Understanding plant-microbe interactions and their impact on plant-host stress resilience through the lens of multi-omics

by

Anna Kazarina

B.S., Alcorn State University, 2018
M.S., Kansas State University, 2021

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

While the implications of plant-microbe interactions are well-established, significant knowledge gaps remain in understanding how plants shape microbial communities and how these microbes affect plant performance. My dissertation explores the role of plants' local adaptation through microbial recruitment and plant–microbe communication using various methods, from broad community fingerprinting to integrated multi-omics.

My first study evaluated the relative importance of plant genetic background and environmental factors in shaping rhizosphere bacterial communities, using the perennial C4 grass *Andropogon gerardii* as a model. Employing a reciprocal garden experiment across a sharp precipitation gradient with distinct regional ecotypes, I characterized ecotypic rhizosphere communities using 16S rRNA amplicon sequencing. The results showed that abiotic conditions and the local soil microbes were the primary drivers of community composition. However, the plant ecotypic effect was also significant, highlighting active microbial recruitment driven by plant genetic background. Interestingly, plants grown at their native sites were more successful at recruiting location-unique microbes, supporting the *"home-field advantage"* hypothesis. These unique microbes may represent microbial specialists linked to plant stress responses. Additionally, ecotype-specific recruitment of congeneric but genetically distinct bacterial variants highlighted the fine-scale influence of host genotype on rhizosphere microbiome assembly.

The second study builds on the first one and explores potential mechanisms of *"home-field advantage."* Using gene-centric and genome-centric metagenomic approaches, I investigated the functional potential of root-associated bacteria and their genetic capacity to support plant stress resilience. I also profiled plant metabolic products that may govern rhizosphere recruitment and are potentially produced in response to plant-microbe interactions. Findings suggested that plants

adapted to drier environments experience less stress, producing fewer stress-related metabolites while recruiting microbes with genes linked to stress alleviation. Notably, plant-derived trimethyllysine was highly associated with microbial populations that improve nutrient uptake, promote plant growth, and modulate stress responses.

The third study switches focus to validation and application, exploring the enhancement of plant-host resilience through the creation of a plant-oriented SynCom. Using culturomics, I single-cell sorted and cultured microbes from rhizosphere soils and assembled their draft genomes. I then evaluated their potential communication and beneficial functional potentials, as well as provided insights into the microbial metabolites. The results revealed that bacterial isolates from both arid and wet environments have the potential to positively influence plant growth, resilience, and support other microbes through the exchange of signaling molecules and metabolic products. Notably, arid-environment isolates produced distinct metabolites associated with stress tolerance and support of plant growth under drought conditions, including lyso-phosphatidylethanolamine 17:1 (LPE 17:1). While the exact function of LPE 17:1 is unknown, I hypothesized that it could enhance the plant host's tolerance to drought stress through priming the host immune system.

This dissertation advances our understanding of the complex factors influencing plant adaptation to local environments. Furthermore, it sheds light on the mechanisms underlying plant-microbe communication, rhizosphere recruitment, and the complex interactions within the holobiont system, offering a foundation for future research.

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Table of Contents

List of Figures xiii

List of Tables xix

Acknowledgements xx

Dedication xxiv

Chapter 1 - Introduction 1

1.1 Importance of the Plant-Microbe Interactions 2

1.1.1 The Evolution of Human Understanding in Plant-Microbe Interactions 2

1.1.2 Holobiont system 3

1.1.3 Rhizosphere functions 5

1.1.4 Plant-microbe communication and the plant immune system 7

1.2 Plant-microbe interactions: past and present 13

1.2.1 First sequencing and Fingerprinting 13

1.2.2 Amplicon sequencing: microbial community composition and diversity 18

1.2.3 SynComs 23

1.2.4 -omics technologies in studying holobiont 26

1.3 The structure of the dissertation 33

Chapter 2 - Home-field advantage affects the local adaptive interaction between *Andropogon gerardii* ecotypes and root-associated bacterial communities 39

2.1 Abstract 40

2.1.1 Importance 41

2.1.2 Keywords 41

2.2 Introduction 41

2.3 Materials and Methods 44
2.3.1 Study sites, samples collection and processing 44
2.3.2 Soil chemistry and soil properties analyses 47
2.3.3 Sequence data processing and analyses 48
2.3.4 Oligotyping analyses 50
2.4 Results and discussion 50
2.4.1 Differences in precipitation are associated with the distinct soil chemistry and texture parameters across sites 51
2.4.2 Site had a strong impact on the ecotypic root-associated bacterial communities' richness and diversity 55
2.4.3 Ecotypes shaped the rhizosphere inhabiting bacterial richness and diversity . 59
2.4.4 Matching of the plant host ecotype and root-associates bacterial communities was evident at the ecotype homesite 64
2.5 Conclusion 70
2.6 Acknowledgements and Funding 71
2.7 Author Contributions 71
2.8 Conflict of Interest 72
Chapter 3 - Interaction of plant-derived metabolites and rhizobiome functions enhances drought stress tolerance 73
3.1 Abstract 74
3.1.1 Background 74
3.1.2 Results 74
3.1.3 Conclusion 74

3.1.4 Keywords 75
3.2 Background 75
3.3 Results and Discussion 78
3.3.1 Distinct metabolic profiles between the Dry and Wet ecotypes reflect the Dry ecotype's adaptation to the “home” environment 80
3.3.2 Microbial communities differ in functional potential and their impact on the plant host 83
3.3.3 Distinguishable microbial populations and pathways were associated with ecotype and precipitation 87
3.3.4 Ecotype–microbial interactions resulted in enhanced plant host resilience under drier conditions 95
3.4 Conclusions 103
3.5 Methods 104
3.5.1 Selection of rhizosphere samples and shotgun metagenomics 104
3.5.2 Bioinformatic workflow of gene-centric metagenomic analyses 105
3.5.3 Bioinformatic workflow of MAGs assembly and genome-centric metagenomic analysis 106
3.5.4 Phylogenomic analysis and functional potential of MAGs 107
3.5.5 Metabolomic profiling of the plant host rhizosphere 108
3.5.6 Statistical analysis 111
3.6 Acknowledgments 112
3.7 Author Contributions 112
3.8 Declarations of Interests 113

3.9 Funding 113
Chapter 4 - Native rhizobiome immune priming mechanism increases plant host stress resilience 114
4.1 Abstract 115
4.1.2 Keywords 115
4.3 Introduction 116
4.4 Materials and Methods 119
4.4.1 Culturing of microbes 119
4.4.2 Metabarcode sequencing of soil and microbial consortium 120
4.4.3 Oligotyping analysis 122
4.4.4 Single cell microbial isolates 122
4.4.5 Phylogenomic analysis 124
4.4.6 Microbial metabolomics 124
4.5 Results and Discussion 125
4.5.1 Culturomics of ecotypic rhizobiome revealed the abundance of the plant-growth promoting bacteria 126
4.5.2 Dry ecotypic bacterial isolates showed high number of microbial associated functions that confer benefits to plant host 129
4.5.3 High abundance of antibacterial, communication and plant-host growth promoting microbial metabolites in Dry ecotypic isolates 135
4.5.4 Dry cultured bacterial metabolite showed potential of plant-host immune system priming 139
4.6 Conclusion 141

4.7 Acknowledgements 143
4.7 Author Contributions 144
4.8 Competing Interests 144
Chapter 5 - Discussion and Conclusion 145
References 155
Appendix A - Supplementary Tables 211
Appendix B - Permissions 213

List of Figures

Figure 2.1 Soil chemistry across locations and ecotypes. (A) Soil chemistry and water content among three sites located across the precipitation gradient: Hays, Manhattan, and Carbondale. Soil pH, %C, %N, and the water content. (B) %C and %N concentration across dry, mesic, and wet ecotypes. 52

Figure 2.2 Bacterial alpha and community composition among three sites located across precipitation gradient. (A) Bacterial α-diversity indices among three sites located across precipitation gradient: Hays, Manhattan, and Carbondale. Species observed richness (S_{Obs}), Shannon's H index, and Faith-PD index. (B) Location impact on *Andropogon gerardii* rhizosphere bacterial composition. NMDS plot of *A. gerardii* rhizosphere communities associated with three site locations across the precipitation gradient: Hays, Manhattan, and Carbondale. NMDS ordinations were obtained from Bray–Curtis similarity matrix (P-values: * −0.05, ** −0.01, *** −<0.01)..... 57

Figure 2.3 Bacterial alpha and community composition among three ecotypes. (A) Bacterial α-diversity indices among the dry, mesic, and wet ecotypes across all locations. Species observed richness (S_{Obs}), Shannon's H index, and Faith-PD index. (B) Ecotype impact on *Andropogon gerardii* rhizosphere bacterial composition. NMDS plot of *A. gerardii* rhizosphere communities associated with three ecotypes across three site locations. NMDS ordinations were obtained from Bray–Curtis similarity matrix (P-values: * −0.05, **–0.01, ***–<0.01)..... 61

Figure 2.4 Venn diagrams representing the overlapping and unique ASVs (numbers in the circle) among (A) all locations, (B) all ecotypes, and (C) ecotypes in Carbondale, ecotypes in Hays, and ecotypes in Manhattan. The circle with a red edge represents the home ecotype for that

location. Inserts represent the top three families. Partial information on unique ASVs is shown here, full information is in Supplementary Table 16. Higher numbers of unique ASVs suggest the "home-field advantage" of the *Andropogon gerardii* ecotypes. 63

Figure 2.5 Relative abundance of each oligotype within the Pseudomonadales and Rhizobiales diversity for each sample among three sites located across the precipitation gradient. The proportion of the relative abundance of the (A) Pseudomonadales (red, top) and Pseudomonadales-Oligos (bottom) and (B) Rhizobiales (purple) and Rhizobiales-Oligos (bottom) suggest the impact of locations and ecotypes on rhizobiome strain-level composition. 69

