enrichments in certain bacteria that are commonly associated with AMF but are not otherwise enriched in the plant roots.

Changes in the root exudation profile of the plant may also come into play, especially in increasing the fungal diversity of the root. To initiate AMF associations, plants release strigolactones, which can also serve as a signal to attract other types of fungi to colonize the root compartment (López-Ráez, Pozo, and García-Garrido 2011). Plants also reduce the exudation of defensive chemicals like phenolic compounds which could make the root more vulnerable to colonization by a more diverse set of organisms (Frew, Heuck, and Aguilar-Trigueros 2023). Overall, AMF are crucial members of the plant microbiome and play a major role in shaping the root and rhizosphere microbiome.

We also observed that the interactions between inoculation and soil and between inoculation and drought among others led to significant changes to the root and rhizosphere beta-diversity (Table 3.5). This could indicate that AMF inoculation has varying effects on the rhizosphere and root communities given different bulk soil communities and different watering conditions.

## 5.3 AMF inoculation and drought led to the enrichment of different taxa

The inoculation and drought treatments led to the enrichment and depletion of certain taxa. As predicted, drought led to the enrichment of oligotrophic bacteria like Actinobacteria and to the depletion of sensitive copiotrophs like Acidobacteria, Bacteroidetes, Proteobacteria, Chloreflexi, Firmicutes, Gemmatimonodales, Plantomycetes, and Verrucomicrobia.

Actinobacteria have a high inherent drought tolerance due to their thick cell walls and their ability to produce osmolytes as part of their baseline metabolism (Niu et al. 2022; Santos-Medellín et al. 2017). Some of the depleted bacterial taxa, like members of the Proteobacteria phylum are generally enriched in the plant environment under normal conditions but can be depleted under drought conditions due to the direct effects of drought stress limiting reproduction and survival and due to plant responses to drought stress including the accumulation of ROS and other compounds that are harmful to sensitive bacteria (Mai-Prochnow et al. 2016).

Inoculation resulted in a depletion of Actinobacteria, Acidobacteria, and Chloreflexi, and an enrichment of Firmicutes, Proteobacteria, and Verrucomicrobia in the rhizosphere. In the roots, bacteria from several phyla were enriched including Acidobacteria, Bacteroidetes, Proteobacteria, Plantomycetes, Firmicutes, and Verrucomicrobia. The changes in the rhizosphere may be due to a combination of the AMF inoculant itself and changes to the soil properties brought about by the inoculation, for example, increases in soil organic matter and moisture and reductions in available phosphorus (Rillig and Mummey 2006; Duan et al. 1996; M. Qin et al. 2020). In the roots, bacteria from several phyla were enriched including Acidobacteria, Bacteroidetes, Proteobacteria, Plantomycetes, Firmicutes, and Verrucomicrobia. This was surprising and may be an indication that the plant reduced its immune response, allowing more bacteria to colonize the root compartment, or that the AMF enriched certain bacteria, including known AMF endobacteria like members of the Proteobacteria phylum (Jiang et al. 2021; Emmett, Lévesque-Tremblay, and Harrison 2021; Lastovetsky et al. 2024).

Drought reduced Ascomycota and Glomeromycota in the rhizosphere and Ascomycota, Glomeromycota, Morteirellomycota and Basidiomycota in the roots. This was surprising given that fungi are generally considered more resilient to drought than bacteria (Evans and Wallenstein 2012). However severe drought can have lasting impacts on fungal relative abundance and AMF (Vilonen et al. 2023; Fu et al. 2022).

Inoculation enriched Basidiomycota in the rhizosphere and Ascomycota, Basidiomycota and Glomeromycota in the roots. The broad enrichment of fungi in the roots was also observed as an increase in alpha-diversity in the inoculated compared to non-inoculated roots. This finding demonstrates that the addition of an AMF inoculant can result in other microbiome changes. The enrichment of non-symbiotic fungi in the inoculated treatment may explain some of the reductions in plant size we observed.

### 5.4 AMF inoculation transformed rhizosphere co-occurrence networks in low diversity soil and had marginal impacts in high diversity soil

Overall, the rhizosphere networks changed because of AMF inoculation and these changes were highly variable with soil type (Figure 3.6; S3.6). In the lowest diversity soil, ARDEC, AMF inoculation drastically transformed the network architecture. In higher diversity soils, there was a new module of highly positively correlated unknown fungi that had very little impact on the network as a whole. This suggests that in the ARDEC soil the inoculant changed the architecture of the rhizosphere communities whereas in the other soil types of AMF was present but not as instrumental in driving community changes. These observations could indicate that the addition of the AMF inoculant in the ARDEC soil filled a niche that was previously unoccupied, leading to transformative changes in network architecture. The ARDEC soil had a lower diversity of fungi, and likely also a lower diversity of AMF. It is expected that soils with a higher status of available nutrients would have lower AMF abundance as the plant does not need to invest in AMF associations for nutrient acquisition (Branco et al. 2022). Due to the higher initial fungal diversity in the WYO and Fruita soil, it is more likely that there was already a reasonably high diversity of AMF in the soil without inoculation. Therefore, the addition of the AMF inoculant resulted in fewer changes to the network as AMF were already functionally present in the system.

### 5.5 AMF nodes were hubs in root networks in the AMF inoculated treatments and the non-inoculated controls

In general, AMF were well connected and frequently appeared as possible keystone species or hubs within the root networks (Figure 3.7; S3.7). This was observed in both the inoculated and non-inoculated control treatments and was consistent across soil and drought treatments. This could indicate that AMF has correlations with several bacteria and other fungi in the roots, regardless of whether that AMF occurs natively in the soil or is added as an inoculant. AMF inoculation increased the number of AMF nodes in some but not all networks. Interestingly, the drought treatment had a greater impact on the prevalence of AMF in the networks than the inoculation treatment. This could indicate that the plant response to drought is a greater driver of AMF colonization than the abundance of AMF in the system. This finding is consistent with other studies that found that site had a greater impact on AMF communities than AMF inoculation treatments (Li et al. 2021). Plants are selective of their AMF partners and are the primary drivers of whether or not an AMF will colonize the root system (Chialva et al. 2024;

Frew, Heuck, and Aguilar-Trigueros 2023). Plants will often choose ideal AMF partners if available but will make compromises and form associations with non-ideal AMF partners if ideal partners are not available (Frew, Heuck, and Aguilar-Trigueros 2023; Kaur, Campbell, and Suseela 2022). Similarly, AMF have some discretion when choosing plant partners. If multiple plants are available, AMF will associate with plants that deliver the greatest amount of carbon to their rhizospheres but will colonize with non-ideal plant partners in monocultures (Faghihinia and Jansa 2022). Based on the reductions in plant size in the inoculated treatment compared to the non-inoculated control, the AMF inoculant strain may be a non-ideal partner for these wheat genotypes. The increases in AMF inoculation and plant fitness sacrifices were more pronounced in the low diversity soil, indicating that the high diversity soil may have had more ideal AMF partners present and were either able to selectively enrich ideal AMF partners, or had a higher diversity of AMF colonizing their roots, which has been shown to lead to greater outcomes for plant fitness (van der Heijden et al. 1998; Turrini et al. 2018).

### 5.6 Inoculation did not increase AMF root colonization

Our results showed substantial impacts of AMF inoculation on the plant growth parameters (Figure 3.1; Table 3.1), soil properties (Figure 3.2; Table 3.2), and soil enzymatic activities (Figure 3.3; Table 3.3). In addition, we also observed a significant effect of AMF inoculation on the diversity (Figure 3.4; Table 3.4), composition (Figure 3.5; Table 3.5), and co-existence networks of plant-associated microbiota (Figure 3.6; Table 3.6). In fact, in many instances, AMF inoculation significantly impacts the measured variables more than soil type—for example tiller number, chlorophyll, plant biomass, bacterial alpha-diversity of the rhizosphere and fungal alpha-diversity of the root—or drought treatments—for example aboveground biomass, chlorophyll, tiller number, alpha-diversity of the rhizosphere, and beta-diversity of the rhizosphere and roots. We therefore expected to see differences in the AMF colonization and increased abundance of *R. irregularis* in inoculated vs non-inoculated treatments. However, our analysis using multiple approaches, including qPCR and microscopy, didn't reveal any such increments (S3.2). This mismatch between the effect of AMF inoculation on soil, plant, and microbial variables and the colonization potential of the inoculant can be mediated by various factors. For example, the competition between the native microbiota and the inoculant can significantly impact the establishment of the inoculant. A recent study has suggested that the establishment success is insufficient to explain the impact of AMF on plant and soil variables (Lutz et al. 2023). In fact, the major predictors of AMF colonization and their effects of plants were the community composition of native microbiota (Lutz et al. 2023). Svenningsen et al. (2018) have also reported microbial-mediated suppression of AMF colonization. Plant-based selection of ideal AMF partners could also be one of the reasons for the non-establishment of the AMF inoculant (Carbonnel and Gutjahr 2014; Kaur, Campbell, and Suseela 2022). Plants can also induce an enhanced defensive strategy known as mycorrhiza-induced resistance (Cameron et al. 2013) that limits the colonization by introduced species. As plant defense response also plays a critical role in sculpting plant-associated microbiota (Hacquard et al. 2017; Trivedi et al. 2020; Lebeis et al. 2015), we postulate that the immune response plays a vital role in influencing the AMF-mediated impact on plant-associated microbiota. In addition, the variable quality of the commercial inoculant could also lead to inconsistent colonization (Salomon et al. 2022) that limits the colonization by introduced species. As plant defense responses also play a critical role in sculpting plant-associated microbiota

(Trivedi et al. 2020) we postulate that plant immune responses may also play a vital role in influencing the AMF-mediated impact on plant associated microbiota.

## 6. Conclusion

Plant microbiomes are complex and multifaceted systems. Inoculation by a single AMF strain may result in different effects on plant phenotype and the plant microbiome given different initial soil microbiomes and different environmental conditions, like drought. Our findings highlight the complexity of the plant microbiome and indicate a need for greater mechanistic studies into multi-kingdom interactions between the plant and its associated bacterial and fungal communities.

# CONCLUSIONS

In this dissertation, we sought to improve our understanding of the community composition, formation, and patterns of multi-kingdom co-occurrence of plant microbiomes under drought stress. In chapter 1, we demonstrated that water deficit conditions in the field have drastic impacts on the bacterial, fungal, and protist communities of plant rhizospheres, and provide evidence that protists are important regulators of rhizosphere communities. In chapter 2, we examined the impact of drought on microbial community composition and patterns of co-occurrence, the ecological processes governing assembly, and source-sink patterns across multiple plant compartments in the field. We found that drought not only drives compositional changes throughout the plant, but also alters the dominant assembly processes driving microbial community formation, and the path by which microbes colonize plant compartments. In chapter 3, we tested the impact of AMF inoculation, drought, and unique preexisting bulk soil microbial communities in the greenhouse on the soil, plant fitness, and rhizosphere and root microbiome community and patterns of co-occurrence. We found that AMF inoculation did not improve plant fitness under these circumstances and had vastly different impacts on the plant microbiome depending on the starting soil community and drought treatment. Overall, this dissertation demonstrates the importance of microbial interactions and drought on plant-microbiome communities, by showing that environmental conditions can significantly alter plant microbiomes, and that microbial inoculants can have very different impacts on the host and the microbiome depending on stress and the preexisting microbial community of the system. Further, we demonstrate the value of considering multiple microbial kingdoms and cross-kingdom interactions in plant-microbiome research.

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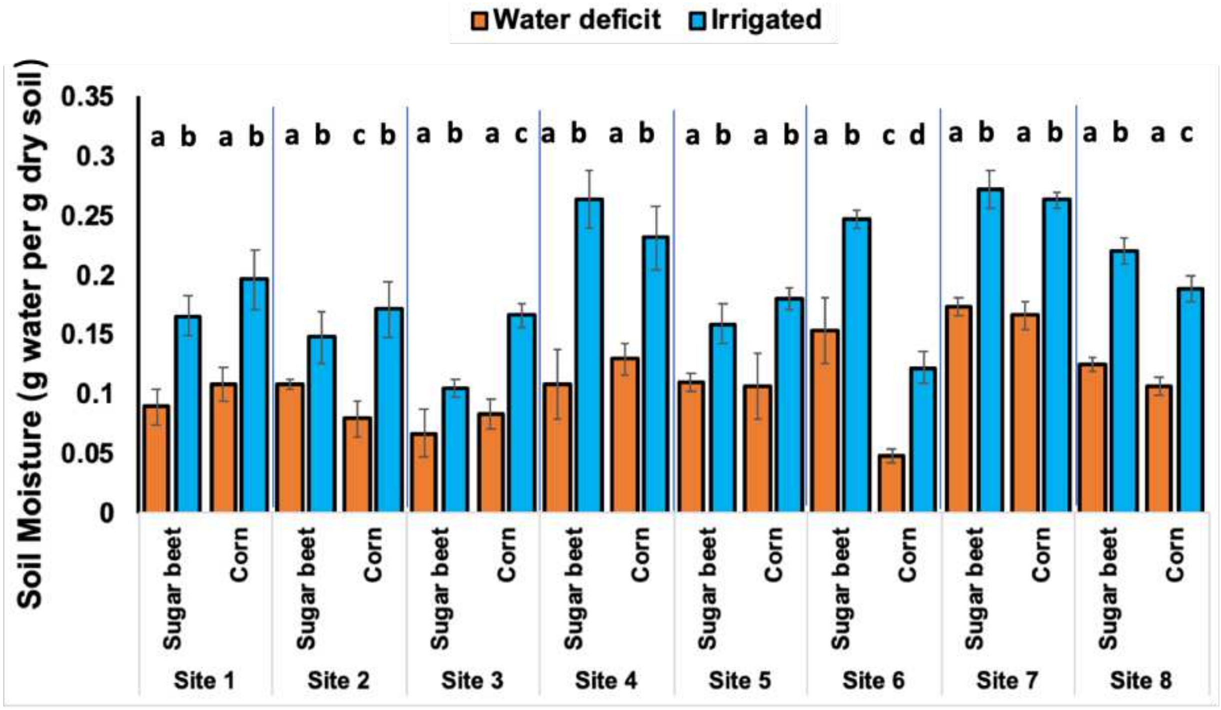
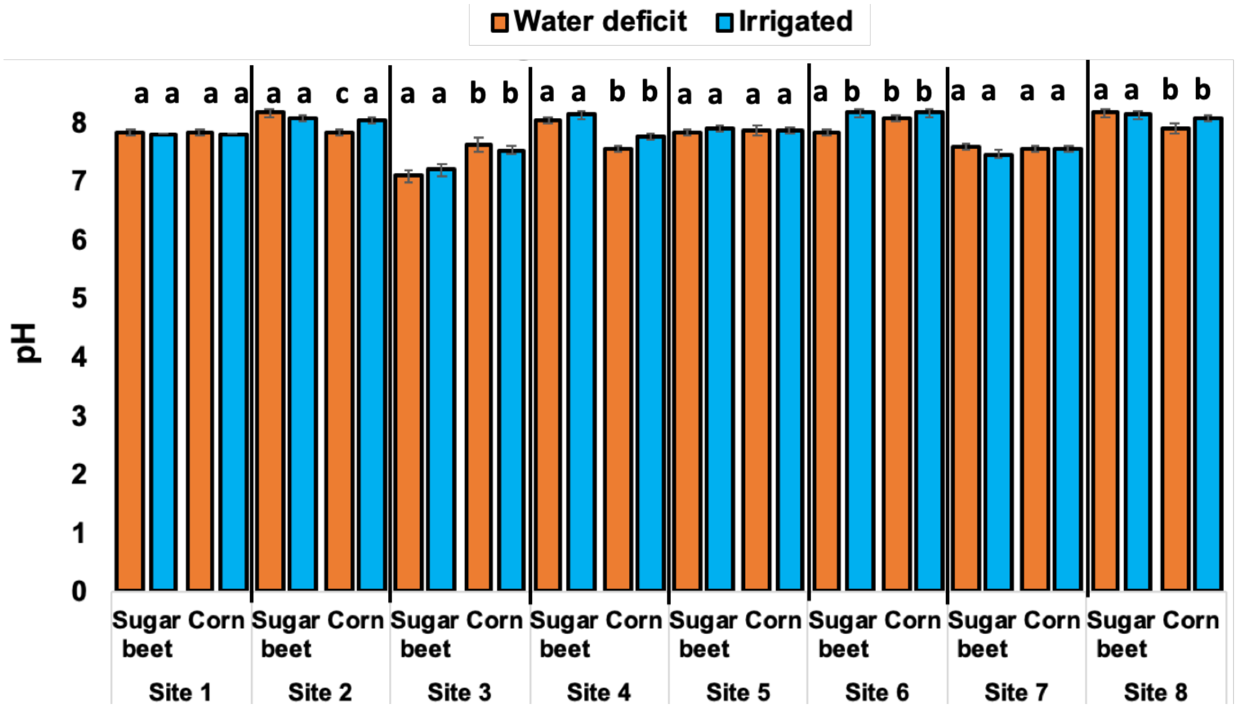
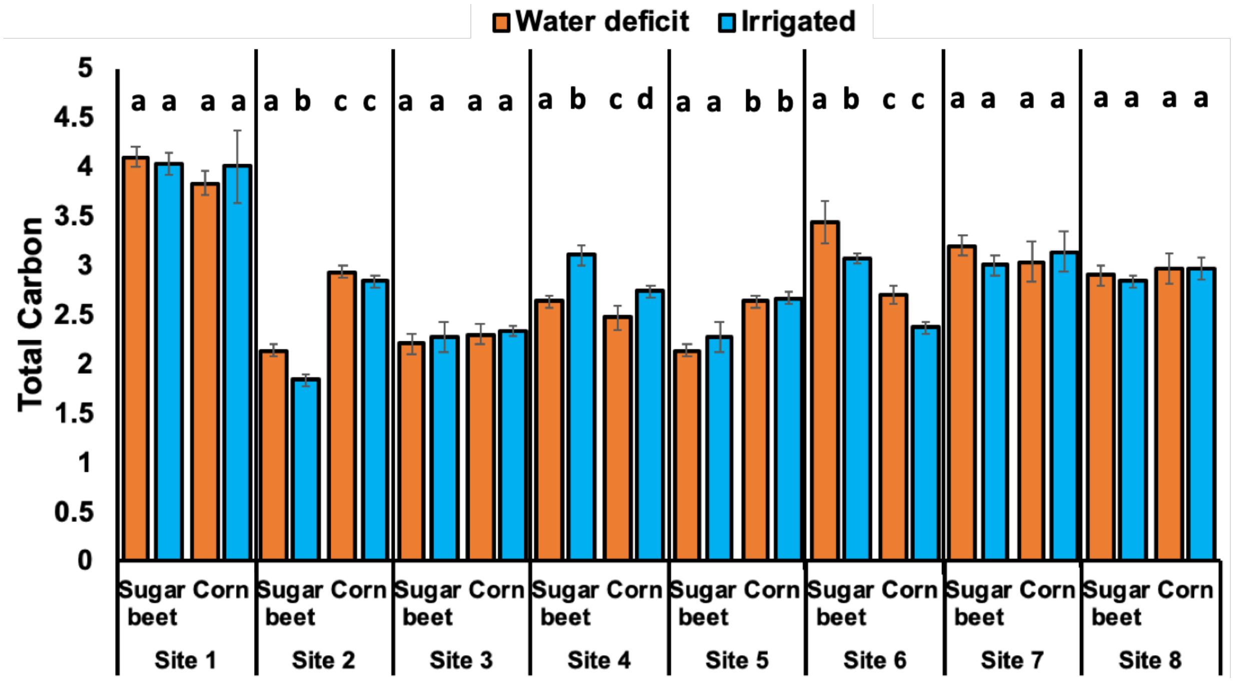
APPENDIX 1 CHAPTER 1



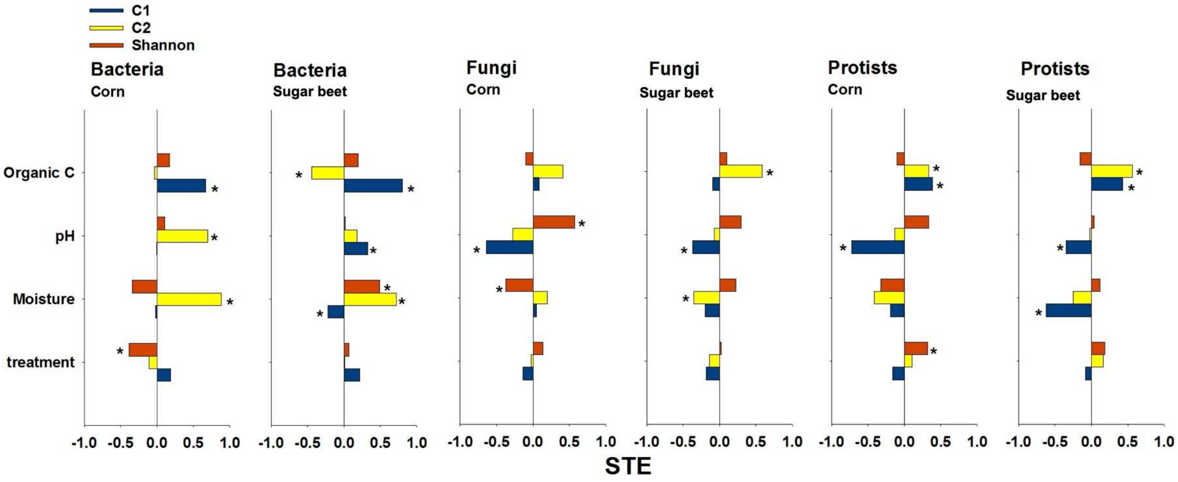
**S1.1:** Differences in soil volumetric water content (VWC, %) between irrigated (blue) and water deficit (brown) treatments for sugar beet and corn at different sites. Different letters mean statistically significant differences at $p < 0.05$ within each site. Error bars indicate Standard error.



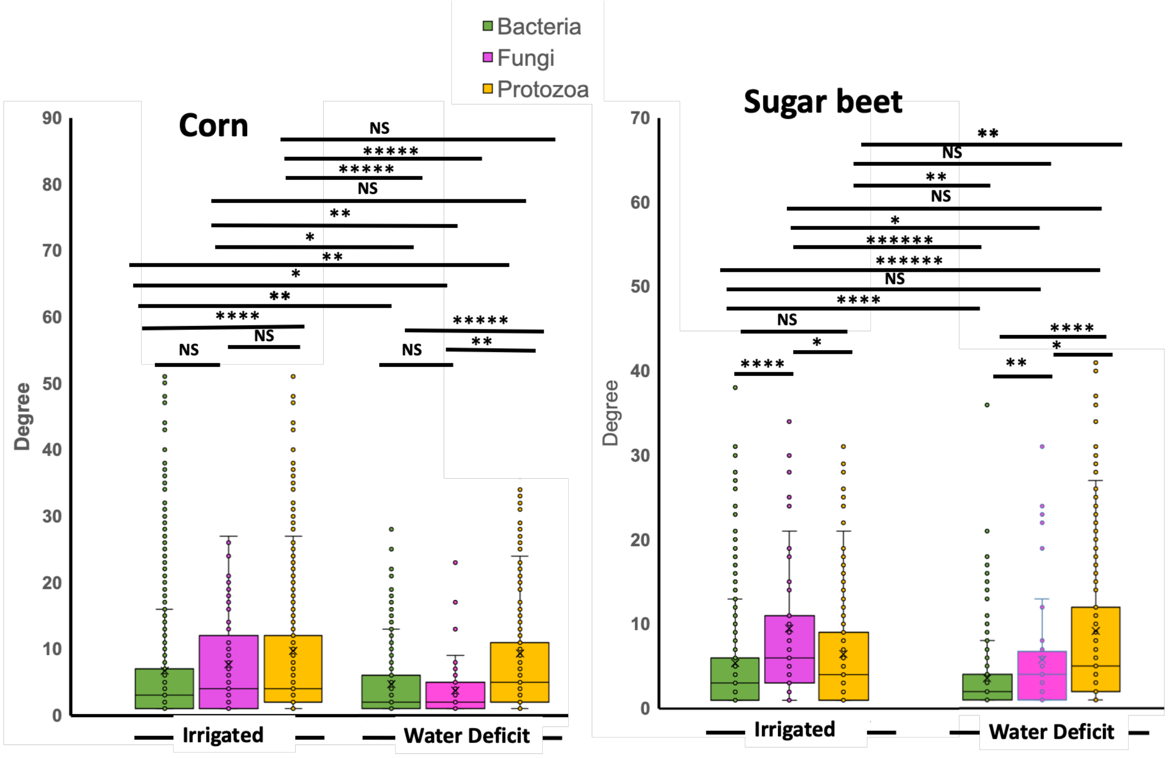
**S1.2:** Differences in soil pH between irrigated (blue) and water deficit (brown) treatments for sugar beet and corn at different sites. Different letters mean statistically significant differences at $p < 0.05$ within each site. Error bars indicate Standard error.