Figure 3.1 The schematics of the experimental design highlighting the flow of the project. Briefly, we collected rhizosphere samples from reciprocal gardens in Carbondale, Illinois, and Hays, Kansas, growing Dry and Wet regional *A. gerardii* ecotypes. We conducted shotgun metagenome sequencing to evaluate the microbial functional potential of these rhizosphere samples using gene- and genome-centric approaches. First, we performed gene-centric taxonomic community profiling (MetaPhlAn) and metabolic pathways profiling (HUMAnN). Next, we assembled 34 non-redundant metagenome-assembled genomes (MAGs) and performed genome-centric profiling of the MAGs. To assess the host metabolic output, we performed plant rhizosphere metabolites profiling. Finally, we conducted a correlation analysis to identify key omics variables across the datasets. 79

Figure 3.2 Plant hosts displayed different metabolic profiles across ecotypes and reciprocal garden locations. (A) The relative abundance of the plant host metabolites within Hays and Carbondale. The Venn diagrams with the number of metabolites significantly different (not overlapped; $p<0.05$) across two locations and bar plots with four metabolites (shortlisted

metabolites) significantly higher in Hays ($p<0.05$). (B) The relative abundance of the plant host metabolites within Dry and Wet ecotypes. The Venn diagram with the number of metabolites significantly different across two ecotypes and three metabolites that were significantly higher in the Dry ecotype. (C) The two-sided bar plot represents the split of the metabolic classes across two ecotypes between the two locations. The Venn diagram with the number of metabolites significantly different between the two ecotypes in Hays and the three metabolites that were significantly higher in the Dry ecotype in Hays. 81

Figure 3.3 The gene-centric profiling of potential bacteria taxa (MetaPhlAn) and metabolic pathways (HUMAnN) corroborated the *"home-field advantage"* hypothesis, showing the Dry ecotype displaying resilience in the drier environment of Hays. (A) Stacked bars with the relative abundance of the taxonomic phyla within two locations and two ecotypes. (B) The abundance of four *Kribbella* species significantly differed between the Dry and Wet ecotypes in Hays. (C) Two metabolic pathways thant significantly differed between the Dry and Wet ecotypes in Hays, with the higher counts associated with the Wet ecotype. Significant p-values marked by asterisks * represent $p < 0.05$. 85

Figure 3.4 The phylogenomic placement of the MAGs and the detection of MAGS across collected rhizosphere samples. (A) Phylogenomic tree based on alignment of six bacterial ribosomal genes: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. The tree included 34 MAGs and 11 unique closest strains to identify relationships and the confirmation of the identity of MAGs. (B) Clustered heatmap with rows corresponding to 34 MAGs and columns corresponding to rhizosphere samples. The heatmap was clustered by rows and columns to observe the highest detection similarity within samples. Precipitation was the main driver of changes in the detection of the MAGs. 89

Figure 3.5 Genes/subsystems (subset) presence (red) and absence (gray) in selected MAGs. Rows correspond to the subset of genes/subsystems related to microbial mechanisms highly detected in Hays potentially unitized to survive in the drier environment, and columns correspond to the MAGs these genes/subsystems were observed in. 92

Figure 3.6 Plant host-derived metabolite, lower trimethyllysine (TML) positively correlated with five MAGs and was highly associated with Dry ecotype populations in Hays. (A) Network analysis of plant–host metabolite (gray) couples with MAGs detection levels (red). A correlation cutoff of 0.83 was used to visualize only strong associations, with (Green) lines indicating negative correlations and (Red) lines marking a positive correlation of MAGs with the host metabolites. (B) The detection heatmap with rows corresponding to rhizosphere samples collected in Hays and columns corresponding to the five MAGs of interest. (C) The detection of the TML among Dry and Wet ecotypes in Hays. Not significant p-value marked by "ns." (D) The detection of TML among Dry and Wet populations in Hays. The p value marked asterisks * represent $p < 0.05$. 98

Figure 3.7 Genes/subsystems (subset) presence (red) and absence (gray) in the MAGs-of-interest. Rows correspond to the subset of genes/subsystems related to microbial mechanisms detected in five MAGs (columns) of interest, potentially promoting plant host survival and environmental performance. 101

Figure 4.1 The schematics of the experimental design highlighting the flow of the project. Briefly, we used rhizosphere soil from Dry and Wet regional *A. gerardii* ecotypes to culture associated bacterial communities. We then performed single cell sorting of the microbial populations, followed by functional profiling of isolates, and metabolic profiling of the resultant consortium, to unveil functional mechanisms of microbes..... 119

Figure 4.2 The effect of the sample type and ecotype on the bacterial community composition. (A) Sample type has the highest impact on the bacterial community composition. NMDS plot of microbial communities associated with rhizosphere soils from Dry and Wet regional *A. gerardii* ecotypes, and microbial consortia that were prepared from the ecotypic soils. NMDS ordinations were obtained from Bray–Curtis similarity matrix. (B) Relative abundance of taxa within Dry and Wet consortium samples. Relative abundance of each oligotype in (C) *Pseudomonas* and (D) *Acinetobacter* diversity for each consortium sample. 127

Figure 4.3 Phylogenomic tree based on alignment of six bacterial ribosomal genes: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. The tree included 320 microbial isolates and 11 unique similar strains to identify close relationships and identity of isolates. Additional data layers, i.e. source, ecotype, phylum, family and the position of *Pseudomonas fuscovaginae, Acinetobacter venetianus* and *Arthrobacter globiformis* are incorporated in the figure according to the key 129

Figure 4.4 Metabolic profiling of the Dry and Wet consortiums. (A) Distribution of microbial metabolites (n=1093) found predominantly in the Dry and Wet ecotype consortiums, but not R2A media control. Venn diagram shows the number of shared and unique metabolites across consortia, and stacked bars show the relative abundance of metabolite classes across those groups. (B) The abundance of metabolites of interests detected across Dry and Wet consortia as well as R2A media control. Metabolites of interest represent critical functions in Dry consortium: survival, communication and function. 136

Figure 4.5 LPE17:1 and LPE18:0. (A) Heat map of lyso-phospholipid (Lyso-phosphatidylcholines (LPC) & Lyso-phosphatidylethanolamines (LPE)) metabolites detected across Wet and Dry consortiums. Uninoculated R2A media used for the microbial consortium growth was used

as a control. (B) The schematics showing the glycerophospholipid metabolism pathway converting PE to LPE. Phospholipase A2 (PLA2) is the enzyme that removes a fatty acid group, and carries out partial hydrolysis of PE. (C) PLA2 enzyme observed across all Dry consortium isolates (D) The structure of the LPE17:1 and LPE18:0. 141

List of Tables

Table 2.1 Soil Chemistry ANOVA. Statistical analyses (ANOVA and pairwise test) reveal locations (C-Carbondale, H-Hays, MHK-Manhattan) had a strong influence on the soil chemistry and water content parameters. 54

Table 2.2 Alpha Diversity ANOVA. Statistical analyses (ANOVA and pairwise test) reveal locations (C-Carbondale, H-Hays, MHK-Manhattan) had a strong influence on the rhizobiome diversity and richness..... 58

Table 4.1 Functional potential of the Dry and Wet consortium isolates. The values represent the number of isolates possessing all the necessary genes to perform the potential plant-host beneficial function. Abbreviation keys are in the Appendix A..... 131

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Dedication

To my family

Chapter 1 - Introduction

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1.1 Importance of the Plant-Microbe Interactions

1.1.1 The Evolution of Human Understanding in Plant-Microbe Interactions

Humans have exploited plant-microbe interactions for centuries, often embedded in Indigenous Knowledge and cultivation practices passed down through generations [1]. American Indian communities, for example, employed the "Three Sisters" technique, recognizing that certain plants, such as squash, corn, and beans, grow better together. This technique unknowingly harnesses the power of N-fixing rhizobia bacteria [1,2]. Rhizobia are unique bacteria that can establish mutualistic relationships with the Leguminosae (Fabaceae) family of plants and convert atmospheric nitrogen gas (N_2) into ammonia (NH_3), which, upon entering the soil, rapidly reacts with water to form soil-bound plant usable ammonium ($NH4^+$) [3,4]. While farmers may not have the tools to understand these processes at the molecular level, they recognize that certain plant combinations lead to improved soil fertility and enhanced plant growth and productivity.

The scientific method was first used to explore plant-microbe interactions about 200 years ago [5]. Initially, research primarily focused on beneficial microbes that promote plant growth, such as arbuscular mycorrhizal (AM) fungi [6], atmospheric nitrogen-fixing bacteria [5], and plant growth-promoting rhizobacteria (PGPR), such as *Bacillus* spp., *Azotobacter* spp., or *Burkholderia* spp. [7,8]. This knowledge solidified that microorganisms increase plant fitness, enhance growth, and enable plants to perform better under stressful environmental conditions [9–12].

Over time, advances in sequencing and pure culture technologies have revolutionized the study of plant microbial ecology. High-throughput sequencing, for example, allowed us to explore the composition of entire microbial communities without the need for culturing [13–17]. Additionally, advances in culturing techniques have allowed us to gain insights into bacterial lifestyles and nutritional requirements, as well as focus on the specific mechanisms of plant-

microbe interactions [18,19]. We recognize that a plant is not a singular organism but a host to a complex microbiome [20–23]. In addition, the plant microbiome is not merely composed of a few beneficial microbes. Still, it comprises rich and dynamic communities across all plant tissues at all life stages influenced by various factors [9].

1.1.2 Holobiont system

Over 450 million years of coevolution, plant-microbe interactions have created a complex and integrated symbiotic system known as the *"holobiont"* [22,24–27]. The term *"holobiont"* was first introduced by Lynn Margulis in 1991 [28] and initially referred to a simple biological entity involving a host (generally refers to the larger, eukaryotic, multicellular organism in or on which the symbiont resides) and a single inherited endosymbiont. It was extended to define a host and its associated communities of microorganisms. Over time, the definition expanded to encompass the host along with its associated microbial communities—collectively referred to as the microbiota [29,30]. Together, the host and its microbiota form the holobiont. This concept has since been widely adopted in diverse research contexts, with studies focusing on humans [31], animals [32], and plant holobionts (reviewed in [22]).

In the case of plants, the holobiont system is now understood as a highly cooperative association between the plant host and its diverse microbiome, which includes a range of bacteria, fungi, and archaea [33,34]. The plant-host and its beneficial microbiome form a dynamic entity, with both parties co-adapted to the environment they co-inhabit in a mutually beneficial relationship [22]. In the context of the holobiont, the plant host and its associated microorganisms function as a single, multi-organismal, integrated unit, with all parties contributing to the survival and fitness of the whole [34].

Plant microbiomes primarily originate from the local bulk soil microbial communities, which include a wide range of microorganisms that can colonize the plant [35–37]. For instance, just one gram of soil is often estimated to contain ~10^6-10^{11} bacterial cells [38–42]. While the composition of soil microbial communities is largely influenced by various biotic and abiotic factors, such as climate, soil type, and nutrient availability, there is substantial evidence that the microbial communities associated with plants are not simply a random subset of the soil microbes [43–46]. Instead, plant-associated microbial communities change significantly from the surrounding bulk soil microbiome due to filtering and selective processes controlled by the plant host [43,47].

These selective and filtering mechanisms occur in various plant tissues, generally diverging from bulk soil to rhizoplane (zone of soil directly adjacent to the root), then to the endosphere (inside of plant root), and ultimately to stems, leaves, and flowers [48–50]. For example, the abundance and diversity of microbial populations are lower in the plant's interior tissues when compared to the bulk soil [51,52], as up to 10^8 bacteria per g are found on leaves [53]. The rhizosphere of plants contains up to 10^9 bacteria and 10^6 fungi per gram of soil, the highest concentration being attached to the root epidermis [9,54,55]. Additionally, many bacterial taxa—such as Pseudomonadota, Planctomycetes, Actinobacteria, Bacteroidetes, and Firmicutes—tend to have a higher relative abundance in the rhizosphere (combined endorhizosphere and rhizoplane areas) compared to bulk soil [56–59]. All this suggests the plant's role in enriching its immediate microbial environment and the importance of plant-microbe communication mechanisms, as well as microbiome selection, recruitment, and assembly.

1.1.3 Rhizosphere functions

As this dissertation centers on the rhizosphere, the introduction will primarily focus on this root zone. It is well established that plant rhizosphere communities have a significant impact on plant health, development, and overall ecosystem functioning [60]. Beneficial root-associated microorganisms not only influence plant resilience but also contribute to various key physiological processes [61,62]. For example, they can directly enhance plant resistance to biotic stresses, such as pathogen attacks, and abiotic stresses, including drought, salinity, and nutrient deficiencies [63,64]. Rhizosphere microbes can assist plants in acquiring essential nutrients, particularly limiting macronutrients, such as nitrogen (N) [65–67], phosphorus (P) [68,69], potassium (K) [70,71], and sulfur (S) [72–74], facilitating more efficient nutrient absorption by plant roots (reviewed in [75]). For example, some rhizosphere bacteria, such as *Pseudomonas fluorescens*, *P. putida*, *P. taiwanensis*, *Stenotrophomonas maltophilia*, and *Pantoea agglomerans,* can solubilize phosphorus. These bacteria can produce organic acids, such as gluconic, 2-ketogluconic, lactic acid, and citric acid, which lower the pH around the bacterial cell. This, in turn, solubilizes phosphate compounds and makes them available to the plant host [76–79].

Other examples of beneficial rhizosphere functions include the production of phytohormones such as auxins, cytokinins, gibberellins, abscisic acid (ABA), and ethylene, which help regulate plant growth and development [80–83]. For example, *Pseudomonas fluorescens* HP72 produces indole-3-acetic acid (IAA), one of the auxin-related molecules that can promote root elongation and branching, improve plant nutrient uptake and overall growth as well as prime the plant immune system to better cope with abiotic stress [83,84]. Additionally, some beneficial microbes produce siderophores—molecules that chelate iron and sequester it away from pathogens, limiting their access to this essential nutrient [85–87].

Beneficial rhizosphere microbes can also trigger systemic immune responses in host plants, a process known as Induced Systemic Resistance (ISR). For example, some *Bacillus* spp. or *Pseudomonas* spp. release elicitors, including phytoalexins, proteins, and enzymes that activate the plant's immune system and prime it for a more effective response to future pathogen attacks [88–90].

Rhizosphere microbes often produce antimicrobial metabolites that protect plants from pathogenic microorganisms [91]. Plant-beneficial bacteria like *Bacillus subtilis* can release lipopeptides, antibiotics, and volatile organic compounds (VOCs) that inhibit the growth of pathogens in the rhizosphere [92–94]. However, it is also important to note that the production of antimicrobial compounds by microbes is not limited to plant-beneficial microbes [95]. The production of antimicrobial compounds is an evolutionary strategy used by many microorganisms to protect themselves and secure resources in the highly competitive rhizosphere environment [96].

Indirectly, rhizosphere microbes play a critical role in shaping soil properties. By promoting the formation of soil aggregates and organic matter, they improve soil structure and texture, which in turn enhances soil aeration, water retention, and nutrient availability. These microbial activities are particularly important in sustaining soil fertility and ensuring sustainable plant productivity [63,64]. In soils with low fertility, adding beneficial microbial communities can enhance plant growth and recovery [97,98].

It is also important to note that plants engage in a wide range of interactions with soil microorganisms, which encompass various ecological scenarios, including not only mutualism but also competition, exploitation, neutralism, and commensalism [33,99–101]. For example, plants constantly engage with pathogenic microbes that actively attempt to invade plant tissues and suppress host defense mechanisms. Pathogens can disrupt plant metabolic pathways, produce

toxins, cell-wall-degrading enzymes, and virulence proteins that directly harm the plant or inhibit its immune responses and utilize plant resources for their benefit, often negatively affecting the plant's health [26,102]. For example, pathogenic bacteria often secrete metabolites that degrade plant cell walls, suppress immune responses, or manipulate host cellular functions [103]. For example, strains of pathogenic *Pseudomonas syringae* secrete toxins and effector proteins that interfere with plant signaling pathways, compromising the plant's immune response [103–105]. However, it is important to note that plant-pathogen interactions are not necessarily bad for the plant. Similar to the human immune system, the plant immune system has the capacity to learn, evolve, and adapt over time. This process is a part of natural selection, which drives pathogens to evade effector-triggered immunity (ETI) [104]. They do so by either shedding or diversifying recognition effector genes or by acquiring additional effectors that suppress ETI. As a result, the plant's immune system must continually adapt, leading to the evolution of new specificities that can trigger ETI again [104]. For example, Systemic Acquired Resistance (SAR) is a mechanism that induces long-lasting protection against pathogens. SAR acts as a plant's *"immune memory,"* allowing it to mount a stronger and faster defense if the same or a related pathogen infects the plant again. Hence, understanding the mechanisms of pathogenicity and the plant's adaptive immune responses has become a significant focus of plant microbiome research. This knowledge is crucial for understanding how plants adapt to unfavorable environments and the mechanisms of microbiome assembly.

1.1.4 Plant-microbe communication and the plant immune system

The rhizosphere acts as an active interface where plant hosts interact with a diverse community of soil microorganisms and actively recruit beneficial microbes into the root

environment while simultaneously repelling pathogens [26]. Plant-soil microbe interactions occur through a blend of chemical signals, also called exudates or rhizodeposits, released from the plant root [55,106]. These responses are triggered by specific biotic or abiotic factors while stimulating a range of regulatory mechanisms and selective processes. The exudate blend includes primary metabolites (such as sugars, amino acids, organic acids, and fatty acids) and secondary metabolites (including phenolics, flavonoids, and terpenoids) [56,107,108] that the plant host releases into the soil. The composition of root exudates is not uniform or static and varies depending on plant species, developmental stage, root traits, environmental conditions, nutrition, and soil type, among many others [56]. For instance, plants can release *"cry for help"* molecules in response to biotic and/or abiotic stresses, actively recruiting beneficial microbiota to facilitate adaptation and protection [109,110]. For example, during periods of moderate drought, plants release higher amounts of organic acids such as malic acid [111]. While malic acid plays a crucial role in phosphate solubilization, it can also act as a chemoattractant for microbes, potentially enhancing water retention and drought tolerance [111–114]. Hence, these molecular signals act as communication signals, alerting the microbial community to dynamic plant needs and fostering mutualistic relationships that enhance plant resilience [109,110].

In nutrient-deficient soils, plants also may release exudates that stimulate microbial activity involved in nutrient cycling. For example, nitrogen-fixing symbiosis in legumes is a fantastic example of plant-microbe recognition and communication. This process is triggered by nitrogen starvation of the host, prompting the secretion of flavonoid signals, which attract Rhizobia and activate their nodulation genes [4,115–117]. These rhizobial genes produce Nod factors (NFs), lipochitooligosaccharides that trigger nodule development in the plant [117,118]. The NFs are recognized by LysM-type receptors on the plant's surface, which activate a signaling cascade that

induces nodule formation and facilitates bacterial colonization, allowing the establishment of a mutualistic relationship between the plant and Rhizobia [119]. Similar processes for establishing plant-microbe symbiosis are also described for arbuscular mycorrhizal (AM) fungi and *Frankia* bacteria [119].

These exudates can serve not only as a nutrient source for microorganisms but also as chemical signals that shape microbial communities in the rhizosphere by attracting beneficial microbes while repelling potentially harmful ones. For a plant to recognize and differentiate between beneficial and harmful microbes, it relies on the complex signaling pathways of its immune system. This process involves pattern recognition receptors (PRRs) on the surface of plant cells [104]. PRRs detect microbe-associated (MA), symbiont-associated (SA), pathogen-associated molecular (PA), or damage-associated molecular patterns (DA) molecular patterns (MPs), which are conserved molecular signatures found on the surfaces of microorganisms [120–123]. Beneficial microbes often produce specific microbe-associated molecular patterns (MAMPs) or metabolites that the plant can recognize as 'safe' or advantageous, triggering a response that promotes colonization and mutualistic interactions. In contrast, PAMP-triggered immunity (PTI) can halt further colonization, initiating a defense response to limit infection [104,124–126]. PRRs' alerts are followed by recognizing specific effector proteins by nucleotide-binding leucine-rich repeat (NLR) immune receptors [104,127].