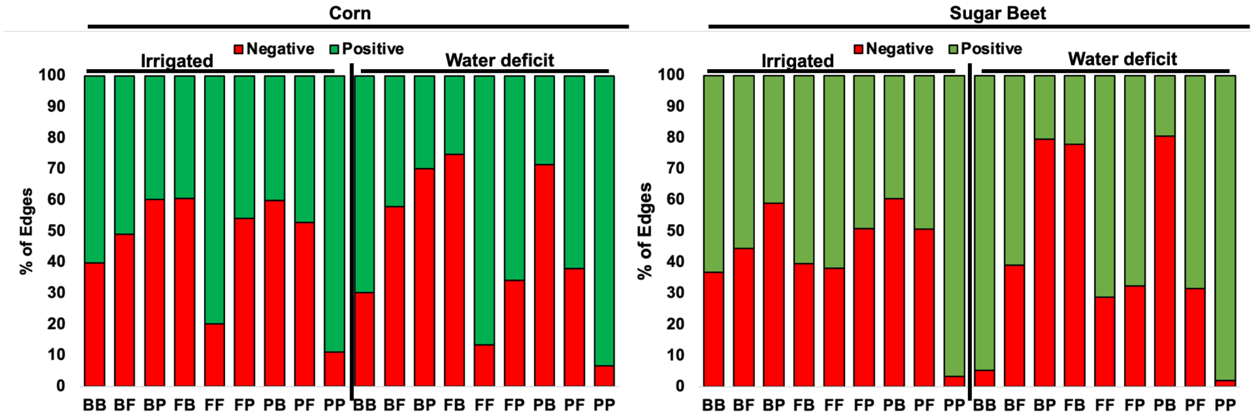
**S1. 3:** Differences in total carbon between irrigated (blue) and water deficit (brown) treatments for sugar beet and corn at different sites. Different letters mean statistically significant differences at $p < 0.05$ within each site. Error bars indicate Standard error.



**S1. 4:** Standardized total effects (STE) showing direct plus indirect effects of organic C, pH, soil moisture and treatment on the community composition (C1 and C2) and Shannon diversity of bacteria, fungi, and protist in the rhizosphere of corn and sugar beet. STEs are derived from the structural equation model presented in Fig. 2.



**S1.5:** Degree values of bacteria, fungi, and protist taxa in irrigated and water deficit networks for corn and sugar beet. The significance of difference was determined by nonparametric Kruskal–Wallis test. *, **, ***, ****, ***** represent significance at *p* values of < 0.05, 0.005, 0.0005, 0.00005, and 0.000005, respectively. NS = non-significant.



**S1.6:** Percentage of edges showing positive (green) or negative (red) correlations in the irrigated and water deficit microbial rhizosphere network of corn and sugar beet. B, bacteria; F, fungi; P, protists.

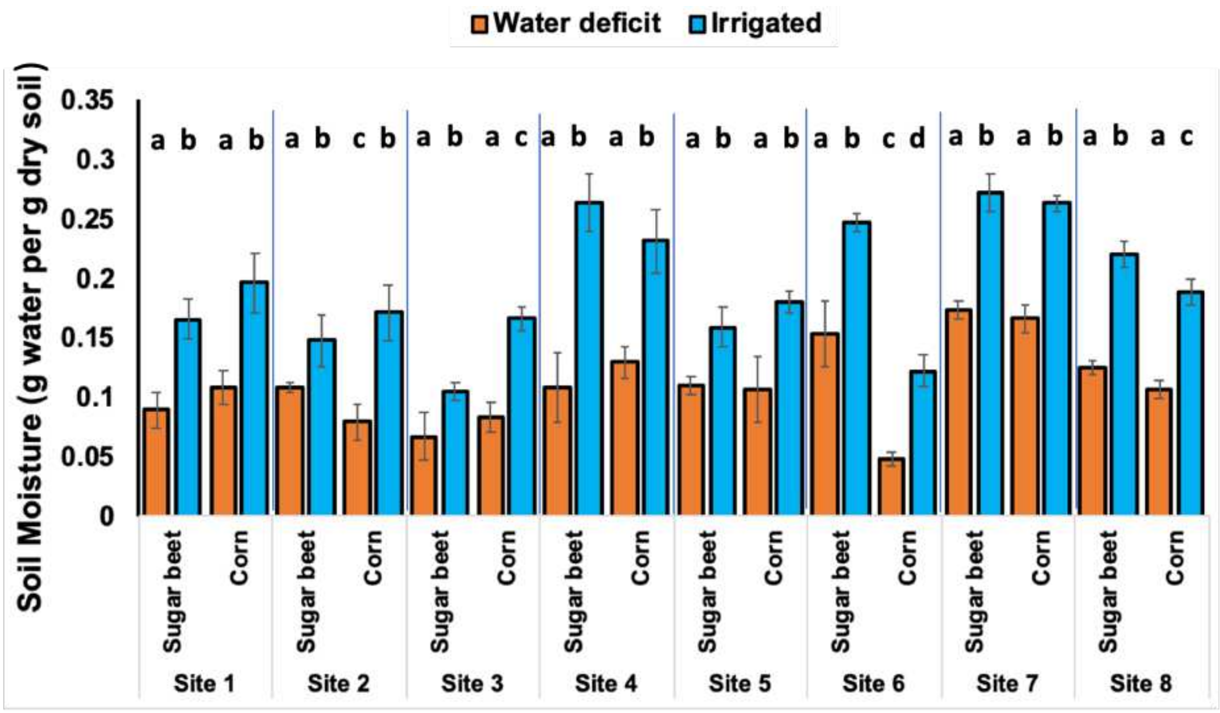
Supplementary Table 1. Site information.

| **Site ID** | **State** | **Latitude** | **Longitude** |
|---|---|---|---|
| NE 4 | Nebraska | 42.278668 | -102.911604 |
| NE 7 | Nebraska | 41.843778 | -103.595356 |
| CO 1 | Colorado | 40.638932 | -105.015802 |
| CO 3 | Colorado | 40.30281 | -104.925196 |
| CO 8 | Colorado | 40.24657 | -104.099522 |
| CO 10 | Colorado | 40.287572 | -103.641678 |
| MT 1 | Montana | 45.43407 | -108.88046 |
| MT 2 | Montana | 45.72424 | -108.63317 |

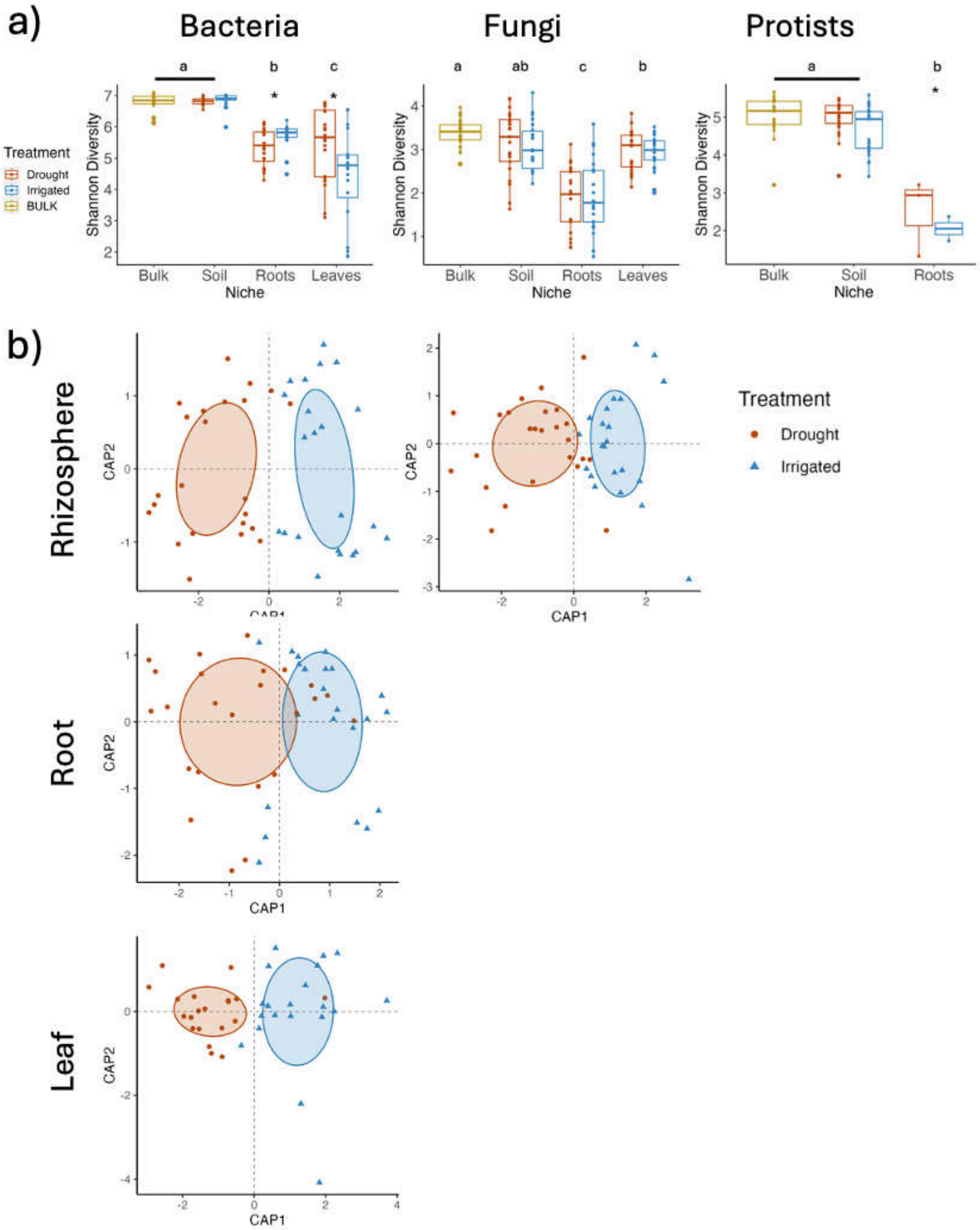
# APPENDIX 2 CHAPTER 2

| Site ID | State | Latitude | Longitude |
|---|---|---|---|
| NE 4 | Nebraska | 42.278668 | -102.911604 |
| NE 7 | Nebraska | 41.843778 | -103.595356 |
| CO 1 | Colorado | 40.638932 | -105.015802 |
| CO 3 | Colorado | 40.30281 | -104.925196 |
| CO 8 | Colorado | 40.24657 | -104.099522 |
| CO 10 | Colorado | 40.287572 | -103.641678 |
| MT 1 | Montana | 45.43407 | -108.88046 |
| MT 2 | Montana | 45.72424 | -108.63317 |

**S2.1:** Information on the sites including a Site ID, the State, Latitude and Longitude. Each site had a Corn field with a directly adjacent Sugar Beet field watered with central-pivot irrigation.



**S2.2:** Differences in soil volumetric water content (VWC, %) between irrigated (blue) and water deficit (brown) treatments for sugar beet and corn at different sites. Different letters mean statistically significant differences at $p < 0.05$ within each site. Error bars indicate Standard error.


a)
Bacteria
Fungi
Protists
a
b
c
ab
*
Shannon Diversity
Niche
Bulk
Soil
Roots
Leaves
Treatment
Drought
Irrigated
BULK
b)
Rhizosphere
Root
Leaf
CAP1
CAP2
Treatment
Drought
Irrigated

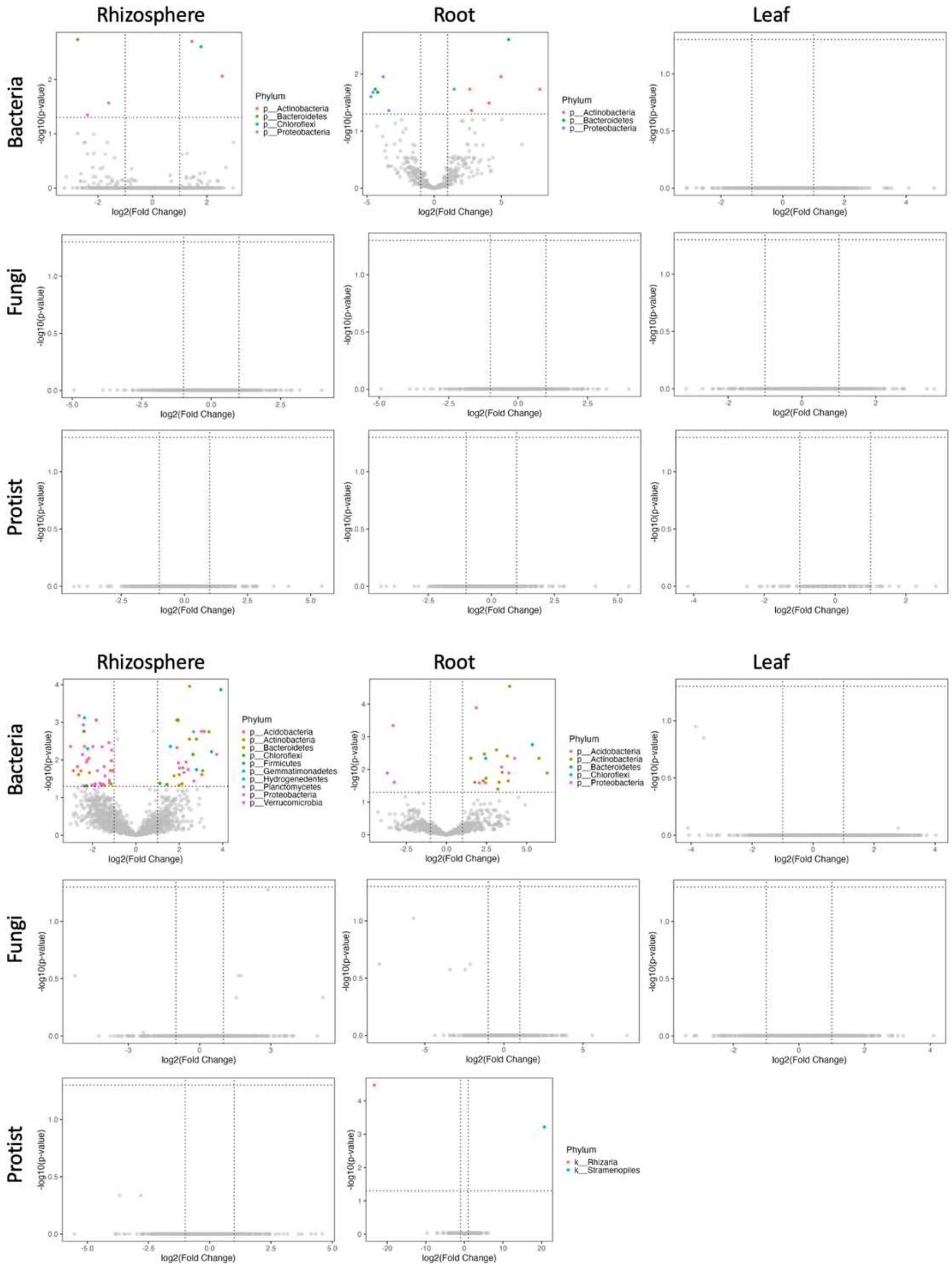
**S2.3:** Alpha and beta-diversity of Sugar Beet. (a) The alpha diversity (Shannon-Index) of the different niche compartments—bulk (bulk soil), soil (rhizosphere soil), roots, and leaves—and the different irrigation treatments (drought and irrigated) for bacteria, fungi, and protists. Asterix indicated significance ($p < 0.05$) by Tukey HSD test between the irrigation treatments. Letters indicate the significance among the various niche compartments. (b) CAPs ordinations showing the beta-diversity of the drought and irrigated treatments for bacteria, fungi, and protists (left to right) within the rhizosphere, root, and leaf compartments (top to bottom). Missing CAPs diagrams for the fungal root and leaf and each protist community are due to insufficient data to perform the analysis.

| | Bacteria | | Fungi | | Protist | |
|---|---|---|---|---|---|---|
| Effect | F-statistic | p-value | F-statistic | p-value | F-statistic | p-value |
| Site | 11.636 | < 0.001 ** | 3.693 | 0.001 ** | 3.855 | 0.003 * |
| Watering | 4.603 | 0.0340 * | 0.121 | 0.728 | 8.504 | 0.006 * |
| Niche | 249.738 | < 0.001 *** | 80.494 | < 0.001 *** | 99.400 | < 0.001 *** |
| Site:Watering | 7.377 | < 0.001 *** | 0.687 | 0.683 | 1.457 | 0.209 |
| Site:Niche | 11.898 | < 0.001 *** | 4.616 | < 0.001 *** | 1.072 | 0.306 |
| Watering:Niche | 35.285 | < 0.001 *** | 0.480 | 0.620 | 0.090 | 0.765 |
| Site:Watering:Niche | 6.559 | < 0.001 *** | 0.961 | 0.497 | 1.4868 | 0.230 |

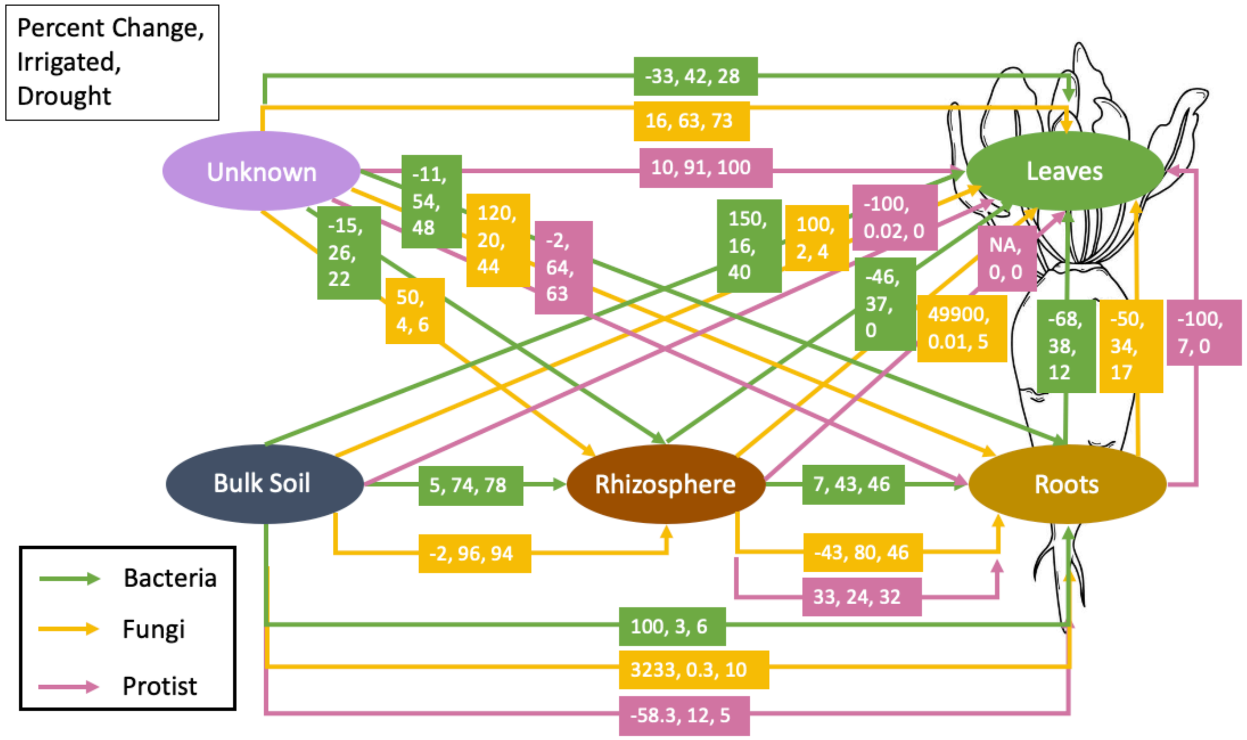
**S2.4:** Analysis of Variance (ANOVA) performed on general Linear Models (GLMs) of the Shannon Indexes for Sugar Beet for bacteria, fungi, and protists.

| | Bacteria | | Fungi | | Protist | |
|---|---|---|---|---|---|---|
| Effect | $R^2$ | p-value | $R^2$ | p-value | $R^2$ | p-value |
| Site | 0.15329479 | < 0.001 *** | 0.16240727 | < 0.001 *** | 0.30700389 | < 0.001 *** |
| Watering | 0.01789356 | < 0.001 *** | 0.01156073 | < 0.001 *** | 0.05072057 | < 0.001 *** |
| Niche | 0.22074993 | < 0.001 *** | 0.28131818 | < 0.001 *** | 0.05670263 | < 0.001 *** |
| Site:Watering | 0.04415864 | < 0.001 *** | 0.03650781 | < 0.001 *** | 0.1351187 | < 0.001 *** |
| Site:Niche | 0.17312223 | < 0.001 *** | 0.21182112 | < 0.001 *** | 0.025947 | < 0.001 *** |
| Watering:Niche | 0.02502659 | < 0.001 *** | 0.01152783 | 0.006 ** | 0.02146657 | 0.002 ** |
| Site:Watering:Niche | 0.07669902 | < 0.001 *** | 0.05037041 | 0.007 ** | 0.0250486 | < 0.001 *** |
| Residuals | 0.28905524 | | 0.23448665 | | 0.37799204 | |

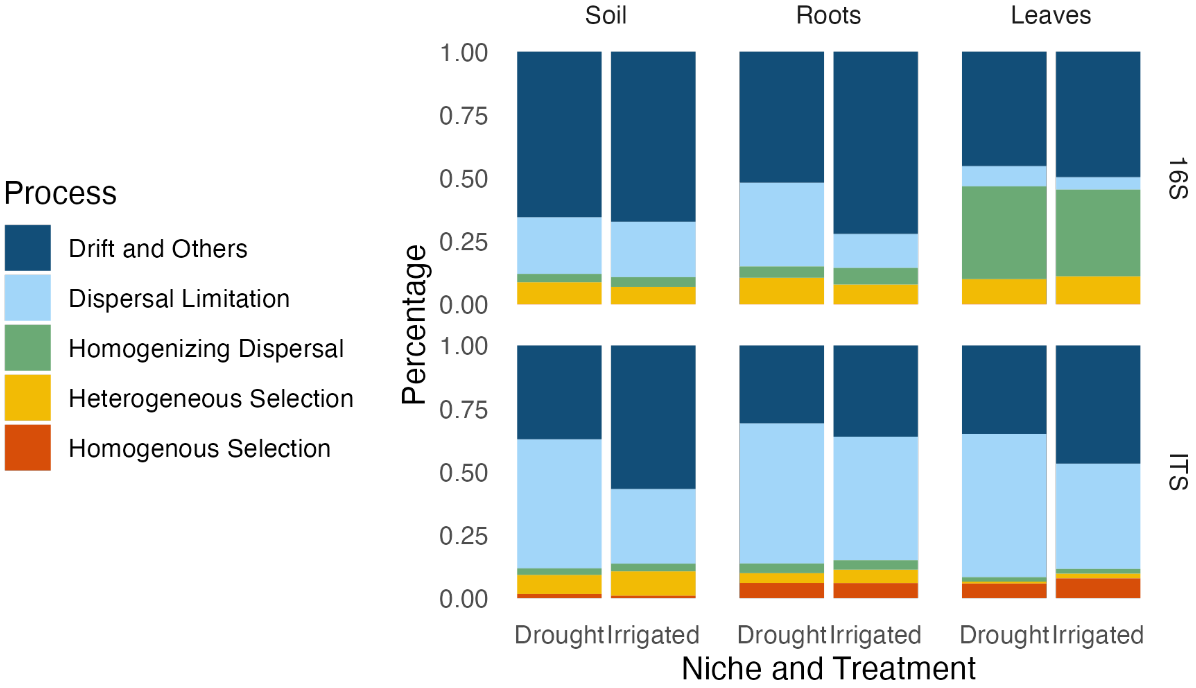
**S2.5:** Permutational Multivariate Analysis of Variance (PERMANOVA) on Sugar Beet for bacteria, fungi, and protists.



**S2.6:** Volcano plots for Corn (top nine) and Sugar Beet (bottom nine) within the rhizosphere, roots, and leaves (left to right), for bacteria, fungi, and protists (top to bottom). OTUs with a |log2(Fold Change)| > 1 and a p-value < 0.01 are considered significantly enriched (right) or depleted (left) in the drought treatement compared to the irrigated control and are color coded by phylum.



**S2.7:** FEAST source tracker analysis showing the path of microbial assembly for bacteria, fungi, and protists in Sugar Beet. Arrows indicated the proposed direction of microbial colonization from a source to a sink. The numbers indicate the percent change between treatments [(percent drought – percent irrigated) / percent irrigated], followed by the percent sourced in the irrigated treatment, followed by the percent sourced in the drought treatment.

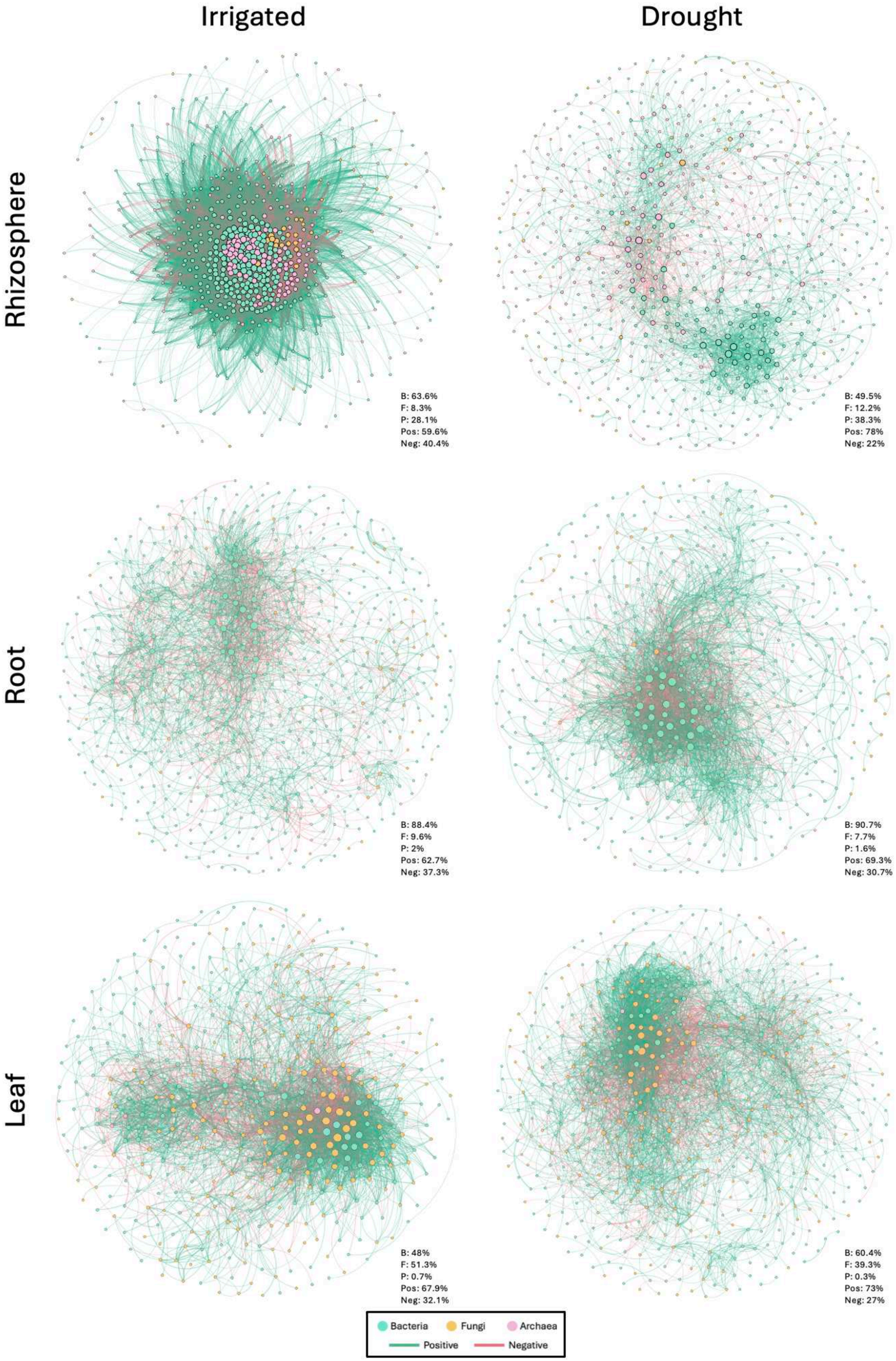


**S2.8:** Stacked bar charts representing the results of the iCAMP analysis in Sugar Beet to determine the prevalence of different microbial community assembly processes in the soil (rhizosphere soil), roots, and leaves (left to right), for the different microbial kingdoms 16S (bacteria), ITS (fungi), and 18S (protists), in the drought and irrigated treatments. The processes tested include stochastic processes (drift and others, dispersal limitation, and homogenizing dispersal) and deterministic processes (heterogeneous selection and homogenous selection).

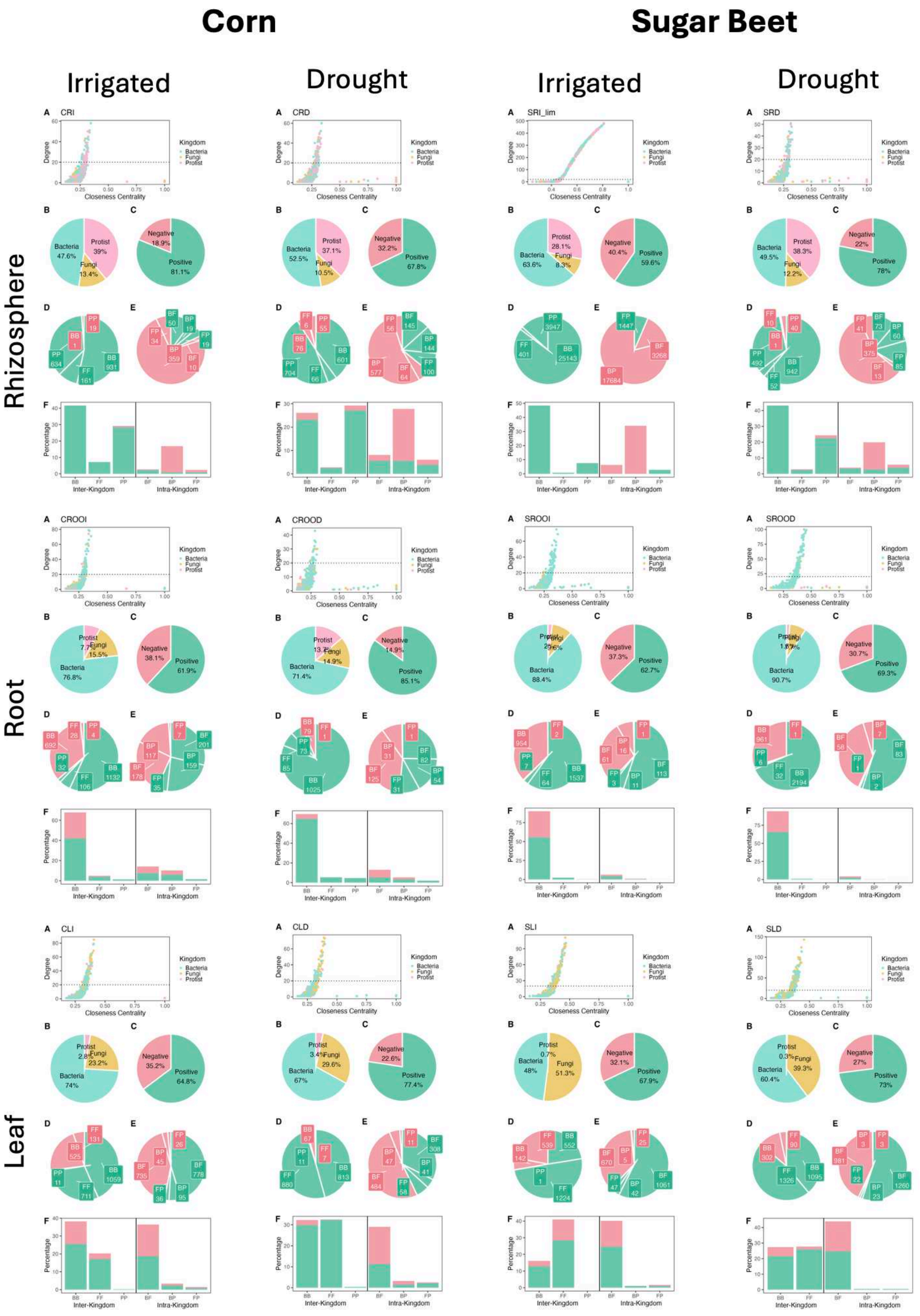
| Amplicon | Host Niche | Treatment | Heterogeneous Selection | Homogenous Selection | Dispersal Limitation | Homogenizing Dispersal | Drift and Others |
|---|---|---|---|---|---|---|---|
| 16S | Leaves | Drought | 0.0610113 | 0.00495825 | 0.19182825 | 0.10800376 | 0.63419844 |
| | | Irrigated | 0.08003124 | 0.00071453 | 0.11124927 | 0.09419057 | 0.71381439 |
| | Roots | Drought | 0.09659111 | 0.00039987 | 0.3642996 | 0.02224655 | 0.51646287 |
| | | Irrigated | 0.06114187 | 0.00024209 | 0.3383984 | 0.02375371 | 0.57646393 |
| | Soil | Drought | 0.08884 | 0.00035493 | 0.23383861 | 0.03754952 | 0.63941695 |
| | | Irrigated | 0.07620681 | 0.0001501 | 0.18491726 | 0.04434206 | 0.69438377 |
| ITS | Leaves | Drought | 0.02184693 | 0.05529846 | 0.38902804 | 0.03184323 | 0.50198335 |
| | | Irrigated | 0.0140428 | 0.07131973 | 0.36402052 | 0.01922318 | 0.53139377 |
| | Roots | Drought | 0.06519805 | 0.02539474 | 0.60204325 | 0.03273405 | 0.27462992 |
| | | Irrigated | 0.02975093 | 0.01781327 | 0.55113855 | 0.02315653 | 0.37814073 |
| | Soil | Drought | 0.12637094 | 0.0145517 | 0.36523442 | 0.02666437 | 0.46717857 |
| | | Irrigated | 0.09759984 | 0.02254332 | 0.3424836 | 0.03040263 | 0.50697061 |
| 18S | Soil | Drought | 0.13321504 | 0.00027591 | 0.7444346 | 0.00187371 | 0.12020075 |
| | | Irrigated | 0.10802645 | 0 | 0.68113913 | 0.00028553 | 0.21054888 |
| | Roots | Drought | 0.01964769 | 0.02926275 | 0.0143656 | 0.17617838 | 0.76054559 |
| | | Irrigated | 0.0102096 | 0.00415388 | 0.00244614 | 0.33994771 | 0.64324267 |
| | Leaves | Drought | 0.0002967 | 0.02891306 | 0 | 0.01853237 | 0.95225788 |
| | | Irrigated | 0.00046541 | 0.0467377 | 0 | 0.06969404 | 0.88310285 |

| Amplicon | Host Niche | Treatment | Heterogeneous Selection | Homogenous Selection | Dispersal Limitation | Homogenizing Dispersal | Drift and Others |
|---|---|---|---|---|---|---|---|
| 16S | Leaves | Drought | 0.09897838 | 0.00096108 | 0.07996851 | 0.36720024 | 0.45289179 |
| | | Irrigated | 0.11025426 | 0.00105484 | 0.04951341 | 0.34273258 | 0.49644492 |
| | Roots | Drought | 0.10502797 | 0.00024245 | 0.33126968 | 0.04489031 | 0.51856959 |
| | | Irrigated | 0.07823455 | 0.00038605 | 0.13465342 | 0.06540259 | 0.7213234 |
| | Soil | Drought | 0.08715609 | 0.00041726 | 0.22434996 | 0.03306872 | 0.65500796 |
| | | Irrigated | 0.06884582 | 0.00018307 | 0.21956218 | 0.03865745 | 0.67275148 |
| ITS | Leaves | Drought | 0.00764174 | 0.05809422 | 0.56668761 | 0.01766676 | 0.34990968 |
| | | Irrigated | 0.01894676 | 0.07846389 | 0.41682011 | 0.01841882 | 0.46735043 |
| | Roots | Drought | 0.0389602 | 0.06018535 | 0.55474713 | 0.03847019 | 0.30763714 |
| | | Irrigated | 0.05328862 | 0.05979803 | 0.4890336 | 0.03708398 | 0.36079578 |
| | Soil | Drought | 0.07580892 | 0.01655361 | 0.51097052 | 0.02587386 | 0.37079309 |
| | | Irrigated | 0.09692433 | 0.00948947 | 0.29562611 | 0.03033461 | 0.56762548 |

**S2.9:** Percentage (as decimals) of contribution of each community assembly process as determined by iCAMP for Corn (top) and Sugar Beet (bottom) for each amplicon 16S (bacteria), ITS (fungi), 18S (protists) in three niche compartments, leaves, roots, and soil, in the drought and irrigated treatments.


Irrigated
Drought
Rhizosphere
B: 63.6%
F: 8.3%
P: 28.1%
Pos: 59.6%
Neg: 40.4%
B: 49.5%
F: 12.2%
P: 38.3%
Pos: 78%
Neg: 22%
Root
B: 88.4%
F: 9.6%
P: 2%
Pos: 62.7%
Neg: 37.3%
B: 90.7%
F: 7.7%
P: 1.6%
Pos: 69.3%
Neg: 30.7%
Leaf
B: 48%
F: 51.3%
P: 0.7%
Pos: 67.9%
Neg: 32.1%
B: 60.4%
F: 39.3%
P: 0.3%
Pos: 73%
Neg: 27%
Bacteria
Fungi
Archaea
Positive
Negative

**S2.10:** Co-occurrence networks of the bacterial, fungal, and protistan communities of Sugar Beet in the irrigated and drought conditions (left to right) for the rhizosphere, root, and leaf compartments (top to bottom). Green points represent bacterial nodes, yellow points represent fungal nodes, and pink points represent fungal nodes. Green lines represent positive correlations between nodes and pink lines represent negative correlations between nodes. The percents at the bottom right of each network show the percent of bacterial (B), fungal (F), and protistan (P) nodes in each network and the percent of positive (pos) and negative (neg) edges. Correlations were performed in SparCC on OTUs with at least 150 reads in the total dataset. Correlation cutoffs were adjusted based on niche compartment to allow for clearer comparison between irrigation treatments. For the rhizosphere networks we kept correlations where |SparCC| > 0.7, for the roots |SparCC| > 0.65, and the leaves |SparCC| > 0.5.



**S2.11:** Analysis of the co-occurrence networks for Corn (left) and Sugar Beet (right) in the irrigated and drought treatments (left and right) in the rhizosphere, root, and leaf compartments (top to bottom). Within each subset, (a) scatterplot with the degree vs. closeness centrality, where high degree (> 20) and high closeness centrality indicate possible network hubs or keystone species, (b) pie chart with the percentages of each type of node (bacterial, fungal, or protistan) within each network. (c) pie chart with the percentages of positive and negative edges, (d) pie chart with the number of positive (green) and negative (pink) inter-kingdom edges in the network, (e) pie chart with the number of positive (green) and negative (pink) intra-kingdom edges in the network, and (f) bar chart with the percentages of positive (green) and negative (pink) inter-kingdom and intra-kingdom edges between bacterial (B), fungal (F), and protistan (P) OTUs.

### **S2.12:** Library Preparation:

50 uL PCR reactions were performed for each amplicon. For soil the 16S mix was 25 uL Platinum hotstart 2x, 4 uL of mixed forward and reverse barcoded primers, 4 uL of DNA, and 22 uL of nuclease-free water, and the ITS and 18S mixes were 25 uL Platinum hotstart 2x, 4 uL of mixed forward and reverse barcoded primers, 6 uL of DNA, and 20 uL of nuclease-free water. For the plant tissue the 16S mix was 20 uL of Platinum hotstart 2x, 4 uL of mixed forward and reverse barcoded primers, 4 uL of DNA, 17 uL of nuclease free water, 2.5 uL of pPNA, 2.5 uL of mPNA, both with a concentration of 10 uM. pPNA is a sequence specific peptide nucleic acid for preventing the amplification of chloroplast DNA and mPNA prevents the amplification of mitochondrial DNA. For ITS, the mix was 20 uL of Platinum hotstart 2x, 4 uL of F and R barcoded primers, 6 uL of DNA, 20 uL of nuclease-free water. For 18S, the mix was 20 uL of Platinum hotstart 2x, 4 uL of F and R barcoded primers, 6 uL of DNA, 12.5 uL of water, 7.5 uL of PNA at 10 uM. The PNA sequence was designed to block the amplification of plant host DNA, particularly wheat (Taerum et al. 2020). All amplicons were amplified by heating to 94 degrees Celsius for 2 minutes, followed by 20 replications of 94 degrees for 45 seconds, 54 degrees for 1 minute, 72 degrees for 1 minute 30 seconds, followed by 72 degrees for 5 minutes. All libraries were sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, California) at the Next Generation Sequencing Facilities at Colorado State University.

### **S2.13:** Statistical Analysis:

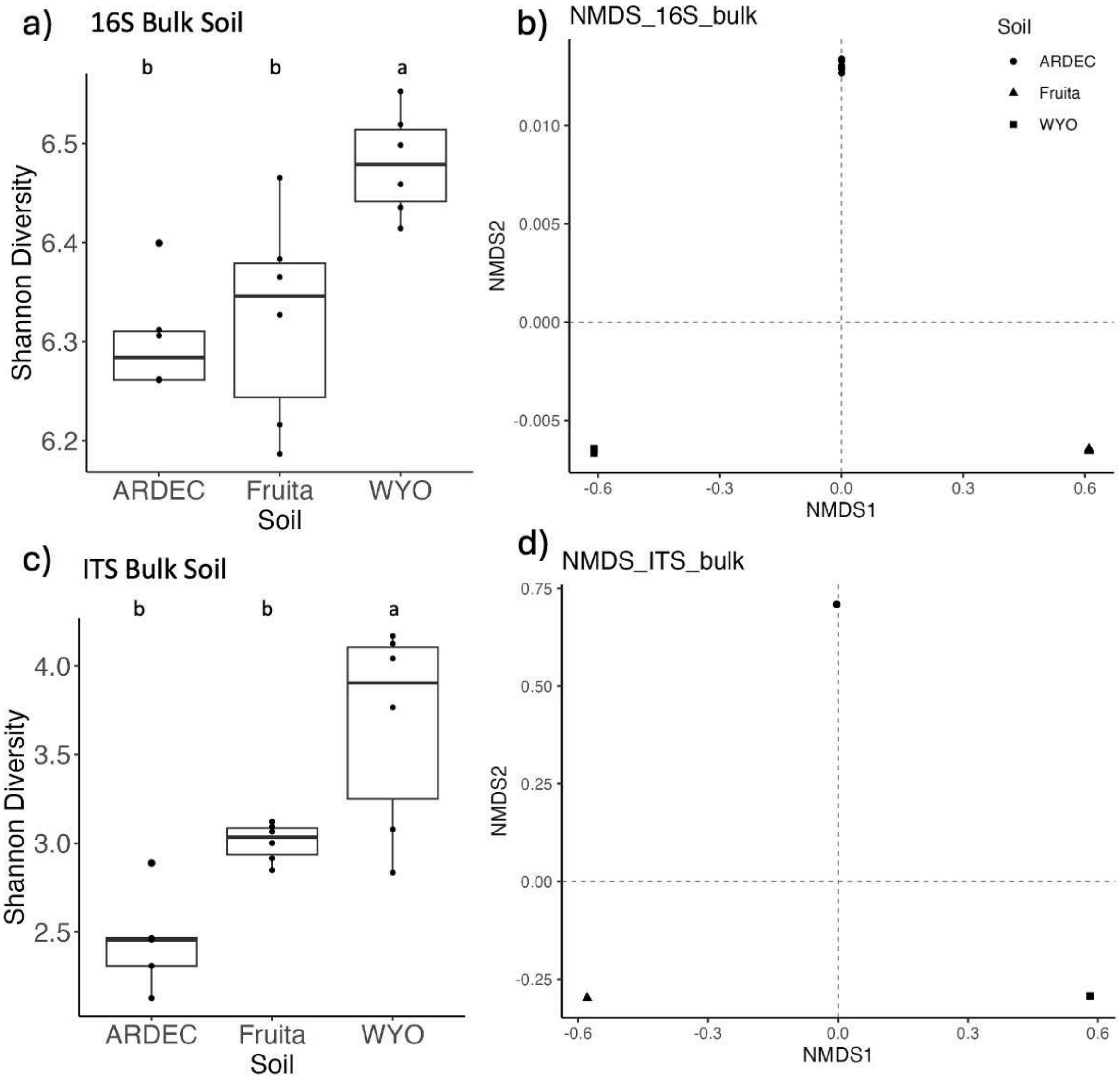
To determine the indicator taxa enriched or depleted under water deficit conditions, we performed Linear Discriminant Analysis Effect Size (LefSe) using the Huttenhower Lab Galaxy portal (Segata et al. 2011). Taxa with a Log10 LDA score < -2 were considered drought indicators, and taxa with a logarithmic LDA score > 2 were considered indicator taxa for the irrigated treatment. The alpha value for the factorial Kruskal-Wallis tests among classes was set at 0.05.