In addition to the recognition system, plants also maintain a finely tuned balance between their immune responses and the need to keep a functional rhizosphere microbiome [128]. Therefore, before activation of the immune system response, the plant host must distinguish between harmful pathogens and beneficial microbes to ensure that beneficial interactions are not disrupted [129]. Phytohormones such as salicylic acid (SA), jasmonic acid (JA), Ethylene (ET),

and abscisic acid (ABA) have diverse functions, regulating not only growth, stress responses, and microbial recruitment but also pathogen resistance, adding challenging complexity to understanding their role in plant-microbe interactions. However, these hormones can exert different and sometimes opposing effects depending on the context. For example, the Induced Systemic Resistance (ISR) system relies on JA and ET, whereas the Systemic Acquired Resistance (SAR) system relies on SA. At the same time, it is generally believed that SA signaling is linked with plant resistance against biotrophic and hemibiotrophic pathogens, while JA/ET signaling provokes host resistance to necrotrophic pathogens [130–133]. To add to these complexities and context dependencies, ABA, an essential hormone in drought stress response, can both antagonize SA-mediated pathogen defenses and, in other contexts, cooperate with SA to enhance stomatal closure and improve drought tolerance. Similarly, the crosstalk between SA and JA shows a dose-dependent relationship, with cooperation at low concentrations but antagonism at higher levels [134–138]. This illustrates the plant's ability to tailor their defense strategies to combat diverse pathogen types without compromising its immune function [139]. Hence, the rhizosphere recruitment process is complex, and understanding how plants fine-tune their microbiomes in response to environmental cues and microbial threats presents a substantial challenge. Rhizosphere microbial communities are so deeply integrated with the plant that they are often considered an extension of the plant genotype, thus the holobiont concept. Recent research has also suggested that plant-microbe interactions can influence plant epigenetics and gene expression, further strengthening the link between the plant and its associated microbiome [140–142]. The rhizosphere microbial community structure is the result of a complex series of interactions and feedback between plant roots, microorganisms, and the soil's physical and chemical environment. Therefore, it is reasonable to hypothesize that this long-term association between microorganisms and plants

has led to mutual adaptation and recognition. However, our understanding of the mechanism behind rhizosphere recruitment is incomplete, and the assembly of communities remains incomplete.

Another critical aspect to consider in understanding plant-microbe interactions is the adaptation of both the plant and its associated microbial communities to their environment [143]. Over time, plants growing in specific environmental conditions can undergo local adaptation, leading to the emergence of ecotypes—genetically distinct populations within a species that are specialized for particular ecological niches [143–148]. Plants adopt a variety of adaptive strategies shaped by long-term exposure to environmental pressures such as climate, soil characteristics, water availability, and biotic interactions, including those with microbes [149–153]. These include physiological adaptations, such as altering periods of stomatal opening to conserve water, and morphological adaptations, including modified root or shoot architecture and changes in biomass production [146,154–159]. These adaptations also include biochemical ones, such as the induction of specific chemical signaling molecules and the regulation of phytohormones, consequently affecting the microbiome structure [160–162]. For example, an ecotype from a drought-prone area may exhibit a different balance of ABA and SA signaling pathways compared to an ecotype from a more temperate region, influencing how these plants respond to both environmental stress and pathogenic threats [162–164]. As a result, both the plant and its microbiome may co-evolve, shaping distinct interactions that are finely tuned to local conditions [153,165].

As research continues to uncover the depth of plant–microbe interactions, it is becoming clear that microbial communities exert significant selective pressure, shaping plant traits over evolutionary time [23,166]. Studying distinct plant ecotypes offers valuable insights into how genetic variation within a species influences rhizosphere microbial recruitment, immune signaling

pathways, and stress responses [43,167,168]. There is evidence that different ecotypes can host distinct microbial communities, even when grown in similar soil conditions, suggesting that the plant genotype itself plays a significant role in shaping the microbiome [169,170]. However, many key questions remain unresolved. For example, what is the relative contribution of environmental factors versus plant ecotype in shaping the plant microbiome? If a plant that has adapted to a harsh environment is grown in a more favorable setting, how will it perform—will it retain its adaptive advantages, or will its performance change? Will its microbiome shift accordingly? Can beneficial microbes associated with a particular ecotype be harnessed to enhance stress tolerance or the yield of another ecotype? Moreover, in challenging environments, do plants perform better when surrounded by locally adapted microbial communities or when reintroduced to the microbial partners they co-evolved with in their native environment? Do plants and microbes co-evolve to produce distinct chemical or molecular cues that facilitate the selective assembly of the microbiome during long-term environmental stress? What are the signaling pathways and receptor systems involved in this bidirectional communication? Furthermore, what underlying mechanisms drive the microbiome assembly, and to what extent do these mechanisms differ across plant ecotypes?

In this dissertation, I explore plant–microbe interactions, with a particular focus on the mechanisms of communication and microbial recruitment, to shed light on some of these intriguing and unresolved questions. To address these complexities, I employ an integrative multi-omics approach that includes genomics, transcriptomics, and metabolomics from both the plant host's and associated microbes' perspectives. The multi-omics approach provides an in-depth analysis and offers new insights into the mechanisms of microbiome assembly [171,172].

1.2 Plant-microbe interactions: past and present

Over the years, the questions we ask about plant-microbe interactions have evolved, driven by new scientific discoveries, the identification of emerging research gaps, and advancements in technological capabilities. Although the term *"holobiont"* was introduced by Margulis in 1991, the concept itself was not entirely new, as the role of microbes in plant biology had long been recognized [21,28]. What is novel is a relatively recent recognition of the ubiquitous presence of host-associated microbes and their pivotal role in host biology, ecology, and evolution [173]. Studying this diversity has posed significant challenges, yet it is crucial for understanding the ecological roles and functional importance of microbial communities within the plant holobiont and their broader impact on ecosystem functioning [174]. In this section, I will outline the key breakthroughs that have shaped our current understanding of the plant holobiont, all driven by technological advancements. These milestones have opened new avenues for research on the complex relationships between plants and their microbiomes, transitioning from the initial recognition of plants as holobionts to understanding the complex biological processes involved in holobiont system interactions.

1.2.1 First sequencing and Fingerprinting

One of the fundamental advancements was the development of the first DNA sequencing techniques, such as Sanger sequencing. This method involved amplifying and sequencing the entire ~1,550 base pairs (bp) in length 16S rRNA gene to identify individual bacterial species within a community [175–179]. 16S rRNA gene is a highly conserved bacterial gene that is widely favored in microbial ecology due to its stability, high copy number, and universal presence across bacterial species [180–182]. This process required cloning of the gene and assembling two to three

reads per clone to allow taxon assignments. However, these approaches are limited by shallow sequencing depth, high costs, and labor-intensive processes, making them particularly challenging for analyzing complex polymicrobial samples when compared to next-generation sequencing (NGS) technologies such as Illumina MiSeq/HiSeq, PacBio, or Oxford Nanopore platforms [183]. In contrast, early profiling studies often generate only a few hundred sequences per sample, which fails to capture the full microbial diversity [184–186]. As a result, early sequencing efforts were primarily focused on individual species, rather than providing a comprehensive view of microbial populations or communities as a whole [187].

Despite their limitations, early DNA sequencing techniques sparked foundational breakthroughs in plant-microbe research and even reshaped research approaches. Traditional biology focused on specimen collection, observation, and experimentation, such as plant-microbe challenges or identifying pathogen-induced symptoms [188,189]. In contrast, the advent of DNA sequencing marked the beginning of the bioinformatics era, emphasizing large-scale data collection, storage, and computational mining to uncover patterns and generate knowledge from vast datasets rather than direct experimentation alone [190]. Additionally, these early sequencing efforts enabled the detection and taxonomic identification of previously unculturable or unknown microbial taxa associated with plant tissues and soils [191,192]. This fundamentally shifted understanding of the plant microbiome—from a narrow focus on a few culturable pathogens or symbionts to a more complex view of plants as hosts to rich and diverse microbial communities [190]. For example, researchers could catalog environmental bacterial diversity at a phylogenetic level, revealing taxa such as Acidobacteria or Verrucomicrobia, which were virtually absent from culture-based studies [193–196]. However, despite these foundational advances, early sequencing efforts provided a limited view of the plant-associated microbial community. Without a

comprehensive, systems-level perspective, several critical questions about the plant holobiont remained unresolved. For instance, how diverse is the plant microbiome, and is it compositionally consistent across different species, environments, or developmental stages? How do microbial communities assemble over time, and how do they respond to biotic factors such as pathogens or symbionts and abiotic factors like soil type, nutrient availability, or climate? Additionally, to what extent does the host plant's genetic background shape the composition and function of its microbiome?

Some other early microbial DNA utilization methods, including Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Amplified Ribosomal DNA Restriction Analysis (ANDRA), and Terminal-Restriction Fragment Length Polymorphism (T-RFLP), have been crucial in studying microbiome diversity by identifying microbial genetic markers and revealing differences among communities [178,197–202]. These early DNA fingerprinting methods helped answer several critical questions in plant-microbe research. They demonstrated that microbial communities associated with plants are distinct and dynamic, varying between different plant species, plant tissues, environmental conditions, and developmental stages [198,200,203–209]. Moreover, they revealed that microbial communities could shift over time or respond to external factors such as fertilizer application, pathogen exposure, or environmental stressors [210–212]. These fingerprinting methods provided a comprehensive snapshot of entire microbial communities, including uncultured or unknown microbes, offering insights into microbial diversity and dynamics [203,213]. Most importantly, they allowed scientists to track microbial community changes and identify potential biomarkers of plant health, disease, or beneficial microbial activity [198,199,203,207]. For example, Smalla et al. [198] used DGGE and T-RFLP

to monitor rhizosphere microbial communities of sugar beet. They found that bands representing specific taxa were consistently associated with healthy or pathogen-infected plants. Similarly, Kandeler et al. [199] used T-RFLP to assess microbial community responses to heavy metal contamination in soil. They showed that specific microbial populations disappeared or were significantly reduced under stress, suggesting the potential use of microbial community profiles as early bioindicators of environmental disturbance. These findings showed that microbial fingerprints reflect ecologically meaningful changes in the plant-microbe interface and served as a steppingstone toward recognizing that plant-associated microbial communities respond predictably to external stressors [199].