Source tracker analyses were performed using Fast Expectation Microbial Source Tracking (FEAST) (Shenhav et al. 2019) to determine the proportion of the different potential sources of microbes of each given sink environment. Like Source Tracker, FEAST assumes each sink is a convex combination of sources but infers the model parameters via fast expectation-maximization, which is much more scalable than Markov Chain Monte Carlo used by SourceTracker (X. Wang et al. 2023). We aimed to determine the impact of irrigation treatment on the relative importance of each source in determining the microbial community of each sink for bacteria, fungi, and protists. We examined bulk soil as a source for rhizosphere communities, bulk soil and rhizosphere soil as a source for the roots, and bulk soil, rhizosphere soil, and roots as sources for the leaf communities. Unknown sources include microbes from any other source not accounted for in the study including airborne microbes, microbes transferred by insects, and the seed microbiome.

The iCAMP package (community assembly mechanisms by phylogenetic bin-based null model analysis), developed by (Ning et al. 2020), was used to determine the relative importance of community assembly processes. Phylogenetic trees were generated for the 16S, ITS, and 18S reads. Sequences were manually filtered so that only ASVs with at least 300 total reads that were present in at least 20% of samples were included in the phylogenetic tree. This data subset was then aligned with Muscle (R. C. Edgar 2004). Trees were generated with IQ-TREE 2 (Minh et al. 2020). iCAMP analysis was performed on each phylogenetic tree to evaluates the importance of homogenous selection (HoS), heterogeneous selection (HeS), homogenizing dispersal (HD), dispersal limitation (DL), and drift and others (DR) based on beta nearest taxon index (bNTI) and a modified Raup-Crick metric (RC). Deterministic processes were dominant in any given phylogenetic bin where the absolute value of the bNTI was greater than one and stochastic processes were dominant when the absolute value of the bNTI was less than one. From the predominantly deterministic bins, HoS was considered dominant if the bNTI was less than -1.96, and HeS was considered dominant if bNTI was greater than 1.96. In the primarily stochastic bins, HD was considered dominant if the RC was less than -0.95, DL was considered dominant if the RC was greater than 0.95, and DR was considered dominant if the RC was between -0.95 and 0.95. We examined the differences in the relative importance of these processes in determining the bacterial, fungal, and protist communities associated with the different microhabitats of Corn and Sugar Beet under drought stressed and irrigated plants.

Co-occurrence networks were developed based on correlations among and between bacterial, fungal, and protistan OTUs with greater than 20 reads present in the data subset. The SparCC correlations were calculated using Python 3 (Friedman and Alm 2012). The SparCC correlations between every OTU were calculated 100 times. We evaluated the two-sided p-values for each of these 100 datasets with 100 bootstraps. Strong ($r > 0.70$) and robust ($p < 0.01$) correlations were selected and visualized in Gephi (Bastian, Heymann, and Jacomy 2009).

APPENDIX 3 CHAPTER 3



**e)**

| | | 16S | | ITS | |
|---|---|---|---|---|---|
| Term | df | F stat | *p* | F stat | *p* |
| Soil | 2 | 10.17 | ** | 14.17 | *** |
| Residuals | 14 | | | | |

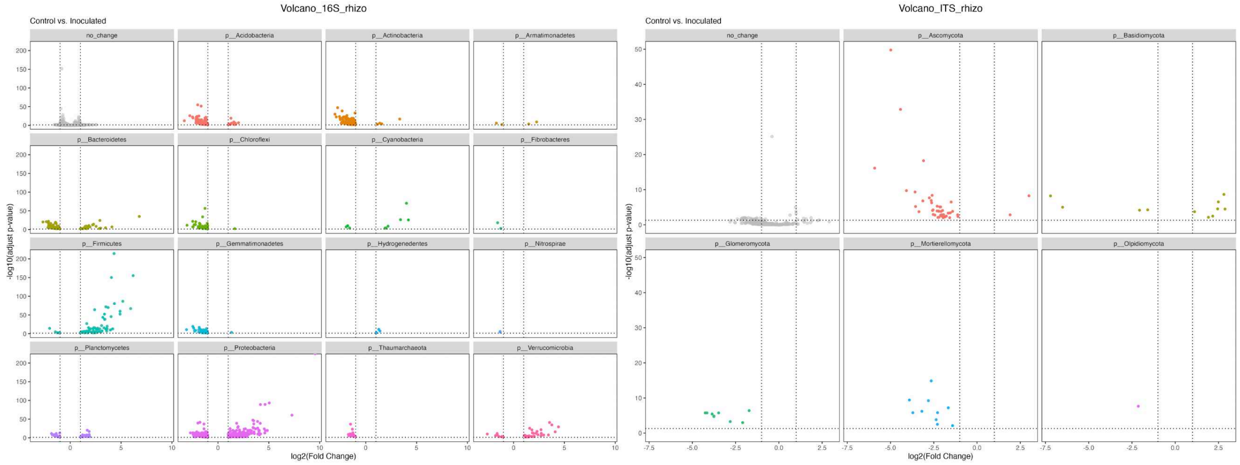
* p < .05, ** p < .01, *** p < .001

**f)**

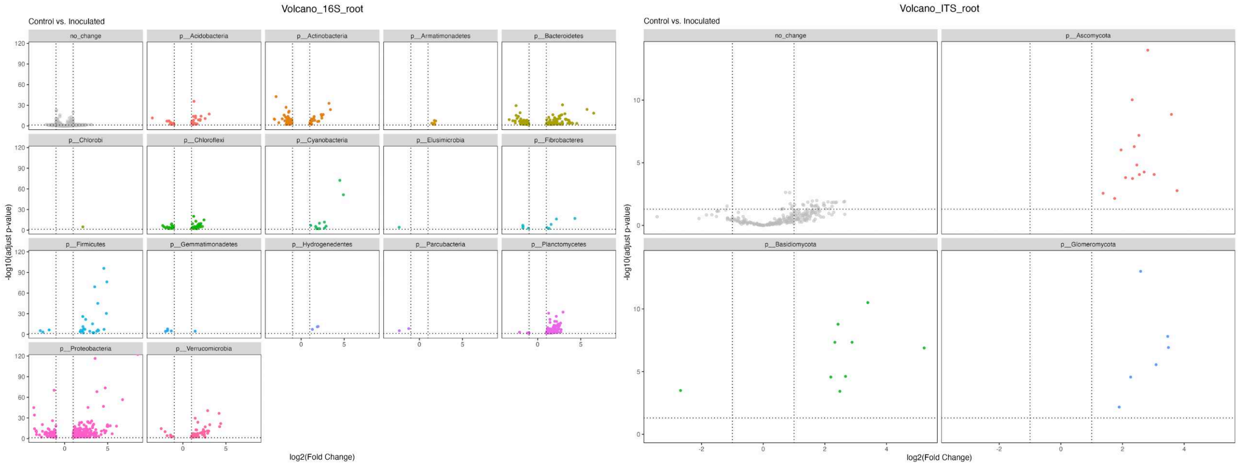
| | | 16S | | ITS | |
|---|---|---|---|---|---|
| Term | *df* | *R²* | *p* | *R²* | *p* |
| Soil | 2 | 0.7 | *** | 0.68 | *** |
| Residual | 15 | 0.3 | | 0.32 | |
| Total | 17 | 1 | | 1 | |

Note. * p < .05, ** p < .01, *** p < ***

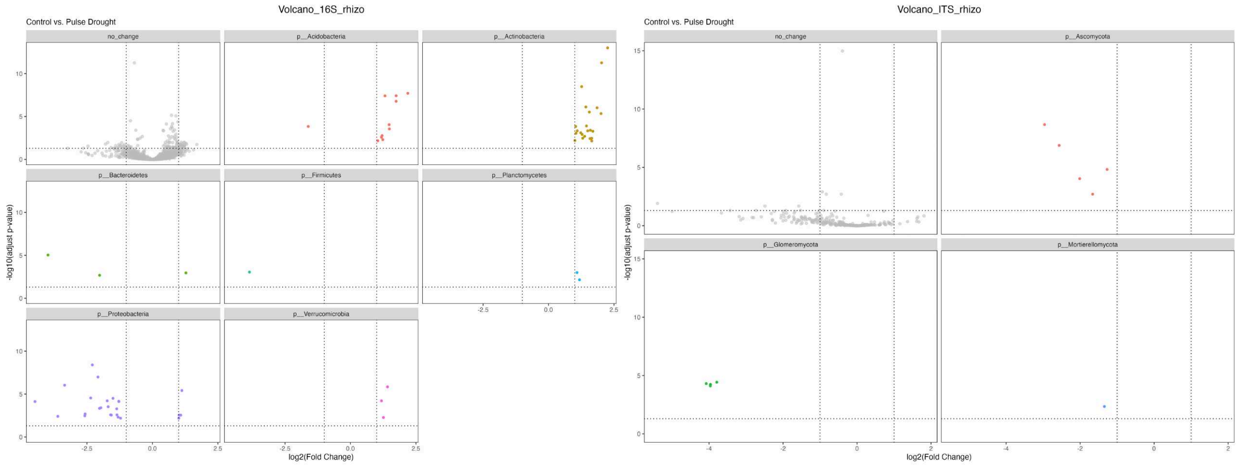
**S3.1:** Soil treatment bacterial and fungal biodiversity as determined by 16S and ITS amplicon sequencing. (a) bacterial Shannon-diversity of the soil types with letters representing significant differences as determined by Tukey HSD test, (b) NMDS of the bacteria, (c) fungal Shannon-diversity, (d) NMDS of the fungi, (e) ANOVA performed on the GLM of the bacterial and fungal Shannon diversity, (f) PERMANOVA performed on the bacterial and fungal libraries.



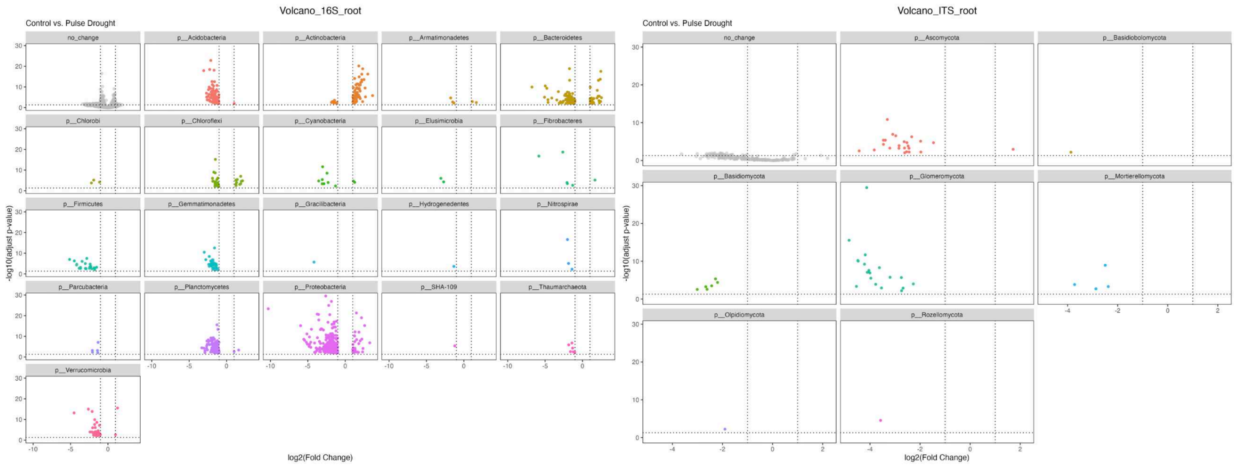
**S3.2:** Volcano plots examining patterns of relative abundance in the rhizosphere for the 16S sequencing (left) and ITS sequencing (right). OTUs with a |log2(foldchange)| > 1 and a p-value < 0.01 are shown in color. Plots are faceted by phylum. Colored points on the left side of each subplot represent OTUs with a higher relative abundance in the control treatment compared to the AMF inoculated treatment while OTUs on the right side of each subplot have a higher relative abundance in the AMF inoculated treatment compared to the control.



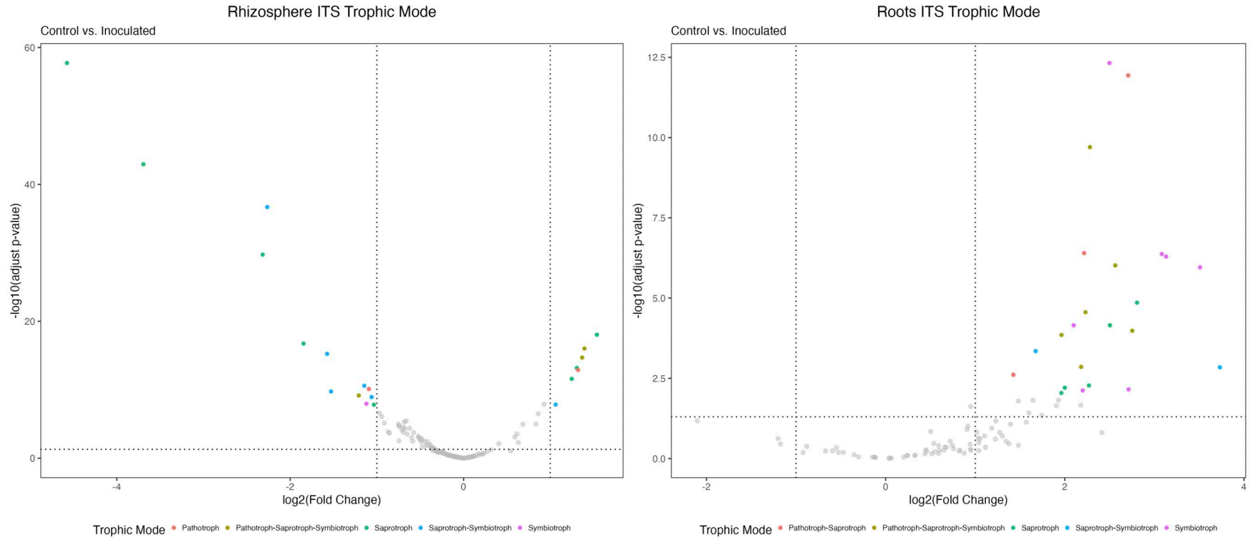
**S3.3:** Volcano plots examining patterns of relative abundance in the roots for the 16S sequencing (left) and ITS sequencing (right). OTUs with a |log2(foldchange)| > 1 and a p-value < 0.01 are shown in color. Plots are faceted by phylum. Colored points on the left side of each subplot represent OTUs with a higher relative abundance in the control treatment compared to the AMF inoculated treatment while OTUs on the right side of each subplot have a higher relative abundance in the AMF inoculated treatment compared to the control.



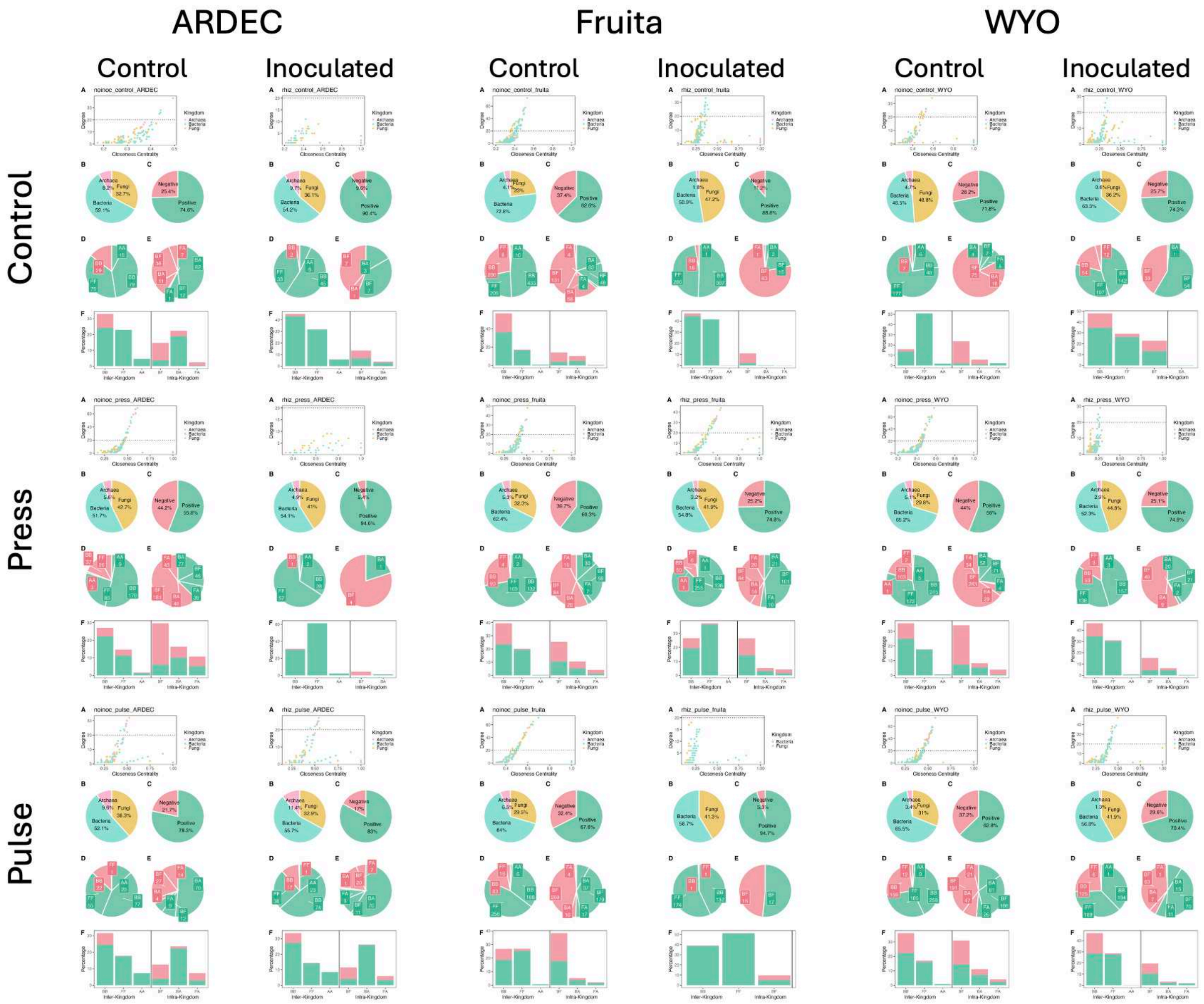
**S3.4:** Volcano plots examining patterns of relative abundance in the rhizosphere soil for the 16S sequencing (left) and ITS sequencing (right). OTUs with a |log2(foldchange)| > 1 and a p-value < 0.01 are shown in color. Plots are faceted by phylum. Colored points on the left side of each subplot represent OTUs with a higher relative abundance in the control watering treatment compared to the Pulse drought treatment while OTUs on the right side of each subplot have a higher relative abundance in the Pulse drought treatment compared to the watering control.



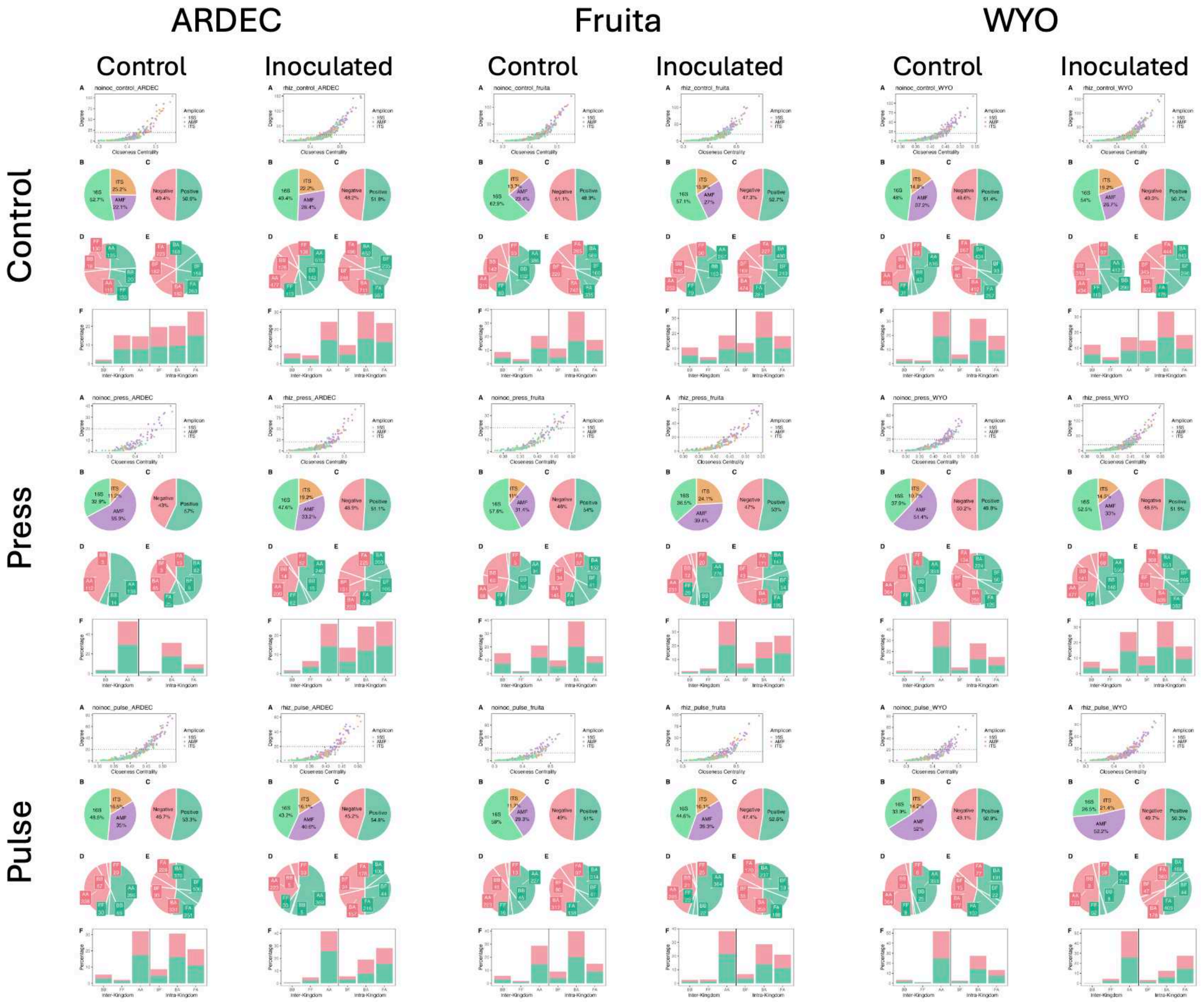
**S3.5:** Volcano plots examining patterns of relative abundance in the roots for the 16S sequencing (left) and ITS sequencing (right). OTUs with a |log2(foldchange)| > 1 and a p-value < 0.01 are shown in color. Plots are faceted by phylum. Colored points on the left side of each subplot represent OTUs with a higher relative abundance in the control watering treatment compared to the Pulse drought treatment while OTUs on the right side of each subplot have a higher relative abundance in the Pulse drought treatment compared to the watering control.