Other techniques, like Phospholipid Fatty Acid (PLFA) and Enzyme Activity Assay (EEA) profiling, do not rely on DNA sequencing [214,215]. Instead, they focus on microbial diversity and activity by analyzing biological products, such as fatty acids and microbial enzyme activity. PLFA profiling relies on phospholipid biomarkers and the abundance of different fatty acids in microbial cell membranes [214,216,217]. Variations in chain length, saturation, and branching serve as distinctive community overview at coarse taxonomic levels [218–221]. PLFA profiling has led to breakthroughs in understanding plant-microbe interactions by revealing shifts in microbial community structure in response to plant developmental stages [222], root exudation patterns [223], environmental stressors [224], and seasonals [225–228]. In contrast, EEAs measure microbial enzyme activity, assessing community functions like cellulose or chitin degradation [229,230]. EEAs have illuminated the functional capacity of microbial communities in real time, showing how enzymatic activity in the rhizosphere correlates with soil nutrient cycling [231] or nutrient mobilization [232]. Together, these techniques highlight the value of microbial biomarkers as indicators of microbial community composition and viable biomass, reflecting microbial

activity, interactions, and dynamics, as well as their influence on host health and productivity. These findings have also provided further evidence that plants actively recruit or suppress specific microbial groups in response to varying biological and environmental conditions, prompting researchers to develop new methods to understand the relationship between structural and functional diversity in the plant microbiome.

Despite these advancements, fingerprinting methods faced technical limitations that left several critical questions unanswered. These included uncertainties about the functional roles of the microbial products, the taxonomic identity of the microbes responsible for producing these products, and, importantly, the role of the plant host in shaping the microbial community dynamics [178,233]. Some open key questions still are included: How many microbial members are in a holobiont system? Which specific microbes drive changes within the community? What functional roles do these microbes play within the holobiont system? Do microbes interact with one another and with their plant host? How does the microbiome evolve over time or in response to different environmental conditions? Do microbial communities vary across different plant species? Addressing these questions became crucial for advancing our understanding of the plant holobiont.

Today, classical fingerprinting and first-generation sequencing methods have largely been replaced by more advanced nucleic acid-based methods and metabolite profiling approaches. However, traditional methods still provide value by offering rapid, cost-effective, and reproducible ways to assess microbial biomass and community composition, sometimes even being more sensitive than nucleic acid-based methods in detecting community shifts [221,229,233,234].

1.2.2 Amplicon sequencing: microbial community composition and diversity

A significant shift in our understanding of the holobiont came in 2002 when Rohwer et al. expanded the concept to include the *"hologenome"*—the combined genetic information of the host and all its symbiotic microorganisms [21,23,235]. Today, the microbiome refers to all microbes associated with a plant host, while the microbiome encompasses their collective genetic material [30].

As tools used to study microbial communities advanced, so did the holobiont research. In the early 2000s, the development of metagenomic methods, including next-generation sequencing (NGS) platforms such as Illumina, Oxford Nanopore, or PacBio, involved recovering genetic material directly from environmental samples [172,236]. This shift enabled a more comprehensive understanding of microbial communities, providing deeper insights into their taxonomic diversity, structure, and functional potential [237].

For example, the Illumina MiSeq platform heavily relied on targeted sequencing of a specific region of DNA or RNA, focusing on the roles of individual genes in plants and microbes [182,238]. The widespread use of bacterial 16S rRNA amplicon sequencing, which focuses on nine variable regions interspersed throughout the gene, has significantly advanced our understanding of the plant microbiome [239–241]. Illumina MiSeq short-reads (2x250bp and 2x300bp) enabled the identification and relative abundance estimation of taxa based on read counts, providing a comprehensive view of bacterial communities in a single run [238,242]. Most importantly, 16S rRNA amplicon sequencing provided datasets that were relatively inexpensive, easy to generate, and straightforward to interpret. As a result, the number of publications and scientists addressing plant holobiont-related questions has increased exponentially since 2010 [243]. Special attention has been given to the rhizosphere microbiome, with growing recognition

of its crucial role in plant–microbiome interactions. The rhizosphere was widely recognized as the primary zone where plants communicate with soil microbes, acting as a key area for interaction, signaling, filtration, and nutrient exchange [244].

The development of robust taxon-assignment pipelines greatly improved the resolution and accuracy of microbial classification, addressing the main limitations of fingerprinting methods [245–247]. For example, the taxonomic assignment of 16S rRNA amplicon sequences relies on databases populated with known bacterial species. Early methods of taxonomic assignment relied on referencing taxonomic outlines to classify sequences into taxonomic bins (phenotypes) [248–250]. This was later complemented by the operational taxonomic unit (OTU) method, which grouped sequences based on their similarity within a dataset [245,251–253]. The OTU method assigned sequences to bins based on similarity, with a standard threshold of 97%, which allowed for the assignment of taxa even when reference sequences did not align perfectly. However, this approach has been criticized for introducing bias and losing resolution due to the use of arbitrary clustering thresholds [254–258].

As a result, many researchers have shifted toward using Amplicon Sequence Variants (ASVs), which represent exact sequence variants identified directly from amplicon sequencing [259]. Unlike OTUs, ASVs are defined by unique nucleotide differences, eliminating the need for clustering based on similarity thresholds [247]. This method offers greater precision and 99% accuracy in taxon assignment, providing better resolution, detecting rare or underrepresented community members, and offering a clearer picture of community structure [247,260]. These advancements have deepened our understanding of microbial diversity, enabling more accurate taxonomic assignments, reshaping taxonomic trees, and highlighting the importance of microbes as essential components of the holobiont system.

However, for both OTUs and ASVs, the universal primers used to amplify the 16S rRNA gene are based on known sequences, limiting the analysis to existing databases and preventing the detection of novel bacterial groups [261–264]. Database-free approaches like oligotyping were introduced, which detect subtle nucleotide differences at high resolution [265]. The oligotyping method relies on identifying information-rich variable sites and discarding low-entropy nucleotide positions within a group of sequencing reads [265]. This technique allows for the identification of closely related, but distinct organisms, which may differ by as little as one nucleotide over the sequenced region of the 16S rRNA gene [265–267]. By focusing on subtle nucleotide differences, oligotyping offers an even higher resolution, making it particularly useful for distinguishing closely related microbial variants or strains within the same species [265]. Unfortunately, oligotyping has technical limitations when applied to datasets with complex microbial communities. Additionally, gene regions used for oligotyping analysis are not universal across bacterial species. Therefore, if analyzed across a complex community, the results might lack biological meaning [265].

Despite its limitations, 16S rRNA amplicon sequencing has been widely used to explore microbiome structure and diversity across environments [268–270]. These studies have provided an understanding of the dynamics of plant microbiomes, which are influenced by various factors such as seasonal variations [271–273] and environmental conditions [274]. For instance, significant interest has emerged in understanding how microbial communities shift in response to temporal changes, nutrient fluctuations, plant growth cycles, and environmental stressors [275–277]. Additionally, researchers have recognized the differences between agricultural and natural systems, such as prairies, regarding microbial diversity [278,279]. 16S rRNA amplicon sequencing is also commonly used in large-scale investigations or as a steppingstone for selecting targeted

samples in broader community studies [280,281]. For example, 16S rRNA amplicon sequencing data may permit the selection of the most distinct or similar samples, depending on the research objectives, thus allowing the choice of the most informative samples from a large pool in a large-scale survey experiment. To this day, 16S rRNA gene sequencing remains the most common method for culture-independent analysis of microbial communities, providing valuable insights into their structure and diversity [14,282–284].

The recognition of the incredible microbial diversity within the holobiont systems, revealed through advances in sequencing technologies such as 16S rRNA amplicon sequencing, has driven researchers to explore the ecological patterns and functional roles embedded within this complexity [285–290]. Sequencing technologies made it possible not only to investigate the microbial taxa present in the community, but also to investigate their dynamics across environmental gradients and host systems. With this knowledge, several conceptual frameworks have been developed to explain the ecological and functional significance of this microbial diversity. One such framework is the *"insurance hypothesis,"* which suggests that plants actively recruit diverse microbial communities to ensure functional redundancy [285,291]. This redundancy provides a buffering capacity, allowing key ecosystem functions such as nutrient cycling, pathogen suppression, and stress tolerance to persist even when some microbial taxa decline or are lost due to environmental stressors [287,292,293]. Once seen as noise or background variation, microbial diversity is now viewed as a reservoir of functional potential crucial to host resilience and adaptability [294]. At the same time, the ability to analyze large microbiome datasets across time, space, and different host species led to the concept of the core microbiome. Rather than viewing the diversity as a form of ecological insurance, researchers have focused on identifying a low-complexity microbial community consistently found across diverse rhizosphere

environments and host genotypes [43,295,296]. Due to the consistency of these microbes across hosts and environments, these core microbial members are believed to form stable, co-evolved associations with their hosts, supporting essential physiological functions and contributing to plant health and development [22,43].

In both the *"insurance hypothesis"* and the *"core microbiome,"* the focus was on explaining diversity at the broader community level. This was largely shaped by the capabilities and limitations of available DNA sequencing technologies, which tended to highlight the most abundant taxa due to limitations in resolution. As a result, the role of low-abundance bacterial populations in shaping plant root microbiomes was frequently overlooked [297–301]. These rare taxa were commonly viewed as part of a microbial *"seed bank"*—a reservoir of mostly dormant organisms that can become metabolically active in response to environmental shifts [302,303]. However, around 2010, there was a shift from focusing on broad community fingerprinting to investigating finer-scale changes in microbial community composition. Despite their minimal representation in sequencing datasets, several studies have shown that low-abundance microbes can act as *"keystone"* species, playing critical ecological roles across diverse environments [304–306]. For example, essential functions have been attributed to rare taxa in the ocean [307], peatland soils [305,308], bulk soils [40,309–311], and the rhizosphere [306]. Dawson and colleagues [306] demonstrated that 91% of plant-specific microbial interactions involved bacteria with a relative abundance of less than 0.1%, highlighting the substantial influence of the low-abundance members in shaping rhizosphere communities. This growing recognition of the functional importance of rare taxa reflects a shift in the focus of microbial ecology. Techniques like 16S rRNA amplicon sequencing have been valuable for cataloging the presence or absence of microbial taxa. Still, they

fall short in addressing some questions, particularly those concerning the genetic and molecular mechanisms that shape microbial community structure and drive ecosystem function.

1.2.3 SynComs

As our understanding of plant–microbiome interactions has deepened, interest has grown in leveraging this knowledge to promote sustainable agriculture, support ecosystem restoration, and strengthen climate resilience. Through the strategic manipulation of these interactions, researchers have aimed to develop microbiome-informed strategies that enhance crop resilience, improve nutrient uptake efficiency, and reduce reliance on synthetic chemical fertilizers and pesticides [37,312–315]. This deepened knowledge of the plant holobiont system led to the development of the first synthetic microbial communities (SynComs) [47,316–320]. SynCom is a selected group of microbes designed to mimic the structure and functions of the natural system while replicating or enhancing their functional roles [321,322]. The selection of the microbes for the SynCom consortia is commonly based on the representation in the natural system, culturability, compatibility, or selection based on the desired functions [47,295,323–327]. Numerous experimental studies have investigated the integration of SynComs into plant systems, particularly under controlled laboratory or greenhouse conditions [47,320]. The vast majority of these studies have focused on plant model organisms such as *Arabidopsis thaliana* [47,328], as well as agriculturally relevant plants like maize [320], soybean [329], tomato [330], or germ-free plants [322,331,332]. While these experiments have yielded promising results in the controlled conditions, the transition to the natural field environments has proved far more challenging [324,329,333,334]. To date, no successful SynCom addition experiment has demonstrated effective establishment or performance under field conditions. A significant barrier is the low

survival, establishment, and functional persistence of introduced microbial consortia, largely due to the unique and highly dynamic interplay of biotic and abiotic factors within complex and competitive native soil microbial communities [335,336].

Emerging alternative approaches shift the focus from sustained SynCom colonization to the functional outcomes of SynCom activity [337,338]. For example, by leveraging the knowledge of the genetic and metabolic potential of organisms, model SynComs can be engineered to include both chitin degraders and non-degraders, with predicted ecological niches informed by their functional traits [337]. Other approaches prioritize bioactive microbes or functional genes to design SynComs [339–341]. For example, function-based SynCom can be designed to produce beneficial microbial compounds or act as priming agents to enhance plant defense responses [342–344]. In this scenario, SynCom could serve not as a permanent colonizer, but as a biological input that prepares plants for environmental stress before field transplantation. These strategies offer promising avenues for enhancing plant performance in challenging conditions, even without a long-term microbial establishment. As a result, ongoing research focuses on improving SynCom design by concentrating more on microbe-microbe interactions, incorporating more competitive and ecologically robust microbial strains, and engineering consortia with adaptive traits and enhanced communication with the plant host to support colonization and persistence [345].

Another critical challenge in SynCom research is the lack of robust, widely accepted model systems across the scientific community [345–347]. In other fields, there has been a consensus around the use of standardized model organisms, such as *Arabidopsis thali*ana for plant studies [348], mice for human-related research [349], or *Drosophila melanogaster* for genetic research [350]. While the focus may now be shifting toward alternative models, such as human or plant cell systems, these standardized model organisms have historically enabled reproducible experiments

and allowed findings to be validated and expanded upon by different research groups, leading to a cohesive and cumulative body of knowledge [351,352]. In contrast, microbiome research still lacks standardized, reproducible model systems, despite the growing recognition of the fundamental roles that microbial communities play in biological systems [351,352]. Moreover, existing studies often fall short of probing the mechanisms of host-microbe interactions, particularly in plant-associated communities under environmental stress or perturbation.

Selecting a standardized SynCom also presents its own set of challenges. Every SynCom study involves a fundamental trade-off between tractability and ecological relevance [345]. Simple communities are easier to design, manipulate, and analyze, but they are often less representative of natural systems [330,353]. This simplicity can result in the loss of emergent properties and context-specific outcomes, such as higher-order competitive interactions, priority effects, or the impact of rare keystone community members [354]. In contrast, while more complex models might capture these effects, they can be harder to implement, show lower reproducibility, and offer significant challenges when it comes to data interpretation [355–358]. For example, suppose oligotyping analysis reveals considerable diversity among *Pseudomonas* congeneres in the sample. In that case, researchers must decide whether to select representative variants or to explore the functional potential of each variant in greater depth. Similarly, when datasets include thousands of rhizosphere taxa—and oligotyping effectively doubles the resolution of standard 16S rRNA sequencing—the question arises: where is the cutoff? Is it even realistic to include this level of diversity in a SynCom?

The design of a SynCom must be guided by the specific goals of the study, which can vary widely across systems. To ensure that SynCom aligns with the research questions, it is crucial to validate its performance using appropriate evaluation methods. For instance, is it sufficient to

confirm that the community presence within the system overtime after inoculation — a question that can be addressed with 16S rRNA sequencing—or should we also assess microbial growth dynamics, the potential products of this inoculation, interspecies interactions, host phenotypic responses and productivity, mechanisms by which the SynCom influences the system, or even broader ecosystem-level functions? The answer ultimately depends on the intended outcomes of a specific study, highlighting the limitations of relying solely on sequencing-based methods and emphasizing the importance of selecting evaluation criteria that align with the experimental objectives.

1.2.4 -omics technologies in studying holobiont

Beyond focusing on microbial communities, significant efforts were made to understand the host's role in the holobiont system. Historically, the phenotypic variation in plants has been attributed to the interplay between genomic properties and environmental factors [359,360]. However, over the last two decades, with the rapid development of high-throughput sequencing technologies, it has become increasingly evident that host-associated microorganisms also shape plants phenotype as a third factor [361–365]. These microorganisms act not only as passive inhabitants but also as active contributors to the host’s physiology, development, and stress responses [43,366–368]. The concept of the holobiont, therefore, extends beyond a simple coexistence to a functional unit shaped by the dynamic interactions between the host genome and its associated microbiota [369]. For example, specific microbial taxa, such as Actinobacteria and Pseudomonadota, have consistently been found in association with plants [56,370–372]. This consistency suggests that plants have evolved mechanisms for selectively recruiting and interacting with microbial partners. These mechanisms include molecular communication

pathways and a finely tuned immune system capable of distinguishing among mutualists, commensals, and pathogens [373–375]. Building on this foundation, researchers began to explore how plants sense and respond to microbial signals. The concept of the *"immune threshold"* emerged, proposing that plants integrate molecular cues to tolerate beneficial microbes while activating defense responses only against potential threats [104]. However, this concept raises fundamental questions: How can we measure or define this immune threshold? What molecular mechanisms enable the plant to distinguish between beneficial and harmful microbes?

Answering these questions requires tools that go beyond microbial community profiling. While 16S rRNA gene sequencing has been instrumental in characterizing the composition and diversity of plant-associated microbiomes, it provides limited insight into functional interactions or host responses [376]. Furthermore, the 16S rRNA gene represents only a small portion of the bacterial genome, which can span from 100 Kbp to over 16 Mbp, thus omitting vast functional information [377–382]. Additionally, methodological biases introduced during DNA extraction and PCR amplification can also skew relative abundance estimates and underrepresent rare taxa [221,383–385]. As discussed earlier, low abundance does not necessarily imply limited ecological relevance—rare microbial members can exert disproportionately large functional effects [376]. It is also crucial to recognize the limitations of relying solely on DNA sequencing for interpreting microbial interactions. Sequencing data can imply correlations, such as co-occurrence patterns, but cannot confirm causation [386–389]. Misinterpretation of these associations can lead to erroneous conclusions about microbial roles or interactions [386]. Therefore, sequencing should be viewed primarily as a hypothesis-generating approach, with insights that require validation through experimentation [386,387].

To fully unfold the complexity of the holobiont, DNA-based methods must be integrated with complementary approaches, such as advanced computational modeling, functional assays, and a suite of -omics technologies, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics [390–392]. These approaches allow researchers to move beyond static taxonomic profiling and begin unraveling the chemical and molecular dialogue between plants and associated microbial communities. For instance, metagenomics has facilitated the identification of microbial genes and biosynthetic pathways that are potentially involved in immune modulation, nutrient exchange, microbial recruitment, and signaling processes [393–395]. Shotgun metagenomics aims to capture the entirety of any genomic content present in a sample, enabling also identification not only of microbial taxa that are both known and unknown but also accessing the functional potential of the community as a whole [16,396–399]. This approach enables a deeper exploration of microbial diversity, ecological roles, and the potential metabolic functions that may influence plant phenotypes [397,400]. However, despite these advantages, metagenomics also presents several limitations, particularly in the context of plant holobiont research. One key limitation is that metagenomic data largely reflect the genetic potential of what organisms can do, rather than actual activity in a given context. The presence of a gene does not confirm its expression or activity [401]. Furthermore, the complexity of plant-associated samples poses unique challenges. A significant limitation is contamination of the data with host DNA, especially in the leaf microbiomes, where abundant plant DNA can mask microbial signals and reduce sequencing depth for microbial genomes [402,403]. Moreover, the vast diversity of microorganisms in these environments complicates both taxonomic classification and functional annotation, particularly for uncultured taxa that have not been identified yet [263,264,404]. At this moment, metagenomic sequencing cannot differentiate between DNA of the *"alive"* vs *"dead"*

organisms, leading to false interpretations about the presence of specific taxa [405,406]. These limitations contribute to key knowledge gaps. For example, many studies have systematized microbial genes associated with hormone modulation or nutrient metabolism, whereas only relatively few have linked these functions to specific taxa and demonstrated their activity in vivo [47,364,407]. Likewise, while metagenomic datasets can reveal the presence of genes potentially involved in microbe-host signaling, they often fail to capture context-dependent expression patterns that define functional outcomes [408]. As a result, many metagenomic insights remain speculative until validated by complementary approaches.