**S3.6:** Volcano plots examining patterns of relative abundance of fungal trophic modes assigned to each OTU with ITS sequencing and FUNGUILD analysis in the rhizosphere (left) and the roots (right). Trophic modes with a |log2(foldchange)| > 1 and a p-value < 0.01 are shown in color. Colored points on the left side of each subplot represent trophic modes with a higher relative abundance in the non-inoculated control compared to the AMF inoculated treatment while trophic modes on the right side of each subplot have a higher relative abundance in the AMF inoculated treatment compared to the non-inoculated control.



**S3.7:** Analysis of the co-occurrence networks for the rhizosphere samples. ARDEC, Fruita, and WYO soil (left to right), control, Press, and Pulse watering (top to bottom), and the non-inoculated control and inoculated treatment (left and right within each soil type). Within each subset, (a) scatterplot with the degree vs. closeness centrality, where high degree (> 20) and high closeness centrality indicate possible network hubs or keystone species, (b) pie chart with the percentages of each type of node (bacterial, fungal, or protistan) within each network. (c) pie chart with the percentages of positive and negative edges, (d) pie chart with the number of positive (green) and negative (pink) inter-kingdom edges in the network, (e) pie chart with the number of positive (green) and negative (pink) intra-kingdom edges in the network, and (f) bar chart with the percentages of positive (green) and negative (pink) inter-kingdom and intra-kingdom edges between bacterial (B), fungal (F), and archaeal (A) OTUs.



**S3.8:** Analysis of the co-occurrence networks for the root samples. ARDEC, Fruita, and WYO soil (left to right), control, Press, and Pulse watering (top to bottom), and the non-inoculated control and inoculated treatment (left and right within each soil type). Within each subset, (a) scatterplot with the degree vs. closeness centrality, where high degree (> 20) and high closeness centrality indicate possible network hubs or keystone species, (b) pie chart with the percentages of each type of node (bacterial, fungal, or protistan) within each network. (c) pie chart with the percentages of positive and negative edges, (d) pie chart with the number of positive (green) and negative (pink) inter-kingdom edges in the network, (e) pie chart with the number of positive (green) and negative (pink) intra-kingdom edges in the network, and (f) bar chart with the percentages of positive (green) and negative (pink) inter-kingdom and intra-kingdom edges between bacterial (B), non-AMF fungal (F), and AM fungal (A) OTUs.

# APPENDIX 4: Plant–microbiome interactions under a changing world: responses, consequences and perspectives

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## 1. Summary

Climate change is increasing global temperatures and the frequency and severity of droughts in many regions. These anthropogenic stresses pose a significant threat to plant performance and crop production. The plant-associated microbiome modulates the impacts of biotic and abiotic stresses on plant fitness. However, climate change-induced alteration in composition and activities of plant microbiomes can affect host functions. Here, we highlight recent advancements in our understanding of the impact of climate change (warming and drought) on plant–microbiome interactions and on their ecological functions from genome to ecosystem scales. We identify knowledge gaps, propose new concepts and make recommendations for future research directions. It is proposed that in the short term (years to decades), the adaptation of plants to climate change is mainly driven by the plant microbiome, whereas in the long term (century to millennia), the adaptation of plants will be driven equally by eco-evolutionary interactions between the plant microbiome and its host. A better understanding of the response of the plant and its microbiome interactions to climate change and the ways in which microbiomes can mitigate the negative impacts will better inform predictions of climate change impacts on primary productivity and aid in developing management and policy tools to improve the resilience of plant systems.

## 2. Introduction

The global mean surface temperature is predicted to increase by 2.6–4.8°C by the year 2100 if no mitigation efforts are made (IPCC, 2014). Elevated temperatures will reduce soil water content and increase the frequency, severity and duration of drought in many regions (Samaniego et al., 2018). Heat and drought affect plant communities, influencing all aspects of plant biology including growth, reproduction, migration and resilience. The exact impacts of climate change on natural vegetation and agriculture are difficult to predict but could have devastating consequences for humankind. Climate variability has already reduced the production of major crops world-wide (Zhao et al., 2017; Tito et al., 2018), and its negative impacts are expected to intensify in coming decades. Therefore, new approaches to mitigate the negative effects of climate change on plants are urgently required.

Plants are intimately associated with diverse, taxonomically structured communities of microorganisms. The plant microbiota includes bacteria, fungi, protists, nematodes and viruses that colonize all accessible plant tissues (Trivedi et al., 2020). The microbiome (microbiota and their genomes) inhabiting the soil, rhizosphere, roots and other plant tissues establishes complex and dynamic interactions with the host plant. These interactions are highly influenced by the environment and can improve plant resilience to environmental stresses (Naylor & Coleman-Derr, 2018; Singh et al., 2020a; Trivedi et al., 2020, 2021). Despite growing recognition of the microbiome's importance to plant growth and health, harnessing the microbial interactions and traits to improve plant resilience to climate variability remains a significant challenge. A better mechanistic understanding of the plant–microbiome relationship is needed to develop future tools to predict and mitigate the impacts of climate change on primary productivity and plant diversity.

This article examines many aspects of the impact of climate change on the interaction between nonwoody crop plants and the microbiomes that are tightly and directly associated with hosts. We discuss how climate change will impact the community composition of the plant microbiome and highlight the response of functional properties, including metabolic and signalling interactions, and the genes driving plant–microbiome interactions. Possible mechanisms of eco-evolutionary processes on climate adaptation are described that can be harnessed for the development of microbial tools for climate-proof primary production. A number of climate change factors such as drought, elevated temperature, increasing CO2 and changes in the freeze/thaw cycles affect plant–microbiome interactions impacting plant performance. In this article, we focus on warming and drought, two abiotic stresses that are postulated to increase in occurrence and severity across the globe and have direct significant impacts on plant productivity and ecosystem sustainability. In proposing new concepts, we have been deliberately provocative, but our arguments are embedded in strong scientific frameworks. Our aim is to initiate new discussions on the topic and shape future research in this discipline.

## II. Impact of climate change on the assembly of the plant microbiome

Plant microbiome assembly is a complex ecological process driven by coevolution over millennia (Dini-Andreote & Raaijmakers, 2018; Trivedi et al., 2020). The seed microbiome (heritable) helps the plant germinate and, along with the seed exudates, shapes the host microbiome. Exudate signals attract desired soil microbes to colonize plants and modulate their immune systems, forming the plant core microbiome. Some 'social cheaters' and pathogenic microbes also use this pathway. The concepts of core-and-hub microbiota are gaining increasing evidence in host–microbiome research (Singh et al., 2020b). They include the microbiota present in a particular species, irrespective of growing seasons, environmental conditions and management practices, and provide key host functions (Singh et al., 2020b; Trivedi et al., 2020). Given their importance, it is critical to decipher the impact of climate change on the ‘core-and-hub’ microbiota that potentially organize community-scale processes in plant–microbiome relations. Increased knowledge of the ecological principles that govern the response of microbiome structure and function to climate change will advance our understanding of microbiome properties (e.g. resistance and resilience) that improve plant fitness under novel environmental conditions.

Microbes differ in their physiology, metabolism and sensitivity to temperature and moisture. Therefore, climate change can have direct impacts on the assembly of the plant microbiome. The direct impact of climate change is likely to be more pronounced on communities that occupy the plant surface (e.g. the phyllosphere), where environmental conditions fluctuate more rapidly as compared to the relatively stable internal plant tissue environments (i.e. the endosphere) (Trivedi et al., 2020). The majority of the bulk soil microbiome (a reservoir of available microbes that can colonize the plant) are directly impacted, while rhizosphere microbiomes are influenced not only by external climatic factors but also indirectly by host responses, which include changes in plant physiology, morphology, immune response and root exudation (Fig. 1). Some reports suggest a consistent response of the plant-associated microbiome to climate change (Naylor et al., 2017; Xu et al., 2018; Vescio et al., 2021; Wipf et al., 2021). Under drought conditions, many plant species selectively recruit and enrich monoderm (or Gram-positive bacteria that are tolerant to desiccation due to thicker cell walls) and deplete diderm (or Gram-negative) bacteria in the rhizosphere and roots (Naylor et al., 2017; Naylor & Coleman-Derr, 2018; Xu et al., 2018). Elucidating how climate change impacts plant–microbiome assembly is a challenge due to the complexity and interconnectedness of the factors that govern this process. Plant–microbiome interactions under climatic stress appear to be largely modulated by chemical communications (Fig. 1). For example, plants have evolved an exudation-mediated ‘cry for help’ response when exposed to stressful environmental conditions, leading to the recruitment of a stress-relieving microbiome (Liu et al., 2020). We have limited knowledge about the indirect influence of climate change on plant–microbiome assembly via changes in root exudation patterns.

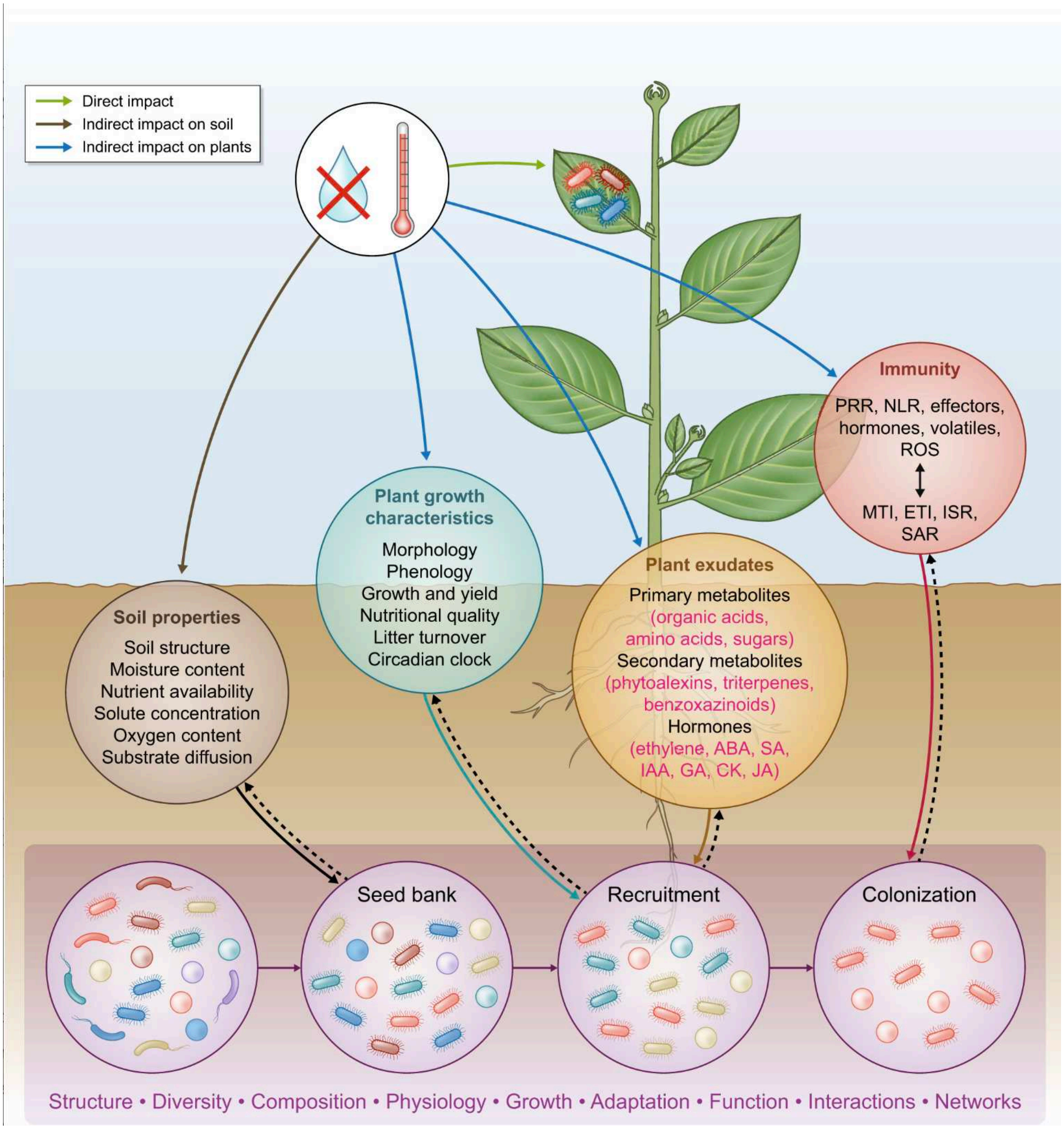

Figure 4.1. Impact of climate change on the plant-associated microbiome. Climate change can have a direct (light green arrow) and indirect impact (brown and blue arrows representing the impacts on soils and plants, respectively) on the plant–microbiome. Different climate change drivers will have a variable impact on the microbiome. For example, the direct impact of warming is likely to be stronger on the aboveground epiphytic microbiome than drought. In comparison, drought will have a stronger indirect impact on the soil microbiome than warming. The indirect impact of climate change on the microbiome can be mediated by changes in soil properties (brown circle), plant growth characteristics (green circle), exudation (yellow circle) and immunity (pink circle). Climate change can impact the bulk soil 'seed' microbiome directly or indirectly via affecting soil properties (black arrow). These impacts will select tolerant (circular) and opportunistic (oblong) groups while reducing sensitive (curved) groups. As the initial soil microbiome is the source for the plant microbiome assembly, a variation in the composition and functioning of the soil microbiome can determine the outcomes of plant–microbiome interactions under climate change. Plants undergo a set of physiological responses that allow them to adapt to short- and long-term environmental fluctuations (green and yellow circles). Recent reports suggest that plant exudates (yellow circle) are critical to the ecosystem responses to climate change (William & de Vries, 2020). It is likely and equally important that the quantity and quality of root exudate directly affects climate resilience via its impact on the plant-associated microbiome. Growing evidence for the 'cry for help' hypothesis posits that plants recruit specific microbes that can alleviate plant stress in a given situation by regulating root exudation patterns. Changes in plant growth characteristics and exudation patterns will impact the microbial recruitment process (green and light brown arrows), selecting the microbial groups (green-, red- and yellow-coloured ovals and circles) that can adjust to the new conditions and metabolize stress-induced communication signals. Plant colonization of microbes (red-coloured circles and ovals) will be determined by changes in the plant immune responses (pink arrow) that are postulated to be significantly impacted by climate change drivers. Plant immune-mediated impact on microbial colonization will be stronger for endophytes than for epiphytes. Direct and indirect impacts mediated by climate change can alter many plant-associated microbiome features (purple text in shaded box). Climate change-induced alteration in plant-associated microbiome assembly will have a strong impact on many aspects of plant–microbiome interactions. These interactions will further influence plant growth characteristics, exudation patterns and immune response (dashed black lines). Successive changes in the microbial community composition are shown by purple arrows. ABA, abscisic acid; CK, cytokinin; ETI, effector-triggered immunity; GA, gibberellic acid; IAA, indoleacetic acid; ISR, induced systemic resistance; JA, jasmonic acid; MTI, microbe-associated molecular pattern-triggered immunity; NLR, nucleotide-binding domain leucine-rich repeat receptors; PRR, pattern recognition receptor; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic induced resistance.

Impact of climate change on the plant-associated microbiome. Climate change can have a direct (light green arrow) and indirect impact (brown and blue arrows representing the impacts on soils and plants, respectively) on the plant–microbiome. Different climate change drivers will

have a variable impact on the microbiome. For example, the direct impact of warming is likely to be stronger on the aboveground epiphytic microbiome than drought. In comparison, drought will have a stronger indirect impact on the soil microbiome than warming. The indirect impact of climate change on the microbiome can be mediated by changes in soil properties (brown circle), plant growth characteristics (green circle), exudation (yellow circle) and immunity (pink circle). Climate change can impact the bulk soil 'seed' microbiome directly or indirectly via affecting soil properties (black arrow). These impacts will select tolerant (circular) and opportunistic (oblong) groups while reducing sensitive (curved) groups. As the initial soil microbiome is the source for the plant microbiome assembly, a variation in the composition and functioning of the soil microbiome can determine the outcomes of plant–microbiome interactions under climate change. Plants undergo a set of physiological responses that allow them to adapt to short- and long-term environmental fluctuations (green and yellow circles). Recent reports suggest that plant exudates (yellow circle) are critical to the ecosystem responses to climate change (William & de Vries, 2020). It is likely and equally important that the quantity and quality of root exudate directly affects climate resilience via its impact on the plant-associated microbiome. Growing evidence for the 'cry for help' hypothesis posits that plants recruit specific microbes that can alleviate plant stress in a given situation by regulating root exudation patterns. Changes in plant growth characteristics and exudation patterns will impact the microbial recruitment process (green and light brown arrows), selecting the microbial groups (green-, red- and yellow-coloured ovals and circles) that can adjust to the new conditions and metabolize stress-induced communication signals. Plant colonization of microbes (red-coloured circles and ovals) will be determined by changes in the plant immune responses (pink arrow) that are postulated to be significantly impacted by climate change drivers. Plant immune-mediated impact on microbial colonization will be stronger for endophytes than for epiphytes. Direct and indirect impacts mediated by climate change can alter many plant-associated microbiome features (purple text in shaded box). Climate change-induced alteration in plant-associated microbiome assembly will have a strong impact on many aspects of plant–microbiome interactions. These interactions will further influence plant growth characteristics, exudation patterns and immune response (dashed black lines). Successive changes in the microbial community composition are shown by purple arrows. ABA, abscisic acid; CK, cytokinin; ETI, effector-triggered immunity; GA, gibberellic acid; IAA, indoleacetic acid; ISR, induced systemic resistance; JA, jasmonic acid; MTI, microbe-associated molecular pattern-triggered immunity; NLR, nucleotide-binding domain leucine-rich repeat receptors; PRR, pattern recognition receptor; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic induced resistance.

Plants have also developed a multilayered microbial management system to incorporate the most favourable microbes into plant tissues and to distinguish friend from foe (Hacquard et al., 2017; Teixeira et al., 2019). These protective barriers include: (1) first immune layer, where pattern recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs), such as bacterial flagellin or fungal chitin, resulting in the plant MAMP-triggered immunity (MTI); and (2) second immune layer, where nucleotide-binding leucine-rich repeat (NLR) proteins recognize pathogen effectors, leading to the plant effector-triggered immunity (ETI). Both warming and drought-induced changes in plant immunity may shape the plant–microbiome, particularly inside plant tissues. Warming is reported to cause both an increase (Cheng et al., 2013) and a decrease (Janda et al., 2019) in MTI and to suppress ETI in plants (Cheng et al., 2019; Desaint et al., 2021). Suppression of ETI can disrupt host-mediated control of microbial colonization and can cause dysbiosis in microbial communities living inside plant tissues. Effector-triggered immunity suppression may also act as a novel mechanism by which plants reduce their immune response to allow colonization by beneficial microbes, which can then tune their genetic machinery to provide stress relief. Overall, strong evidence is emerging that the reciprocal interplay between the microbiota and the plant immune response shapes plant–microbiome assembly.

In rapidly changing environments, plants modulate immunity through dynamic but finely orchestrated changes in many hormone pathways, including abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (Li et al., 2021). Both drought and warming decrease SA production, which is involved in both epiphytic and endophytic microbiome assembly (Lebeis et al., 2015). Salicylic acid may act via canonical signalling pathways, via interaction with other hormones such as JA, or directly on microbial community members, promoting or inhibiting their growth. Drought-induced ABA production acts antagonistically to SA-mediated immune signalling. It should be noted that climate-induced changes in plant hormones can vary with plant developmental stage and tissue type. For example, under drought conditions, maize stimulates benzoxazinoid defences in aboveground tissues, while terpenoid phytoalexins are stimulated belowground (Vaughan et al., 2018). Changes in the allocation or distribution of different classes of defence metabolites or signalling molecules under climate change have the potential to further impact microbiome assembly.

## III. Plant–microbiome interactions under a changing climate

### Pathogen–plant interactions

Plant health and productivity are impacted by tripartite environment–host–pathogen interactions that operate on a continuum from resistance to disease. Climate change can alter pathogen abundance and behaviour, change the host–pathogen interactions and facilitate the emergence of new pathogens (Cohen & Leach, 2020). A proportion of many plant pathogens are predicted to increase as global temperatures rise (Delgado-Baquerizo et al., 2020), and to compound the problem, many widely used approaches fail to control diseases at high temperatures (Burdon & Zhan, 2020). Simultaneously, pathogens can adopt new invasion strategies by modifying their virulence system potentially leading to the breakdown of R gene-mediated plant resistance. Both elevated temperatures and drought can break down ETI and promote disease in many plant pathosystems (Cheng et al., 2019). Most studies on the effect of climate change on host–pathogen interactions have used simplified models composed of a single host plant interacting with a single pathogen. However, in their natural environment, plants interact with a wide variety of potentially pathogenic microbes (pathobiota) (Bartoli et al., 2018) wherein the pathogen establishment depends on cooperation or competition between the pathobiota and members of the plant microbiome. We currently have no understanding of how the interaction between pathobiota and plant microbiome will respond under exposure to long-term abiotic stresses.

### Beneficial plant–microbe interactions

Climate change will have variable effects on beneficial plant–microbe interactions (Cheng et al., 2019). Warming can decrease belowground photosynthate allocation, leading to limited root development in both length and diameter (Qiu et al., 2021). Consequently, root colonization by arbuscular mycorrhizal fungi (AMF) is reduced and/or AMF species with lower carbon (C) requirements are favoured (Ma et al., 2018; Bergmann et al., 2020). Certain members of the plant microbiome have traits that alleviate the effects of abiotic stresses on plants (Trivedi et al., 2020, 2021). These include the following: (1) the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which increases stress tolerance by regulating plant ethylene levels; (2) the production of extracellular polymeric substances (EPS) resulting in hydrophobic biofilms that can protect plants from desiccation; (3) the production of phytohormones that can stimulate plant growth, induce accumulation of osmolytes and/or detoxify reactive oxygen species; (4) directly influencing nutrient and water uptake by increasing root surface area; and (5) modulating the plant's epigenetic regulation leading to acclimation and adaptation to new environmental conditions. For example, root endophytic bacterium Enterobacter sp. SA-87-induced thermotolerance is shaped by a novel mechanism in which constitutive expression of heat shock factor A2 (HSFA2) via the ethylene signalling pathway and the transcription factor EIN3 results in methylation of heat stress memory genes that enhance thermotolerance in plants (Shekhawat et al., 2021). Some plant growth-promoting bacteria can even help plants to cope with multiple stresses (Lata et al., 2018; Bokhari et al., 2019). It is validly postulated that improved plant performance under stress is the net result of multiple microbiome mechanisms that may be activated simultaneously. However, we have a limited understanding of the intertwined molecular mechanisms that open the cascade of interactions between plants and their associated microbiome under climate change. Identifying these mechanisms and the factors that influence them will allow for the development of new tools to predict (Box 1) and mitigate the impacts of climate change on primary productivity.

Box 1. Modelling and predictive framework

Accurate projections of climate change impacts on plant productivity require modelling approaches to understand and predict the dynamics and properties of the microbiome systems and microbiome–host–environment interactions. Although making a direct link between plant–microbe interactions and ecosystem productivity in novel environments is a significant challenge, many connections at intermediate scales can be predicted with the integrated application of new systems biology approaches and powerful analytical and modelling techniques. Hormones and the plant immune response play critical roles in the assembly of the plant-associated microbiome and influence host–microbiome–environment interactions. A combination of 'multi-omics', hormone profiling, immune output and advanced statistical methods (including network analysis and neural and machine learning) to link multi-omics data with plant growth responses has the potential to generate quantitative models of molecular processes that drive plant–microbiome interactions in different climate change scenarios. In the absence of quantitative dynamic models of molecular plant–microbiome interactions, correlative network approaches can be powerful tools in identifying modules, pathways, components and system-level patterns of host–microbe molecular interactions. For example, using multi-omics and integrated informatics, Ichihashi et al. (2020) have revealed complex interactions in the agroecosystem showing multiple network modules represented by plant traits heterogeneously associated with soil metabolites, minerals and microbes. The plant's 'cry for help' response to recruit a microbiome that can provide stress relief is mediated by metabolic exchange. New approaches using spatiotemporal metabolic modelling with genome-scale metabolic networks have the potential to translate genomic information into predictions of metabolic phenotypes, including growth capability, intracellular reaction rates, and the associated nutrient uptake and excretion of intermediate metabolites (by-products). Common to these modelling approaches is their ability to describe trophic dependencies of microbial species. This resource provides a foundation for building models of community metabolism, spatial relationships, and regulatory and interaction networks. Constraining these models by measuring the level of key stress-induced metabolites and simulating the microbiome to examine its physiological responses, such as sporulation, production of osmolytes, extracellular polymeric substances (EPS) and extracellular enzymes, we can obtain a more precise picture of the metabolic changes induced in the plant and in the microbiome in the context of colonization under stress conditions. These insights have the strong potential to enable a predictive framework of the physiology, taxonomic structure and spatiotemporal dynamics of microbial communities under various climate change scenarios and their impact on plant fitness. Although mathematical modelling of plant–microbiome interactions can lead to the evaluation of different hypotheses, one of the major challenges is model validation. Another challenge to predict the microbial impact on processes that influence plant performance is to link information from microbial models (e.g. GEMs) directly to biogeochemical models. Recent breakthroughs in machine-learning and statistical modelling approaches have allowed the integration of information from microbial models into biogeochemical models for improved predictions on ecosystem functions (Saifuddin et al., 2019; Guo et al., 2020). We envision that systems-level understanding of plant–microbiome interactions spanning from molecular levels to ecosystem levels across time and space will provide both the parts and wiring diagrams to refine the models to generate more realistic predictions.

### Plant–microbiome communication

A communication system exists between the microbiome and the host plant. When stressed, plants exude metabolites to selectively recruit microorganisms able to enhance plant resilience (Liu et al., 2020). For example, drought-induced secretion of glycerol-3-phosphate (G3P) in the roots enriches Actinobacteria with the genetic potential to transport and utilize G3P for growth (Xu et al., 2018). Drought causes a reduction in iron and phytosiderophore availability in the rhizosphere, facilitating the enrichment of Actinobacteria, which can thrive in low iron conditions increasing both the fitness advantage and plant growth promotion ability of Actinobacteria (Xu et al., 2021). The plant microbiome also contributes to host phenotypic plasticity, which can impact plant phenology in a changing climate (Dastogeer et al., 2020). For example, rhizosphere microorganisms can modulate flowering time by impacting the nitrogen (N) cycle and converting tryptophan in root exudates to the phytohormone indoleacetic acid (Lu et al., 2018). Furthermore, plants use volatile organic compounds (VOCs) to communicate with insects, nematodes and microbes (Weisskopf et al., 2021). Climate warming is increasing VOC emissions, and we postulate that root exudate-mediated shifts in microbiome composition under drought and warming may be tied to changes in plant immune responses, or to a stress signalling network within the host. Deciphering the molecular dialogues through which abiotic stresses influence metabolites to reshape the composition and function of the plant microbiome is essential for developing strategies to increase plant resilience to climate stresses.

## IV. Evolutionary and eco-evolutionary responses of plant–microbiome interactions under climate change

Climate change may influence the eco-evolutionary interaction between the host and its microbiome. A recent study demonstrated that drought induced the reorganization of hormonal signalling pathways and altered gene expression, resulting in the evolution of virus–plant interactions from pathogenic to mutualistic (González et al., 2021). Further, evolution makes microbes more cooperative with their local host genotype, and cheaters cannot outcompete cooperative microbes (Batstone et al., 2020). It is proposed that the eco-evolutionary interactions between hosts and their associated microbiomes will play a key role in plant resilience to climate change (Fig. 2). Furthermore, studies have provided evidence that while plant habitats strongly shape the microbiome, ecological and evolutionary processes also play a role. For example, drought reduces the host impact on the assembly of the plant fungal community and increases the impact of stochastic forces (e.g. drift or stochastic dispersal) (Gao et al., 2020). The evolutionary interactions between microbial species depend on stress levels. Under moderate stress, microbes display more competitive interactions, whereas under adverse conditions, they display more cooperative or neutral behaviour. Deciphering the principles that underlie the ecological and evolutionary properties of microbial communities can allow for the construction of models that predict the eco-evolutionary dynamics of microbial communities.

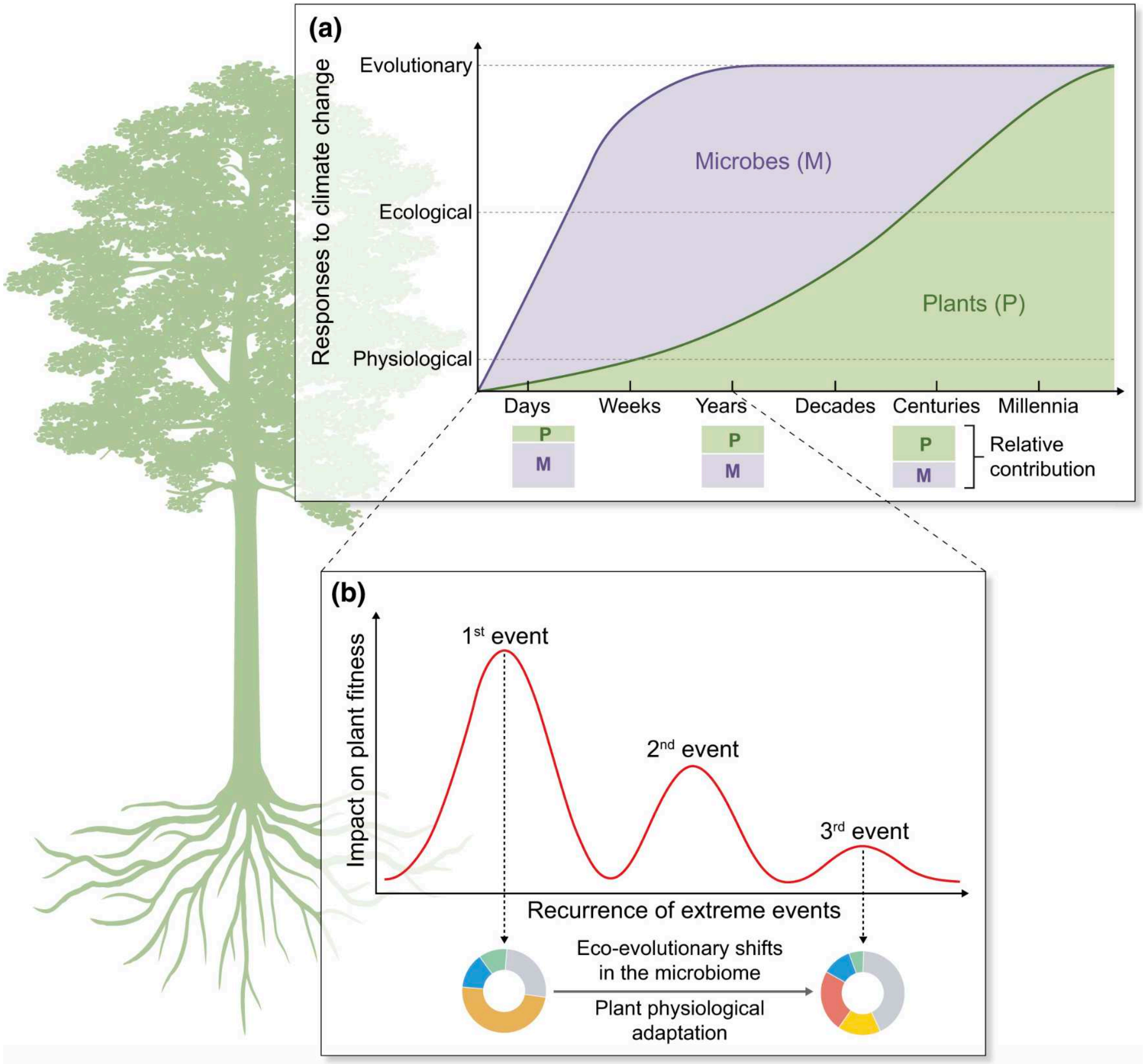

Figure 4.2. Eco-evolutionary response and climate change adaptation. (a) Perennial plants live for centuries to millennia, and significant changes in temperature and water availability may require a compensatory physiological response (e.g. decreased respiration rate; production of stress alleviating hormones and metabolites). The large genome size (up to several thousand times larger than many bacterial genomes) with multiple copies of genes that provide similar phenotypes in plants provides flexibility for physiological adjustment under different environmental conditions (Hartley & Singh, 2018). Microbiomes can also contribute substantially to enzymes, hormones and metabolites for plants to cope with altered environmental conditions. On the contrary, the enormous diversity in the microbiome and the short replication times (minutes to days) mean that microbiomes have an enormous advantage in terms of ecological and evolutionary adjustments. There is evidence that not all microbes are active at the same time, and active and dominant populations vary within a microbial community in different seasons and climatic conditions. There is also evidence that associated microbes are

able to sense their host's stress and respond quickly via changes in population dominance, provision of metabolites and priming the host physiological and immune responses (Liu et al., 2020). Rapid replication rates in microbes mean that ecological responses in terms of a shift in the community can happen within hours to days with dominance of stress-tolerant microbial populations that can help plants to cope with climate changes. Fast replication rates also mean microbial components of the holobiont are more able to acquire new genes (horizontally and vertically) and mutate genomic traits to cope with climate change. Intimate association means some of these microbial phenotypic traits can directly benefit the host. Plant hosts do not have the advantages of such rapid ecological and evolutionary responses in the short and medium terms. However, in the long term, the plant community will also adopt eco-evolutionary mechanisms to adapt to climate change, but this will require centuries to millennia because of the slow rate of community shifts and the development of heritable traits. (b) Previous works have shown that plant and soil microbiome can develop, adapt and contribute towards plant resistance against the re-occurring pathogen attacks (Raaijmakers & Mazzola, 2016). Similarly, it is likely that in future with increasing frequency of drought and heatwaves, as projected under climate change, plant microbiomes can adapt (via eco-evolutionary mechanisms) and confer some resistance to drought/heatwaves to its plant host.

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Eco-evolutionary responses suggest that in short to medium time frames, plant resilience and productivity under global warming are likely driven by host-associated microbiomes (Fig. 2a). Our argument is based on the divergence of timescales for physiological, ecological and evolutionary responses of plants and microbes, the extremely short replication and evolutionary time frame of microbes, and the emerging concept of the ‘holobiont’, that is an assemblage of a host and the many other species living in or around it, which together form a discrete ecological unit (Zilber-Rosenberg & Rosenberg, 2008; Lyu et al., 2021). In addition, recent findings show that the same plant phenotype can be achieved either by altering the plant genome or by manipulating its microbiome (Ravanbakhsh et al., 2021). Further, soil and plant microbiota have been shown to provide relief to plants from recurrent biotic stress events (e.g. pathogen attacks) by inducing plant resistance against invading pathogens. Extreme weather events (e.g. drought, heatwaves) are projected to increase in frequency and intensity. As microbiomes undergo more rapid eco-evolutionary adaptation to these recurrent events than plants, they may provide some immediate resistance and resilience to host plants (Fig. 2b). In the long term, a plant community is likely to achieve a similar level of eco-evolutionary adaptation to climate change (Fig. 2a). It should be emphasized that there are multiple potential pathways for plant microbial adaptation to climate change including those that are summarized in Box 2.

Box 2. Other possible mechanisms of plant microbial adaptation to climate change

This article addresses a specific concept of climate adaptation, but there are other concepts that may also play important roles – some are briefly mentioned below:

Response of biodiversity and its impacts on immunity and productivity. Climate change is expected to have a strong negative impact on plant and soil biodiversity (Maestre et al., 2015). This can have a significant impact on primary productivity as increasing evidence suggests that increasing plant diversity is linked to increased primary productivity. Similarly, given the crucial role of the soil–rhizosphere microbiome and plant diversity on plant immunity and adaptation to abiotic stresses, a climate-linked decline in biodiversity can potentially impact both biotic and abiotic resilience of plant communities with significant consequences for adaptation. We postulate that increased plant diversity provides diverse pattern recognition receptor (PRR) repertoires that can recognize a range of microbe-associated molecular patterns (MAMPs), thus increasing the immune potential within an ecosystem. Systemic signals can then be passed from one plant species to others through volatiles (produced by both plants and microbes) or root exudates that influence plant adaptation and primary productivity (Weisskopf et al., 2021). Legacy impact of management practices influences plant physiology by soil microbiome wherein monocrop and rotational plants showed significant differences in the expression of genes involved in plant hormone and immune response (Li et al., 2019). We postulate that manipulating aboveground biodiversity will impact plant-associated microbiome diversity and create greater immunity and productivity (via enhanced nutrient cycling), thus leading to significant ecological gains. However, we have a limited understanding of the impact of climate change on the molecular interactions that drive the relationship between aboveground and belowground diversity.

Response of ecosystem properties emerging from aboveground and belowground connectivity: both biotic and abiotic properties of the ecosystem are likely to respond simultaneously. How these changes will interact can have a substantial impact on plant adaptation abilities. For example, prolonged drought and warming can encourage plants to invest in root biomass or new and more robust association with mycorrhizal fungi to secure available water from a distance. Such phenotypic and biotic changes can have a significant impact on soil physical properties (e.g. porosity). Understanding how these biotic and physical changes can impact plant adaptability can better predict productivity and resilience. However, this will require integrating understanding at different temporal and spatial scales and across different dimensions of the systems, particularly the physical properties of the ecosystems.

Plant cognitive abilities and gnosophysiology. It is proposed that plants possess cognitive abilities and can acquire, process and memorize information that can modify their response to future natural stimuli (Michmizos & Hilioti, 2019). Plants can extend their cognitive abilities to the environment via root influence and association with mycorrhizal fungi and other microbes that associate with them (Parise et al., 2020). However, in the absence of brain and nervous system the way in which a plant cognitive system operates remains largely unknown. Well-established mechanisms of cellular and molecular learning and memory can provide a potential path for adaptation based on previous events (e.g. prolonged drought, heatwaves). However, a role and relative contribution of plant gnosophysiology in climate adaption needs the development of strong theoretical and experimental evidence.

## V. Conclusion and future perspective

Manipulating the plant–soil microbiome to increase plant productivity in the face of climate change has been recognized as a priority by many national and international policy agencies (Singh et al., 2020b; Trivedi et al., 2021). These interventions can range from direct manipulation of the plant microbiome, functional manipulation via land management practices and/or the use of probiotics. Through breeding, advanced genome-editing tools (e.g. CRISPR) and synthetic biology approaches (Geddes et al., 2019), it will be possible to engineer microbe-friendly plants that release exudates, which promote specific beneficial plant–microbe interactions. The wild relatives of domesticated crops contain a reservoir of genetic diversity (Pourkheirandish et al., 2020) including those traits that promote the assembly of distinct microbiomes that may support the plant adaptation to climate change (Pérez-Jaramillo et al., 2018; Chen et al., 2021). While the effectiveness of transplanting faecal microbiota in humans has been broadly demonstrated, research on the re-colonization of native soil bacteria of domesticated crops and their role in improving plant resilience to climate stress has not been fully explored.

Overall, our knowledge of the intimate and multifaceted plant–microbiome interaction and the consequences of plant fitness and productivity has started to emerge. However, how these interactions will respond to climate change at ecological, evolutionary, biochemical and molecular levels remains poorly understood and, in some cases, completely unknown. Future research should examine how these interactions change over time and space under multiple climate change scenarios (Box 3). A system-based, reductionist approach where both plant and microbial eco-physiological responses are measured with explicit consideration of time, multiple stresses and environmental conditions can unravel these interactions. Further efforts to obtain detailed and potentially predictive insights into plant–microbiome interactions are needed to develop new computational and modelling tools to predict the response of beneficial plant interactions to environmental stresses. This knowledge will facilitate predictions of the impacts of climate change on the plant-associated microbiome and will open new avenues for applied research to harness plant–microbiome interactions to improve the climate resiliency of plant communities.

Box 3. Key future research directions

Better understand the direct impact of climate change on the assembly and functions of the plant microbiome over space and time under multiple climatic scenarios (e.g. warming and drought individual and combined effects).

Decipher the impact of climate change on plant physiology and the immune system: what are key changes in root exudates, photosynthates and immune systems? Obtain experimental evidence on how these changes may affect the plant microbiome assembly, activities, traits and ultimately the host functions.

Identify changes in the composition of key signal and communication molecules and their interaction (in vitro and in vivo tests) and the response of microbes and, ultimately, the consequences for host functions and fitness.

Improve understanding of the underlying genetics, biosynthetic pathways, regulation and precise biological roles of phytohormones modulated by arrays of climatic conditions and how these changes impact the assembly, functions, and plant–microbiome and microbe–microbe interactions.

Define the timescale and frequency of events (e.g. heatwave, drought) required for eco-evolutionary adaptation in plant microbiomes and the degree to which these can provide climate resistance and resilience to the host.

Obtain advanced fundamental knowledge about the molecular interplay driving plant–pathogen or plant–beneficial microbe interactions in a range of climate change scenarios, taking into account the community context.

Develop approaches to manipulate the plant microbiome in situ to reduce the impact of climate change on primary productivity.

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# APPENDIX 5: Management-induced shifts in rhizosphere bacterial communities contribute to the control of pathogen causing citrus greening disease

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## 1. Summary

Citrus greening (aka Huanglongbing, HLB) caused primarily by the bacterial pathogen Candidatus Liberibacter asiaticus (CLas) has devastating effects on the global citrus industry. Agricultural management-induced changes in microbial communities are hypothesised to contribute toward HLB resistance by reducing pathogen titre and increasing root and soil health. However, we have a limited understanding of the impacts of management practices on the soil microbiome, making the extent of HLB management uncertain. Here we investigated the effect of agricultural management practices on reducing CLas titer via changes in rhizosphere-associated bacterial communities. Rhizosphere and root samples were collected from two sites in Florida where different management practices (e.g., metalized reflective mulch ground covers, compost application and microbial inoculations) are currently being implemented to prevent HLB. Management-induced changes in the rhizosphere bacterial community were assessed using amplicon sequencing. qPCR assays were used to quantify the titer of the pathogen CLas in roots. In addition, we measured soil properties and the activities of microbial enzymes involved in soil nutrient cycling. Our results indicated that certain management practices lead to shifts in the community structure of rhizosphere bacterial communities that negatively interact with the HLB pathogen. Management practices improved soil quality and reduced CLas titer. Additionally, we found that Actinobacteria were frequently enriched in the successful treatment sites, suggesting that Actinobacteria taxa could be indicators for HLB suppression properties in the soil. Our

results suggest that microbiome manipulation, either through changes in the management practices or microbial amendment, can increase the suppressive potential of soils, resulting in the reduction in CLas titer and potentially leading to HLB suppression in citrus groves.

## 2. Introduction

Citrus greening disease (aka Huanglongbing, HLB) is caused by the phloem-limited, fastidious α-proteobacteria Candidatus Liberibacter asiaticus (CLas) transmitted by the Asian citrus psyllid (ACP, Diaphorina citri Kuwayama). HLB is one of the world's oldest and most serious citrus diseases (N. Wang & Trivedi, 2009). HLB is a major problem in many citrus-producing countries across the globe, including those from Africa, Asia, Oceania, and the Americas (South, North, and Central Americas, and the Caribbean) (Zhang et al., 2021). It has already devastated the Florida citrus industry and threatens citrus production in California, Texas, Australia, and the Mediterranean region (McCollum & Baldwin, 2017). Infected trees produce several distinct symptoms, including the uneven yellow mottling of leaves, corky veins, branch dieback, small misshapen fruit which remain green, fruit loss by early dropping, and ultimately tree death (Bové, 2006; N. Wang & Trivedi, 2009). To date, neither a cure nor an economically viable strategy for managing diseased trees is available to the industry. Disease management currently depends on the efficient application and adequate distribution of insecticides to reduce the density of ACP. However, recent studies have shown that even advanced insecticide management programs fail to control the spread of HLB (Rehberg et al., 2021). The outlook for the global citrus industry is thus precarious, especially for Florida's citrus industry, which risks losing relevance and economic importance if crop yields continue to fall and trees keep dying.

While there is no cure for HLB, there has been a growing awareness of the importance of root health for HLB management. As much as 30%–50% of root density is lost to the disease before the above-ground symptoms even appear (Graham et al., 2013; Johnson et al., 2014). These observations laid the foundations for near-term therapies that specifically reduce the levels of the pathogen in infected citrus trees and reverse disease impacts by improving root and soil health (Castellano-Hinojosa et al., 2021; Johnson & Graham, 2015; Strauss & Albrecht, 2018; Stokes et al., 2020). Agronomic management practices impact soil health, alter soil microbiome, and activate natural biocontrol mechanisms that lead to the development of disease suppressive soils for managing few plant pathogens (Carrión et al., 2019; Ou et al., 2019; Trivedi et al., 2021). Harnessing the beneficial potential of native microbiota may be one of the few logistically and economically viable solutions for controlling certain phytopathogens that are otherwise difficult to manage, such as Ca. Liberibacter spp. (Blaustein et al., 2018; Munir et al., 2022; Trivedi et al., 2016; Zhang et al., 2021). However, empirical evidence supporting this idea is lacking. Different management practices have variable impacts on the soil microbiome, making the extent of HLB control uncertain.

Herein we investigated the effect of management practices on reducing the titer (population density) of HLB pathogen via changes in rhizosphere bacterial communities. Our central hypothesis is that certain management practices will alter the physiochemical and biological soil variables leading to the buildup of robust populations of beneficial microorganisms that, through their activities, will reduce the pathogen titer thus making plants less susceptible to infection by HLB. To test our hypothesis, we collected samples from two sites in Florida where different management practices (e.g., ground covers, compost application, and microbial inoculations) are currently being implemented to control HLB. We assessed the bacterial community structure using high throughput sequencing and measured soil properties and microbial functional groups that perform essential soil processes in agroecosystems (Trivedi et al., 2017). Titers of the HLB pathogen were monitored by specific qPCR assays (Trivedi et al., 2012). We performed network analysis to evaluate the effects of different management practices on microbial coexistence patterns. We then used machine learning approaches to identify microbial groups that are highly correlated with reduced CLas titer (Trivedi et al., 2017).

## 3. Methods

### Study Sites

We investigated the effect of management practices on reducing CLas titer in two independent study sites.

### 3.1.1 Site A

This is situated at Amelia Farms in Venus, Florida. Management treatments include microbial application and metalized reflective mulch cover (mulch treatment). A bare ground with no mulch or microbial application was used for comparison (referred to as uncovered based on the farm manager's recommendation). There were three individual blocks for each treatment. From each block, four trees were randomly selected. The mulch treatment (Shine N' Ripe XL manufactured by Imaflex) was a six-foot wide reflective plastic film installed on top of the soil before planting and anchored into the ground by furrowed soil on each side. The commercially available microbial treatment Pathway's “PowerBlend™” was applied via irrigation (roots and soil) and aerial spraying (foliage) at 16 fluid ounces per acre once a month from March 2015 (ongoing) to the microbial treatment blocks of the grove. The trees were five years old at the time of sampling.