Such approaches, including metatranscriptomics, metaproteomics, and metabolomics, offer critical insights into the realized functions of the holobiont [409]. Metatranscriptomic analyses capture real-time changes in gene expression in both the host plant and its microbial partners in response to various stimuli [410]. Metaproteomics and metabolomics provide further functional resolution by analyzing the actual proteins and metabolites produced and directly involved in biological functions within the holobiont [411–415].

Metabolomics facilitates the detailed tracking of host-derived and microbiome-derived chemical products, including the secretion of root exudates and signaling compounds, which actively shape the microbial landscape [416–420]. These secretions can influence microbial colonization patterns and, in turn, impact plant traits including root architecture, growth rates, and stress responses [416–418,420]. Together, these integrative multi-omics approaches provide a more holistic understanding of microbial functions at various layers of biological information [395,408,421–425].

Despite their valuable contributions to understanding functional activity within the holobiont, metatranscriptomics, metaproteomics, and metabolomics each present significant

technical and interpretative limitations. Metatranscriptomics, for instance, is sensitive to RNA degradation and typically requires high-quality, intact RNA, which can be challenging to obtain from complex environmental samples, such as the rhizosphere [426,427]. Moreover, distinguishing between host and microbial transcripts can be difficult, especially in samples lacking high-quality reference genomes or annotations, which limits the resolution of gene expression analyses [426,427]. Metaproteomics faces similar challenges, including low protein yields from environmental samples, incomplete protein databases, and difficulties in distinguishing homologous proteins across different taxa [390,428]. These factors can limit both the depth and accuracy of protein identification and quantification, making it harder to confidently assign functional roles to community members [408]. Metabolomics, while powerful in profiling small molecules, is often constrained by the immense chemical diversity of metabolites, many of which remain unidentified or poorly characterized. The dynamic and context-dependent metabolite production also complicates interpretation, as metabolite levels can rapidly fluctuate in response to subtle environmental cues.

It is evident that each -omics dataset, although rich in information, represents only one aspect of the biological system, with each layer of data complementing the others [422,429]. This is why integrating diverse multi-omics data is essential for the effective utilization of -omics technologies, allowing the exploration of connections between host and microbes in the holobiont system [390,425,430]. One common approach to multi-omics integration is the *"top-down"* model of data reduction, where metagenomics or metatranscriptomics data serve as a foundation to predict system responses and shifts in key signaling and metabolic pathways. Targeted metabolomics and/or metaproteomics are then used to validate and measure the active functions of these pathways [409,431,432]. Moreover, longitudinal (time-series) multi-omics experimental

designs have proven effective in capturing dynamic shifts in microbial communities and their functional activities [433]. Hence, multi-omics approaches allow for assessing causality in microbiome research, providing system validation and a more nuanced understanding of the mechanisms driving plant holobiont system dynamics [434,435]. One of the main challenges with multi-omics approaches lies in the sheer volume and complexity of the data they generate. Each -omics layer—be it transcriptomic, proteomic, or metabolomic—produces large, high-dimensional datasets that require sophisticated bioinformatics pipelines, powerful computational resources, and specialized expertise for integration and interpretation. Harmonizing these diverse data types across temporal and spatial scales is particularly difficult in plant holobiont systems, where host and microbial signals often overlap and interact in non-linear ways. In addition to analytical complexity, cost remains a significant barrier. Multi-omics studies usually require deep sequencing, multiple parallel assays, and replicates across environmental or experimental conditions to ensure statistical robustness. Despite the technical challenges and costs, these approaches are becoming vital for understanding plant-microbe interactions. While not all research questions necessitate every layer of -omics data, even partial integration, such as combining metagenomics of the microbial community with metabolomics from the host side, can significantly enhance interpretability by linking genetic potential of microbial communities with the actual metabolic outputs observed in the system [436]. This enables researchers to associate specific microbial taxa or functional genes with the production of key metabolites, thereby uncovering the biochemical feedback from the host and potential mechanisms of interaction between the plant host and its microbiome.

A long history of techniques exists for studying the plant holobiont, with each method offering a distinct perspective on microbial community composition, diversity, and function.

Despite this methodological richness, ranging from community fingerprinting to advanced multi-omics integration, several fundamental knowledge gaps remain unresolved. For instance, the relative importance of biotic versus abiotic factors in shaping the structure and function of holobionts is still poorly understood. Have all these microorganisms coevolved with their host? Do horizontally transmitted microorganisms colonize their hosts randomly, or is their establishment shaped by deterministic factors, such as the host's genetic background or environmental conditions? How does the local environment interact with plant genotype to influence microbiome assembly, and can *"home"* environments confer functional advantages to the plant compared to *"away"* conditions? Similarly, while it is well-established that microbes contribute to plant stress tolerance, what are the precise mechanisms underlying these interactions, particularly under environmental challenges such as drought? Do microbes adapted to arid environments possess specialized traits or signaling capacities that enhance plant resilience or facilitate survival and communication within the microbial community? Are there environmental thresholds to these interactions, beyond which conditions become too harsh for plants to recruit beneficial microbes? Are there unique metabolites produced in these interactions that serve as chemical mediators between plants and microbes, or even among microbes themselves? And ultimately, can we use this knowledge to design SynComs that provide functions in the natural environment? These intriguing questions have inspired the focus of my dissertation work, in which I aim to uncover more information on the functional potential of recruited microbial communities, particularly on how these traits influence the resilience of the plant host to drought conditions.

1.3 The structure of the dissertation

The motivation behind my research stems from the significant knowledge gaps and numerous unanswered questions surrounding plant-microbe interactions within the holobiont system. In this dissertation, I explore the mechanisms of microbial recruitment and plant-microbe communication, with a particular focus on the role of local host adaptation in shaping these interactions.

My research centers around *Andropogon gerardii*, a perennial grass native to North America, and widely distributed across the Great Plains of the U.S [295,437–439]. Notably, *A. gerardii* has persisted along a sharp precipitation gradient for over 10,000 years [440,441]. The long-term selection pressure from environmental conditions has driven the process of local adaptation, wherein plants evolve traits that enhance fitness in specific environments by aligning genetic variation with environmental factors [442]. In case of *A. gerardii*, this has led to the development of distinct ecotypes and populations, with individuals exhibiting genetic traits adapted to regional climatic and soil conditions [152,441,443,444]. For instance, the *A. gerardii* ecotype adapted to arid conditions exhibits characteristics such as a higher chlorophyll absorbance, shifts in root to shoot ratio, i.e., lesser above ground allocation with greater below ground allocation, compared to the ecotype adapted to a more water-abundant environment [445].

Given the complex and heterogeneous environmental conditions that shape *A. gerardii* ecotypes, understanding how microbial communities interact with their host plants could provide key insights into local adaptation mechanisms. Therefore, to better decouple the effects of plant genetic background and environment, we used three regional *A. gerardii* ecotypes adapted to the dry, mesic, and wet environments in a reciprocal garden experiment [152]. Reciprocal gardens were located across the precipitation gradient of the Great Plains in the U.S., with each garden

representing the native environment of one of the selected ecotypes [152]. This experimental setup enabled me to isolate the effect of *A. gerardii* ecotypes while controlling environmental variation.

The main objectives of this work are (1) to evaluate the relative importance of plant host genetic background and environmental factors in structuring rhizosphere bacterial communities, (2) to explore the functional potential of the recruited microbes, with a focus on their communication and survival strategies; and (3), to identify the microbes and their metabolites that contribute to the host adaptation to the environment. In this dissertation, I employed various methods, from broad community metabarcoding to integrating multi-omics data. This dissertation is organized into three chapters, each representing a distinct study.

In the first data chapter, ***"Home-field advantage affects the local adaptive interaction between Andropogon gerardii ecotypes and root-associated bacterial communities,"*** we aimed to investigate the relative importance of plant genetic background and the environment in shaping the plant-host rhizosphere microbiome. Plant responses and adaptation to stress depend on plant genotype, environmental factors, and microbial communities [359–365]. Understanding the effect of these factors on plant performance is required to predict species responses to environmental change [359]. Environmental factors—such as soil chemistry, texture, precipitation, and local propagule and organismal pool —have long been recognized as major influences on plant health and productivity [446–448]. However, the relative role of the plant genotype in the rhizosphere recruitment remains poorly understood.

Since local soil microbes are an integral part of the local environment, I hypothesized that plants locally adapted to their environment would have a *"home-field advantage"* in selectively recruiting these local microbes. Furthermore, because local adaptation enables the plant to survive

and thrive in its environment, this adaptation involves recruiting local microbes with functional potential to protect the plant from local environmental stresses.

I employed broad 16S rRNA metabarcode sequencing to characterize the rhizosphere microbial communities. The data showed that the environment was the main driver of microbial communities. However, the small marginal ecotype effect also highlighted the importance of the host genetic background on the rhizosphere recruitment process. Interestingly, we observed that ecotypes planted at their homesites were more successful in recruiting rhizosphere community members unique to the location. The link between the plant homesite and the specific local microbes supported the *"home field advantage"* hypothesis [449,450]. Additionally, our data revealed ecotypic variation in the recruitment of the same bacterial species, yet with distinct genetic variations, emphasizing the nuanced effects of plant ecotype on the rhizosphere microbiome composition. I suggested that the unique microbes associated with each homesite may represent microbial specialists potentially recruited by the plant for their functional roles in specifically helping the plant host to cope with stresses associated with the local environment. In this way, plants that are locally adapted to their environment may communicate more effectively with these microbes, thus facilitating a better match between the local plant ecotypes and microbial functions than those growing in non-native environments.