### 3.1.2 Site B

This site is situated at Florida Research Center for Agricultural Sustainability, Vero Beach, Florida. Management treatments included Urban Plant Debris compost and mulch cover. Bare ground with no mulch or compost application was used for comparison (uncovered). Treatments consisted of a block of trees two rows wide. Samples were collected from four trees within each block. The compost treatment consisted of shredded yard-trimming waste and was broadcast spread two months after planting at a rate of 12 tons per acre. The second compost application was performed when the trees were about one year old. Mulch treatment was like Site A.

#### a. Sample collection and DNA extraction

Sampling followed the same protocol in both experimental sites. The samples were collected from the four ordinate directions, approximately 30 cm from the trunk of each tree. The top 5 cm of soil was removed, and fine roots (about 1 mm diameter) from a depth of 5–15 cm were collected. The roots were removed from the soil with a shovel and then gently shaken to remove the soil that was not tightly attached to the roots. Samples were shipped in ice to the laboratory at Colorado State University. Samples were received within 24 h and processed immediately. The roots from the four locations around each tree were pooled. The rhizosphere soil was carefully and quickly collected by gently brushing the remaining soil adhering to the roots using brush pencils. The soil collection step was performed on ice. This soil was termed the rhizosphere compartment and was used to elucidate the effect of management practices on microbial community structure, functions, and soil properties. DNA extracted from the remaining root samples was used to quantify the CLas titer. We used Qiagen PowerSoil® DNA isolation kit and DNeasy Plant Pro kit to extract DNA from rhizosphere and root samples, respectively, following the manufacturer's instructions. Extracted DNA was quantified using Qubit and stored at −80°C until further use.

### b. Amplicon sequencing and data processing

Amplicons targeting the V4 region of the bacterial 16S rRNA gene using primer-pair 515F-806R (Caporaso et al., 2012) were sequenced at the Colorado State University Next Generation Sequencing facility (Fort Collins, USA) through Illumina MiSeq. 2 × 300 bp paired-end sequencing (Supporting Information: Section S1). Detailed information on the applied workflow to process sequencing data is available in the supplementary information (Supporting Information: Section S2). Briefly, operational taxonomic units (OTUs) were built at 97% sequence similarity using UPARSE (Edgar, 2013) after joining the paired-end reads using FLASH (Magoč & Salzberg, 2011) and several rounds of quality filtering. Singletons and chimeric sequences were discarded as recommended by Edgar et al. (2011). Taxonomies were assigned to OTUs in mothur using the naïve Bayesian classifier (Q. Wang et al., 2007) with minimum bootstrap support of 60% and the SILVA database (v119; Quast et al., 2012) for bacteria. OTUs representing mitochondrial, chloroplast, and other plant-derived sequences were removed. Samples were rarefied to 7700 and 11,800 OTUs for Sites A and B, respectively, corresponding to the minimum number of sequences for a single sample obtained across sites.

### c. Quantification of CLas

CLas titer were determined by quantitative real-time PCR (qPCR) assays in 96-well plates using an ABI Prism 7500 Sequence detection system (Applied Biosystems). Primer/probe set CQULA04F-CQULAP10P-CQULA04R was used to target the β-operon region of CLas, and qPCR reactions were performed using QuantiTect probe qPCR kit (Qiagen Inc.) according to the conditions described in Trivedi et al. (2012; Supporting Information: Materials S2). Target copy numbers for each reaction were calculated from the standard curve and were used to convert individual Ct values into pathogen tite as genome equivalents gram−1 of samples (Supporting Information: Materials S3).

### d. Soil properties and enzymatic activities

Soil pH was assessed using the field-moist soil-to-water ratio of 2.5 using a Delta pH meter (Mettler-Toledo Instruments Co.). Organic matter (OM) was measured after the initial drying of soil samples at 105°C, followed by ignition in a muffle furnace at 360°C (Nelson & Sommers, 1996). The percent weight loss during the ignition step is reported as OM-loss-on-ignition (%). Nitrate was calorimetrically analysed (Sims et al., 1995) from 0.5 M K2SO4 soil extracts using a 1:5 soil: extract ratio. Soil enzymatic activities including β-Glucosidase, β-D-celluliosidase, β−Xylosidase, α-Glucosidase, N-acetyl-β-Glucosaminidase, phosphatase, and Leucine-amino-peptidase (LAP) activities were measured using 4-methylumbelliferyl (MUB) and 7-Amino-4-methylcoumarin (MUC; only for LAP) substrates yielding the highly fluorescent cleavage products MUB and MUC upon hydrolysis (Bell et al., 2013; details in Supporting Information: Section S4).

### e. Bacterial coexistence patterns

Co-existing relationships of bacterial OTUs were predicted via the network analysis using SparCC-based algorithm Fastspar (Friedman & Alm, 2012; Watts et al., 2018). To minimize the spurious correlations from rare taxa, OTUs with less than three occurrences or less than five total reads were excluded from the calculation. We performed 100 bootstraps to control the false discovery rate, and only strong ($r > 0.6$) and significant ($p < 0.01$) correlation between OTUs were retained in the network. Nodes indicate microbial OTUs, and edges indicate correlations between OTUs; the width of each edge is proportional to the robustness of the correlation. The modularity (clusters of intimately related OTUs) of the network was characterized using Gephi, and nodes were colored as per their modules.

### f. Statistical analysis

We used the R package mctoolsr to analyze microbial community structure (Leff, 2019). To examine beta-diversity, Bray-Curtis distances were calculated and then ordinated in multidimensional scaling using a canonical analysis of principal coordinates (CAPs) analysis constrained to management treatments. Permutational multivariate analysis of variance (PERMANOVA) models were generated to determine significant beta-diversity differences correlating with management treatments. Differences in soil properties and CLas titer were evaluated through general linear models, and Tukey HSD tests were used to determine the influence of management practices on different variables. The statistical analyses were completed using (R www.r-project.org), and a significance level of $\alpha \leq 0.01$ was used. We used Spearman correlation analysis to describe the direction and strength of the relationships (positive or negative) between soil and microbial variables and CLas titer. Spearman correlations were conducted in R with the hmisc package (Harrell & Harrell, 2015). To obtain a quantitative index of microbial functioning and activity (i.e., the ability of the soil microbes to maintain multiple processes simultaneously) for each sample, we first normalized (log-transformed when needed) and standardized each of our seven ecosystem functions (activity of soil enzymes) using the approach described in L. Wang et al. (2019). Following this, the standardized ecosystem functions were averaged to obtain a soil functioning index (L. Wang et al., 2019). This function averaging index is widely used in the soil functioning literature and provides a straightforward and easy-to-interpret measure of the ability of different communities to sustain multiple functions simultaneously (Delgado-Baquerizo et al., 2016; Trivedi et al., 2017; L. Wang et al., 2019).

Using information from the management experiment, we performed random forest (RF) analysis to identify keystone microbes involved in reducing pathogen titer. RF is a powerful machine learning tool that offers high prediction accuracy by using an ensemble of decision trees based on bootstrapped samples from a data set (Breiman, 2001). RF analysis was performed with 999 permutations using the RF and rfPermute packages in R (Archer, 2019). The best predictors were identified based on their importance using the importance and varImpPlot functions. Increases in node purity and mean square error values were used to determine the significance of the predictors using the randomForestExplainer package in R (Paluszynska & Biecek, 2017). The significant factors ($p < 0.01$) were selected as predictors of keystone taxa. Analyses were performed independently for the two sites.

## 4. Results and Discussion

### a. Management practices impact pathogen suppression

Management practices at both sites impacted CLas titer (presented as genome equivalents of CLas per $g^{-1}$ of root samples) in citrus roots (Figure 1A,B). In both sites, uncovered plots have the highest CLas titer (log10 8.56 ± 0.53 and log10 9.37 ± 0.12 for Sites A and B, respectively). CLas tier in mulch-covered treatments (log10 6.11 ± 0.47 and log10 7.52 ± 0.12 for Sites A and B, respectively) were significantly lower than uncovered samples in both sites ($p < 0.001$). In Site A and Site B, microbial (log10 5.42 ± 0.36) and compost (log10 6.27 ± 0.20) treatments were the most effective management practices, each reducing CLas titer by 35% compared to standard, uncovered management practices. Reports from other pathosystems have shown that interactions between plants, their associated microbiome, and pathogens can be influenced by environmental factors, including but not limited to management practices, climate, and soil properties, for example, nutrient content (Campos et al., 2016; Carrión et al., 2019; Hannula et al., 2019; Ou et al., 2019; Trivedi et al., 2017; Wei et al., 2019). Management practices such as reducing or eliminating tillage and crop rotations can result in the development of disease-suppressive soils (Campos et al., 2016; Peralta et al., 2018), supporting the view that soil agroecosystems can be modified to improve disease suppression by enhancing the antibiosis abilities plant-associated microbial communities.

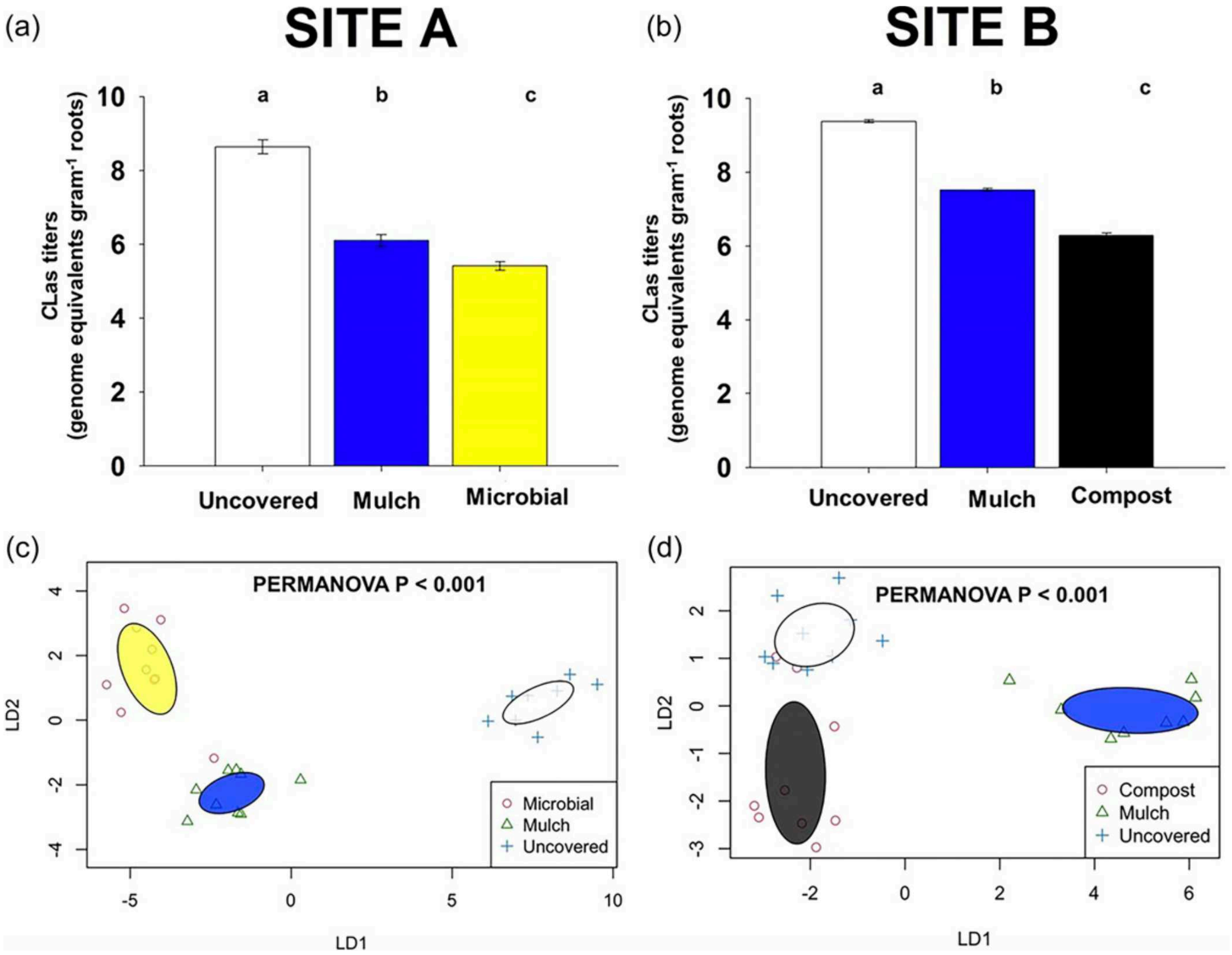


Figure 4.1. Pathogen titer (a, b) in the root and bacterial β-diversity (c, d) in rhizosphere samples from different management practices at Amelia farms (Site A) and Vero Beach (Site B). The panels (a) and (b) show the CLas titer as genome equivalents of CLas g−1 root samples. Different letters represent statistically significant differences at $p < 0.001$. The panels (c) and (d) show ordination biplots for canonical analysis of principal coordinates (CAP) illustrating the impact of management practices on rhizosphere bacterial communities. CAP analysis is based on the Bray-Curtis dissimilarity. White, blue, yellow and black colored bars (a and b) and symbols (c and d) represent uncovered, covered, microbial and compost treatments, respectively.

### b. Management practices impact microbial community structure and composition

Management decisions in agricultural systems can be important drivers of community changes in the soil or plant microbiome leading to the buildup of disease-suppressive soils. To explore whether rhizosphere bacterial community structure and composition correlate with management practices, we visualized and quantified the differences between microbial communities (β-diversity) using CAPs and PERMANOVA on Bray-Curtis dissimilarities (Figure 1C,D). The clear separation pattern indicated distinct microbial community composition in different management practices at both sites. Bacterial community differences were further confirmed by PERMANOVA, which showed a clear distinction among microbial community composition in different managements within both sites ($p < 0.001$). Few studies have reported that compost application influences the rhizosphere microbial community structure and functions. Compost-induced changes in microbial communities were highly correlated with decreased severity of soil-borne diseases and improvement in plant performance (Antoniou et al., 2017; Tsolakidou et al., 2019; Zhao et al., 2018). Compost amendment increases microbial taxon and functional diversity, microbial activities, and the abundance of several groups closely related to biocontrol and plant growth-promoting agents (Antoniou et al., 2017; Tsolakidou et al., 2019; Zhao et al., 2018). Ground cover provided by mulching is reported to alter soil microbiome composition (Dong et al., 2017; Huang et al., 2019; Luo et al., 2019). Since the microbial inoculants applied in Site A consisted of a bacterial consortium composed of several strains belonging to Firmicutes, microbial treatments ($8.90 \pm 2.81$) in Site A had significantly higher ($p < 0.01$) relative abundances of bacteria belonging to Firmicutes compared to uncovered ($2.38 \pm 1.22$) and mulch ($5.89 \pm 2.61$) treatments. However, in line with other studies (Deng et al., 2019; Guo et al., 2019; Schmidt et al., 2014), our results demonstrate that a microbial amendment treatment with nonnative bacteria leads to significant changes in microbial community composition and increases in the abundance of the microbes present in the exogenously applied microbial treatment itself are not the primary driver of this difference. Overall, we observed clear changes in the rhizosphere bacterial community in response to different management practices correlated with reduced CLas titer. In general, our results suggest that microbiome manipulation through selective management practices has the potential for HLB management.

### c. Management practices impact pathogen suppression

Analysis of the collected soil samples showed that management practices significantly impacted soil characteristics ($p < 0.001$; Figure 2). The soils under mulch (both sites), compost (Site B), and microbial treatments (Site A) exhibited higher OM and nitrate content as compared to uncovered. The pH of soil from the uncovered was significantly higher (7.02 ± 0.13 and 7.32 ± 0.13 for Sites A and B, respectively) as compared to other management practices (6.69 ± 0.11 and 6.61 ± 0.14 for mulch and microbial treatments, respectively in Site A and 6.8 ± 0.11 and 6.7 ± 0.17 for mulch and compost treatments respectively in Site B). Soil microbial functioning and activity (used here as a proxy of soil health) was threefold higher in mulch and microbial treatments in Site A and twofold higher in mulch and compost treatments in Site B compared to uncovered treatments. Our results showed that CLas titer is highly correlated ($p < 0.001$) with management-induced shifts in microbial community composition and functions (Figure 3).

Soil functions carried out by soil microbial communities are linked with the inhibition of bacterial and fungal pathogens (Jaiswal et al., 2017; Trivedi et al., 2017). Variation in the composition and functioning of the soil microbiome in response to management practices can lead to microbiota-mediated disease resistance. This occurs due to changes in the abundance of specific taxa that possess multiple traits related to plant protection, including direct antagonism of the pathogen or induction of plant defense responses. We observed significant correlations between management-induced changes in soil pH and soil nutrients with CLas titer (Figure 3). Interestingly, lowering pH through acidification of irrigation water in the central and south-central citrus growing regions of Florida has been shown to reduce the decline in root density associated with HLB (Morgan & Graham, 2019). Similarly, increases in soil OM and N content are reported to increase growth by lowering the CLas titer in both young and old citrus trees (Xu et al., 2013).

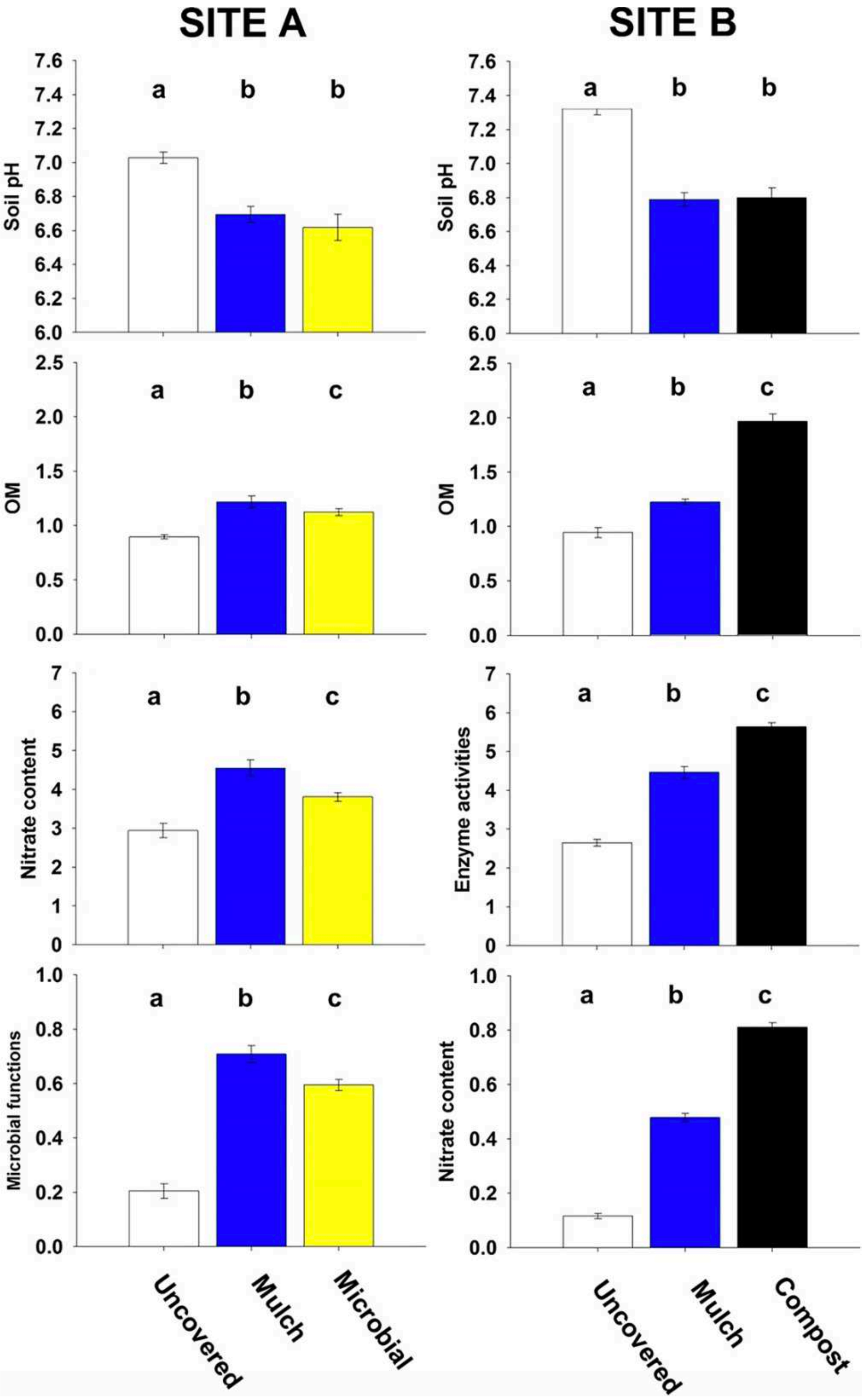
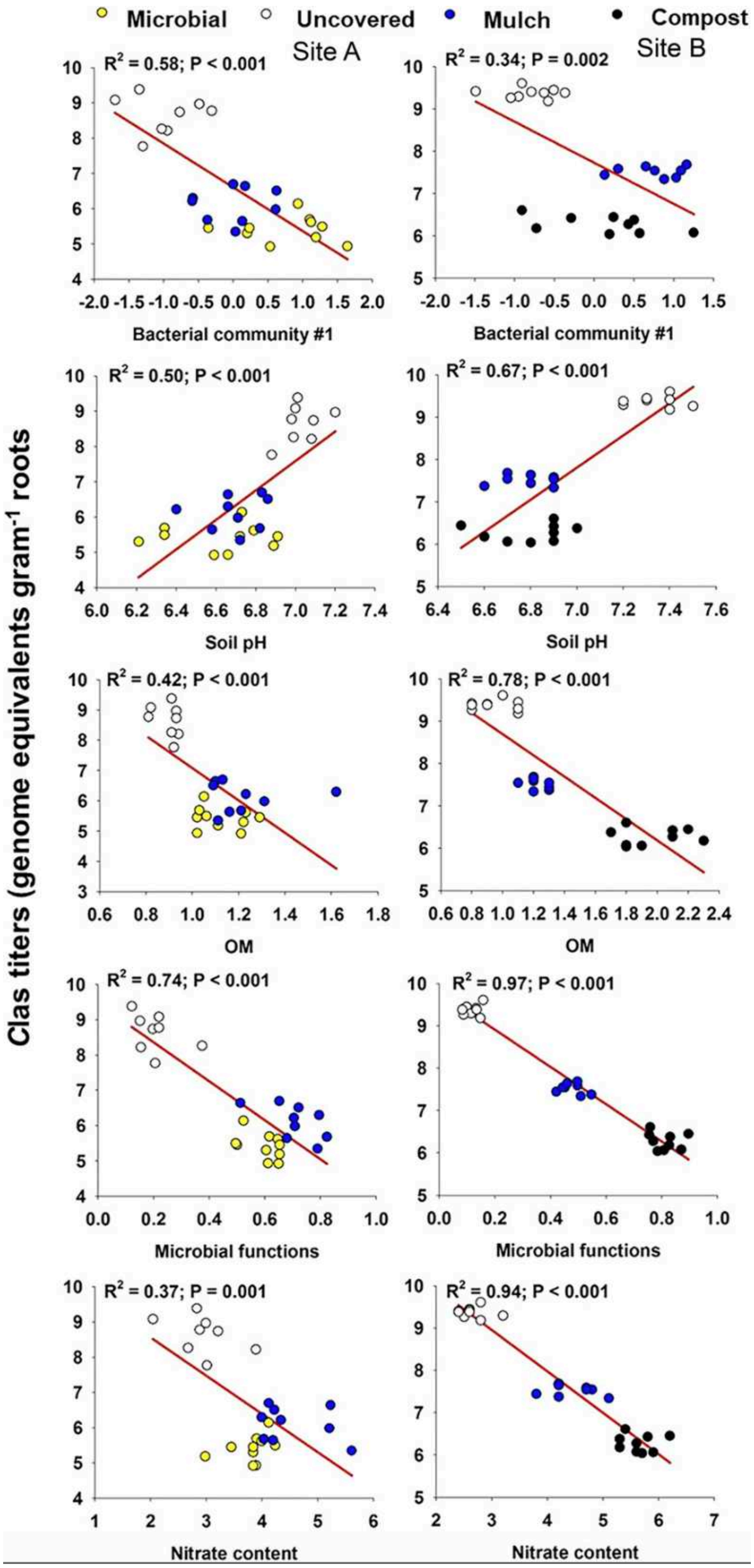

SITE A
SITE B
Soil pH
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
OM
2.5
2.0
1.5
1.0
0.5
0.0
Nitrate content
Enzyme activities
7
6
5
4
3
2
1
0
Microbial functions
Nitrate content
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
c
Uncovered
Mulch
Microbial
Compost

Figure 4.2. Differences in the physicochemical soil properties and microbial functions between different management practices at Amelia farms (Site A) and Vero Beach (Site B). Organic matter (OM) is presented as OM-loss-on-ignition (%). Nitrate content is reported as Nitrate-N ppm N. Microbial functions were calculated by standardising the activity of seven soil enzymes followed by averaging the values to obtain a soil functioning index (L. Wang et al., 2019). Different letters represent statistically significant differences at $p < 0.001$.



Microbial
Uncovered
Mulch
Compost
Site A
Site B
Clas titers (genome equivalents gram-1 roots
R2 = 0.58; P < 0.001
R2 = 0.34; P = 0.002
Bacterial community #1
Bacterial community #1
R2 = 0.50; P < 0.001
R2 = 0.67; P < 0.001
Soil pH
Soil pH
R2 = 0.42; P < 0.001
R2 = 0.78; P < 0.001
OM
OM
R2 = 0.74; P < 0.001
R2 = 0.97; P < 0.001
Microbial functions
Microbial functions
R2 = 0.37; P = 0.001
R2 = 0.94; P < 0.001
Nitrate content
Nitrate content

Figure 4.3. Correlation between physicochemical soil properties and microbial functions with CLas titer under different management practices in two field sites. Organic matter (OM) is presented as OM-loss-on-ignition (%). Nitrate content is reported as Nitrate-N ppm N. Microbial functions were calculated by standardising the activity of seven soil enzymes followed by averaging the values to obtain a soil functioning index (L. Wang et al., 2019).

### d. Management practices influence changes in rhizosphere bacterial coexistence networks

We constructed bacterial networks to identify the impact of management practices on the coexistence patterns of rhizosphere bacterial communities (Figure 4). We observed significant changes in modular and multiple topological properties of the bacterial coexistence patterns in response to management practices for both sites. Higher node and edge numbers, smaller modularity, and average path length represent greater network complexity. There are only a few reports on the impact of the pathogen on the stability of microbial coexistence networks. Our results demonstrate that the bacterial coexistence networks of uncovered treatment with the highest CLas titer were the least complex of the treatments for both sites. In addition, for both sites, the uncovered treatments had the lowest number of nodes and edges compared to mulch, microbial, or compost treatments. In line with our results, other studies have demonstrated decreased network complexity with increased pathogen pressure (Gao et al., 2021; Wei et al., 2019). Increased network complexity in the mulch, microbial and compost treatments may result from enhanced resource availability (e.g., increased availability of water, soil carbon, and nutrients) and increased soil functionality that positively influences microbial diversity and network complexity (Banerjee et al., 2016, 2019; de Vries et al., 2018; Qi et al., 2019). Our results suggest that bacteria under mulch, compost, or microbial management have robust interactions that could potentially increase community stability under pathogen infection compared to control (Wei et al., 2015, 2019). Microbial coexistence networks allow researchers to predict and generate new hypotheses on microbial interactions. While these hypotheses need experimental validation, our results suggest that microbial interactions are important for reducing CLas titer in citrus roots.

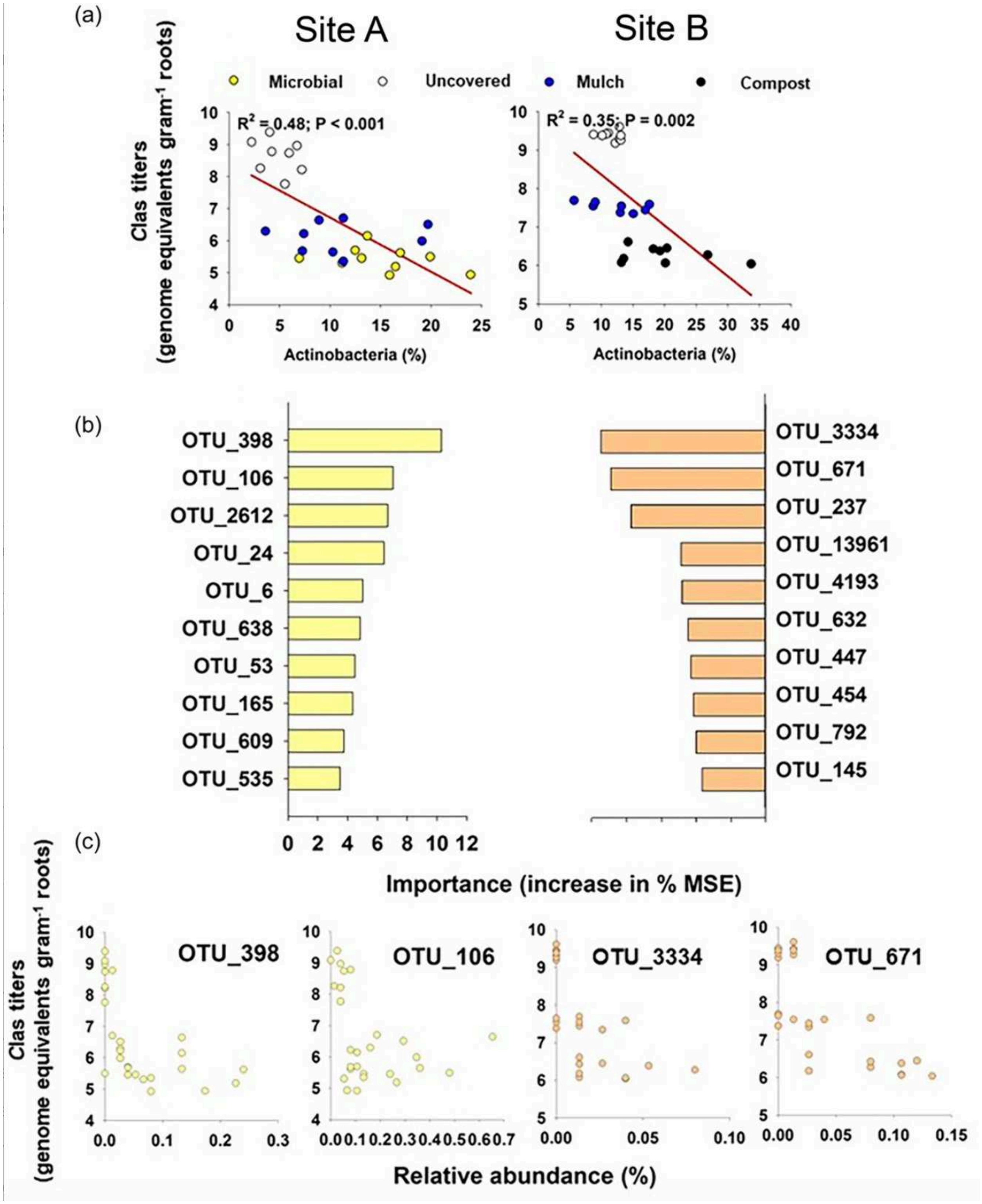


Figure 4.4. Actinobacteria as the modulator of HLB suppression. (a) Correlation between the relative abundance of Actinobacteria and CLas titer under different management practices in Site A and B. (b) The most important groups within Actinobacteria that were related to the reduction

in CLas titer in both sites. Taxonomic details of OTUs are provided in Supporting Information: Table 1. (c) Correlation between CLas titer and the relative abundance of the most effective bacterial groups identified by RF analysis. Yellow and light brown colours indicate the bacterial OTUs from Site A and Site B, respectively. MSE, mean square error.

### e. Microbial Modulators of CLas suppression

The relative abundance (%) of phylum Actinobacteria was significantly higher ($p < 0.01$) in mulch (both Sites A and B), microbial (Site A), and compost (Site B) treatments as compared to uncovered treatment. In Site A, the relative abundance was $4.86 \pm 1.76$, $10.91 \pm 3.34$ and $15.05 \pm 3.60$ in uncovered, mulch, and microbial treatments, respectively. The relative abundance of Actinobacteria was $11.60 \pm 1.54$, $14.36 \pm 2.24$ and $19.37 \pm 2.70$ at Site B in uncovered, mulch, and compost treatments, respectively. Our results demonstrated significant negative correlations between the relative abundance of Actinobacteria and CLas titer ($p < 0.001$ and 0.002 for Sites A and B, respectively; Figure 5A). Both compost amendments (Antoniou et al., 2017; Zhao et al., 2018) and plastic film mulching (Dong et al., 2017) increase the relative abundance of Actinobacteria in a range of soil types. Actinobacteria play an essential role in soil metabolic functioning (Bhatti et al., 2017). The increase in the relative abundance of this group might provide increased soil functions that could be related to plant health and disease suppression. Actinobacteria are recognized as keystone taxa in disease-suppressive soils (Cha et al., 2016; Mendes et al., 2011). The abundance of Actinobacteria at a continental scale can predict soil suppressiveness towards Fusarium oxysporum (Trivedi et al., 2017). Management-induced increase in the relative abundance of Actinobacteria has been reported to reduce pathogen pressure and control soil-borne plant disease (Cha et al., 2016; Cordovez et al., 2015; Lee et al., 2021). Interestingly, several species of citrus-associated Actinobacterial isolates are antagonistic to CLas and various other citrus pathogens (Riera et al., 2017; Trivedi et al., 2011). We postulate that incremental increases in the abundance of Actinobacteria members through artificial inoculation or change in management practices have the potential to control HLB by reducing pathogen titer.

We used RF analysis to identify Actinobacteria phylotypes which are highly associated with the abundance of HLB pathogens. RF analysis identified different groups highly correlated with a reduction in CLas titer (Figure 5B,C, Supporting Information: Table 1). Members of the microbial groups identified in our study, such as those belonging to Gaiellaceae, Streptomycetaceae, and Mycobacteriaceae, are reported to be dominant in soils resistant to soil-borne fungal diseases, including fusarium-induced wilt (Peng et al., 1999) and Panama disease (Xue et al., 2015) of banana. Poudel et al. (2016) identified various families of Actinobacteria, including Gaiellaceae are related to healthy plants. Network analysis of microbial community time series in citrus leaves and root samples identified a mutually exclusive relationship between Liberibacter spp. and members of the Micromonosporaceae and Streptomycetaceae (Blaustein et al., 2017). An option for disease control is to isolate and screen beneficial bacteria that are antagonistic to the pathogen and develop microbial consortiums to suppress the pathogen (Berendsen et al., 2018; Vannier et al., 2019). Our work has the potential to initiate systemic efforts that apply knowledge of the impact of management practices on the native soil microbiome to screen beneficial microbes and design microbial consortiums for HLB control.

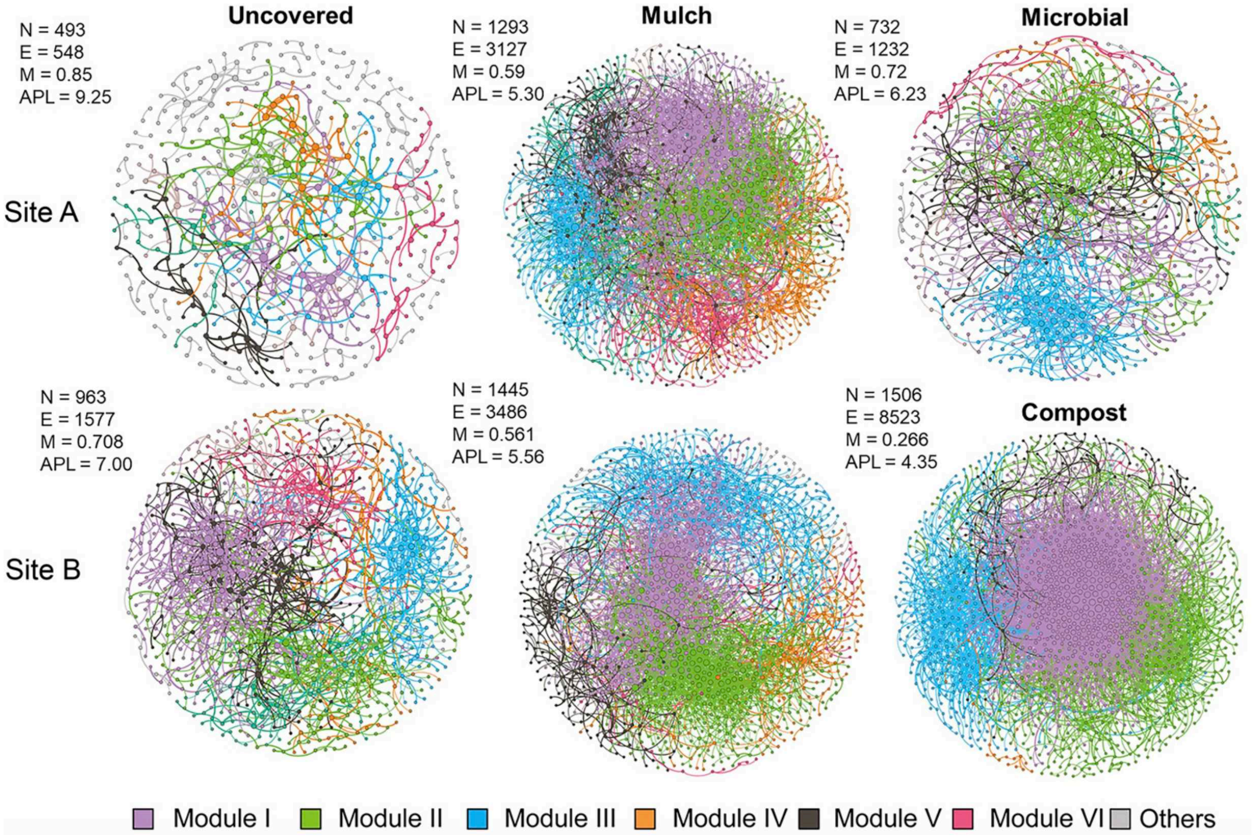


Figure 4.5. Bacterial correlation networks including multiple nodes (taxa) under different management practices. Different colours correspond with different modules. Topological features of individual networks are presented in the top of each network. APL, average path length; E, edge; M, modularity; N, node.

## 5. Conclusion

Management-induced shifts in bacterial community composition, microbial functions, and soil physiological properties interactively shape soils' suppressive potential resulting in the reduction in CLas titer. The similar results reported for two independent field sites suggests that it may be possible to develop a general approach to engineering disease-suppressive soil microbiomes for HLB control. Moreover, the relative abundance of soil Actinobacteria can serve as an effective biological indicator for developing prediction models to identify and characterize management practices that increase HLB suppression. These findings open the door to reducing the incidences of HLB both indirectly through management practices and directly through microbial amendment application. Future studies providing a mechanistic understanding of the interactions between antagonistic microbes with CLas will guide the large-scale adoption of sustainable management practices that leverage the microbiome to reduce HLB progression and plant damage.

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Supplementary Information:

## Material and Methods

### S1. DNA amplification and sequencing

Quality control, library preparation, amplification and sequencing all completed at CSU Next Generation Sequence Core Facility, Fort Collins. Purified DNA concentrations from samples were validated using Qubit Broad Range assay for DNA concentration determination. Illumina adapters and sample identifying barcodes were attached to the individual DNA samples and then amplified, targeting the V4 region, by direct PCR using the primers 515F and 806rB following the Earth Microbiome 16S rRNA Amplification protocol (Caporaso et al., 2012). PCR conditions were 94°C for 2 minutes; 30 cycles 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute followed by a final extension step of 72°C for 5 minutes. Samples were normalized and pooled into a single tube. The total DNA pool gel isolated to purified and remove spurious bands from the library, then validated using an Agilent TapeStation DNA assay (Agilent Technologies, Santa Clara, CA). The pooled samples then sequenced using a 150 base pair pair-ended read on the Illumina MiSeq Platform (Illumina, Inc., San Diego, CA). Resulting sequences demultiplexed, barcode and primer sequences trimmed and output into FASTQ format.

### S2. Sequence data processing

After qualitative assessment of run quality of all Illumina forward (R1) and reverse (R2) reads using FastQC (Andrews 2010), Illumina adapters and 16S primer sequences (forward_16S_505f =TTACCGCGGCKGCTGGCACACAATTACCATAGTGTAGATCTCGGTGGTCGCCGTAT CATT , rev_16S_806R = ATTAGAWACCCBDGTAGTCCGGCTGACTGACT) were removed using default parameters in 'cutadapt' (Martin 2011). Sequences were demultiplexed using the Illumina I1 barcode reference with using a custom Python script in preparation for the UPARSE pipeline (Edger 2013). Forward and reverse reads were merged using usearch v. 11 with a minimum overlap 16 bp and a minimum merge length of 200 bp. Amplicons were filtered to a maximum expected error of 1.0 base over the merged amplicon. A representative set of operational taxonomic units was created by dereplicating and denoising with uNoise3 using the usearch v11 toolkit. Quality filtered amplicons were mapped to this representative set to obtain raw OTU counts using usearch v 11.0 and OTU taxonomy was determined using the RDP Naïve Bayesian classifier (Wang et al., 2007) against the current Silva database. Bacterial OTUs that were unclassified at kingdom and/or phylum level were subjected to a blastn search, and OTUs matching plant-derived sequences were also removed. The 16S rRNA primers used in this study also amplified archaeal sequences (resulting in 68 archaeal OTUs), but these archaeal sequences represented a minority (0.02% of total reads) and therefore were excluded from analysis.

### S3: **Quantification of *C*Las**

The *C*Las-specific plasmid template, pLBA2, was developed by cloning a portion of β-operon DNA sequence of the *C*Las (rplKAJL rpoBC; Hocquellet et al., 1999). A 703 bp fragment was amplified using Liberibacter-specific primers A2 and J5 by using DNA extracted from infected leaf midribs as a template (Hocquellet et al., 1999). Amplification of the DNA was performed using 2× PCR Master Mix (Promega, Madison, WI, USA) in 50 µl total volumes containing 0.4 µM of each primer and 100 ng of DNA template. The PCR conditions were 95°C for 5 min followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C. The PCR products were electrophoresed on 1% agarose gel and the expected size DNA band was excised and purified using Wizard® SV gel and PCR clean-up system (Promega). The purified DNA was ligated to pGEM T- easy vector (Promega) and transformed into chemically-competent *Escherichia coli* (DH5α). The positive clones were confirmed by sequencing of the resultant plasmid (pLBA2; data not shown).

Standard curve for CLas quantification was prepared by isolating plasmid DNA from *E. coli* containing pLBA2 using a Wizard® miniprep DNA purification system (Promega) following manufacturers instruction. The concentration and purity of DNA were determined on a NanoDrop

ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). To identify the detection limit and to develop a standard curve, a known concentration of plasmid in a series of dilutions ranging from $2\times10^{10}$ to $2\times10^{1}$ $\mu l^{-1}$ was used. The reaction mixture without the plasmid DNA was used as a negative control.

All QPCR assays were performed in a 96-well plate using an ABI PRISM 7500 Sequence detection system (Applied Biosystems, Foster City, CA, USA). Primer/probe combinations, CQULA04F- CQULAP10-CQULA04R, were used to target the β- operon region of *C*Las (Wang et al., 2006). The probes were labelled with 56-FAM as a reporter fluorescent dye at the 5′ end and with 3'BHQ 1 as the quencher dye. qPCR reactions were performed according to the conditions described previously in Trivedi et al. (2012). Briefly, qPCR reactions were performed in a 25 µl reaction using a 2× Quantitect Probe PCR master mix (Qiagen, Valencia, CA, USA), 0.8 µM of each primer, 0.4 µM of probe (IDT, Coralville, IA, USA) and an appropriate amount of template DNA for generation of the standard curve. One µl of DNA was used as a template to quantify *C*Las for the root tissue samples. The average Ct values used for estimating *C*Las were determined by using the equation generated from the log curve. The PCR conditions were 50°C for 2 min, 95°C for 15 min, 45 cycles of 94°C for 15 s and 60°C for 1 min. Each individual sample was replicated 4 times on a 96-well plate and the whole reaction is repeated twice to verify the consistency of the method. Results were analyzed using ABI Prism software. Raw data were analyzed using the default settings (threshold = 0.2) of the software.

## S4: Soil enzyme assays

Soil enzymatic activities including β-Glucosidase (BG), β-D-Celluliosidase (CB), β−Xylosidase (XYL), α-Glucosidase (AG), N-acetyl-β-Glucosaminidase (NAG), Phosphatase, and Leucine-amino-peptidase (LAP) activities were measured using 4-methylumbelliferyl [MUB] and 7-Amino-4-methylcoumarin (MUC; only for LAP) substrates yielding the highly fluorescent cleavage products MUB and MUC upon hydrolysis. All the enzyme assays were set up in 96-well microplates as described by Bell et al. (2013). Twelve replicate wells were set up for each sample and each standard concentration. The assay plate was incubated in the dark at 25 °C for 3 h to mimic the average soil temperature. Enzyme activities were corrected using a quench control.

Fluorescence was measured using a microplate fluorometer (EnSpire 2300 Multilabel Reader, Perkin Elmer, Waltham, MA, USA) with 365-nm excitation and 460-nm emission filters. The activities were expressed as nmol $h^{-1}$ $g^{-1}$dry soil.

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**Supplementary Table 1:** Taxonomic information on bacterial OTUs were related to the reduction in *C*Las titers as determined by RF analysis.

| | |
|---|---|
| OTU_398 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_106 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Mycobacteriaceae; g__Mycobacterium; s__ |
| OTU_2612 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Micrococcaceae |
| OTU_24 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Micromonosporaceae; g__; s__ |
| OTU_6 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae; g__Streptomyces |
| OTU_638 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Kineosporiaceae; g__; s__ |
| OTU_53 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae; g__Streptomyces; s__lanatus |
| OTU_165 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales |
| OTU_609 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |

| | |
|---|---|
| OTU_535 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Solirubrobacterales; f__; g__; s__ |
| OTU_3334 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_671 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_237 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Geodermatophilaceae; g__; s__ |
| OTU_13961 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Mycobacteriaceae; g__Mycobacterium; s__ |
| OTU_4193 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__; g__; s__ |
| OTU_632 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_447 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Frankiaceae; g__; s__ |
| OTU_454 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_792 | k__Bacteria; p__Actinobacteria; c__Thermoleophilia; o__Gaiellales; f__Gaiellaceae; g__; s__ |
| OTU_145 | k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Nocardioidaceae; g__Nocardioides; s__ |