The second data chapter, ***"Interaction of plant-derived metabolites and rhizobiome functions enhances drought stress tolerance,"*** builds on the findings of the first one and digs deeper into potential mechanisms of *"home-field advantage."* Plant-microbe coevolution can result in beneficial associations that allow plants to cope with abiotic and biotic stresses across various environments. The interactions between plant roots and local soil microbes are critical for environmental adaptation, as they facilitate nutrient uptake, improve disease resistance, and

improve plant health overall. Given this co-evolution, plants can actively regulate the composition of their rhizosphere, fostering the growth and recruitment of specific microorganisms that enhance their fitness in *"home"* environments. Therefore, I hypothesized that plants adapted to drier environments would experience less stress, resulting in fewer stress-related metabolites, which would impact the recruitment of microbes with the functional potential to provide host stress relief.

While the first chapter explores community dynamics of the rhizosphere using broad 16S rRNA amplicon sequencing analysis, this chapter shifts focus to the metagenomic potential of the rhizosphere community. I analyzed metagenomic data using gene- and genome-centric approaches to assess the functional potential of root-associated microbes. The gene-centric approach allowed me to examine the microbial community as a whole, identifying all functional capacities potentially recruited by the plant host. In contrast, the genome-centric approach enabled me to assemble metagenome-assembled genomes (MAGs), providing insight into the functional potential and taxonomic identities of specific microbial populations reconstructed from the metagenomic data.

To investigate the host side, I profiled plant metabolic products that may govern rhizosphere recruitment and are potentially produced in response to plant-microbe interactions. I then performed correlation analyses to explore relationships between host metabolites and rhizosphere microbes.

The results revealed reduced production of stress-associated metabolites in plants adapted to drier environments, suggesting that these dry-adapted plants experience lower stress. This reduction is influenced by their associated microbial communities, which harbor genes linked to stress-alleviation pathways. I observed a positive correlation between the plant-derived metabolite trimethyllysine and five MAGs, which were frequently detected in the dry ecotype growing at the

"home" environment. Although literature on plant-derived trimethyllysine is limited, it has been implicated in host stress responses. My data suggest that interactions between trimethyllysine and microbial traits such as chemotaxis, biofilm formation, and root attachment may play a more significant role in plant stress resilience than previously recognized. Finally, I demonstrate that plants actively produce metabolites to recruit microbial populations with functional traits that enhance their ability to thrive under stressful conditions.

In the third and final data chapter, "***Native rhizobiome immune priming mechanism increases plant host stress resilience***", I explored the idea of enhancing plant resilience by creating a plant-oriented SynCom. While the first two chapters highlighted the great potential of the rhizosphere microbiome and its impact on the host, much of our knowledge was based on computational analyses. In this chapter, I wanted to focus on the validation of our findings and lay the foundation for developing a microbial-based product with potential real-world applications.

Although the first two thesis chapters provided evidence that plant hosts selectively recruit microbes from the soil, our understanding of the functional potential of these recruited microbes—and the mechanisms through which they communicate with the host—remains limited. I conducted an enrichment experiment to allow self-selection of microbes from dry and wet rhizosphere environments, followed by culturomics techniques to isolate individual cells from these communities. I assembled 320 draft genomes from the single-cell isolated microbes and evaluated their communication strategies and potentially beneficial functional traits. Additionally, I analyzed microbial products from dry ecotype-associated isolates to gain deeper insight into crucial microbial metabolites that may enhance the resilience of the dry *A. gerardii* ecotype under arid environmental conditions

The results revealed that bacterial isolates from both dry and wet environments have the potential to positively influence plant growth and resilience and support other microbes through the exchange of signaling molecules and metabolic products. The metabolic profiles of the Dry isolates showed a distinct capacity to produce compounds that promote stress tolerance and sustain plant growth under arid conditions. These findings suggest that Dry isolates may provide adaptive *"home-field advantages"* that help plants thrive in water-limited environments. Finally, I observed that Lyso-phosphatidylethanolamine 17:1 (LPE 17:1) was one of the most abundant metabolites produced by the Dry ecotype isolates. While the exact function of the LPE 17:1 is unknown, I speculated that it can potentially enhance the plant host's tolerance to drought stress through priming the host immune system.

Although many questions remain, this dissertation advances our understanding of the complex factors that influence plant adaptation to local environments. Furthermore, it sheds light on the mechanisms underlying plant-microbe communication, rhizosphere recruitment, and the complex interactions within the holobiont system, offering a foundation for future research.

Chapter 2 - Home-field advantage affects the local adaptive interaction between *Andropogon gerardii* ecotypes and root-associated bacterial communities

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2.1 Abstract

Due to climate change, drought frequencies and severities are predicted to increase across the United States. Plant responses and adaptation to stresses depend on plant genetic and environmental factors. Understanding the effect of those factors on plant performance is required to predict the species' responses to environmental change. We used reciprocal gardens planted with distinct regional ecotypes of the perennial grass *Andropogon gerardii* adapted to dry, mesic and wet environments, to characterize their rhizosphere communities using 16S rRNA metabarcode sequencing. Even though the local microbial pool was the main driver of these rhizosphere communities, the significant plant ecotypic effect highlighted active microbial recruitment in the rhizosphere, driven by ecotype or plant genetic background. Our data also suggest that ecotypes planted at their homesites were more successful in recruiting rhizosphere community members that were unique to the location. The link between the plants' homesite and the specific local microbes supported the *"home field advantage"* hypothesis. The unique homesite microbes may represent microbial specialists that are linked to plant stress responses. Further, our data support ecotypic variation in the recruitment of congeneric but distinct bacterial variants, highlighting the nuanced plant ecotype effects on the rhizosphere microbiome recruitment. These results improve our understanding of the complex plant host-soil microbe interactions and should facilitate further studies focused on exploring the functional potential of recruited microbes. Our study has the potential to aid in predicting grassland ecosystem responses to climate change, and impact restoration management practices to promote grassland sustainability.

2.1.1 Importance

In this study, we used reciprocal gardens located across a steep precipitation gradient to characterize rhizosphere communities of distinct dry, mesic, and wet regional ecotypes *of the perennial grass Andropogon gerardii*. We used 16S rRNA amplicon sequencing and focused oligotyping analysis and showed that even though the location was the main driver of the microbial communities, ecotypes could potentially recruit distinct bacterial populations. We showed that different *A. gerardii* ecotypes were more successful in overall community recruitment and recruitment of microbes unique to the *"home"* environment, when growing at their *"home site"*. We found evidence for *"home field advantage"* interactions between the host and host-root-associated bacterial communities, and the capability of ecotypes to recruit specialized microbes that were potentially linked to plant stress responses. Our study aids in better understanding of the factors that affect plant adaptation, improve management strategies, and predict grassland function under the changing climate.

2.1.2 Keywords

Rhizosphere, plant microbiome, ecotypic variation, local adaptation, precipitation gradient

2.2 Introduction

The rhizosphere is a dynamic region characterized by the complex interactions between the plant host and associated microbial communities [451,452]. It has been widely recognized that plants directly and indirectly benefit from associated microbial activities and resultant microbial compounds [453,454]. Complex microbe-microbe interactions around the rhizosphere facilitate nutrient transformations, uptake, and cycling as well as alter soil structure and soil water

availability [455,456]. Similarly, plant host-microbe interactions can also greatly affect the overall plant health and productivity [457,458]. Root-associated microorganisms can affect plant resistance to biotic and abiotic stress, help with nutrient uptake and even alter plant morphology and phenology [459,460]. Given the crucial roles of the root-associated bacterial communities, understanding what shapes their assembly, function, and mechanisms, as well as adaptive responses between plant host and associated microbes is critical in predicting the response of this system to changing environmental conditions [461,462].

Numerous studies have demonstrated the need to consider the interactive effects of the environment and plant host genetics in understanding the root-associated bacterial communities. The chemical and physical characteristics of the local soil can directly affect plant function, which in turn can have consequential influence on the root-associated bacterial composition [461,463,464]. Plant hosts actively modulate associated microbial communities by releasing various signaling molecules (phytohormones) and compounds into the soil [461,465]. Phytohormones are structurally diverse secondary metabolites released by the plant to activate the immune system [466,467] in response to microbial pathogens [468] and even insect herbivores [469]. In addition to direct immune responses, plants produce phytohormones and compounds in response to abiotic stress such as nutrient or water deficiency, or to promote symbiotic interactions with soil microbes [470]. Although there are numerous studies on plant host influence on root-associated bacterial community composition, little information is available in the natural ecosystems about the interactive impact of the host-environment on these communities. The root-associated bacterial community is a subset of microorganisms available in the surrounding soil microbial pool [465]. Thus, it is no surprise that the same plants perform differently in distinct locations. In previous studies, the "*home field advantage*" is described as stability of the

performance of an organism at the *"home"* environment, and decrease in performance away from *"home"* [471–473]. Interestingly, some studies have described an increase in efficacy of the plant root host-associated bacterial communities interaction due to the *"home field advantage"* [471,472]. However, questions remain if plants locally adapted to the prevailing environmental conditions can take advantage of the local soil microbes or if plants preferably favor microbiomes similar to their home environment.

Due to climate change, drought frequencies and severity are predicted to increase across the United States [474–476]. In the Great Plains, drought events limit productivity especially in tallgrass prairies [475]. Thus, understanding the effect of drought in shaping the root-associated bacterial communities and the mechanisms of mutual adaptation between plants and associated microbes is critical in the prediction of the ecosystem's response to changing climate. To date, most studies [477–479] on the effect of drought on plant-microbe interactions have been under in vitro conditions using model organisms, lacking the complexity and dynamics of the natural systems [480,481].

To address the question if there is a co-adaptation of the plant host and its associated root-associated bacterial communities in a non-model plant system, our study took the opportunity to investigate the root-associated bacterial composition of three locally adapted big bluestem (*Andropogon gerardii* Vitman) ecotypes planted in reciprocal common gardens across the precipitation gradient in the Great Plains. *Andropogon gerardii* is a perennial C4 grass that is widely distributed across the Great Plains of North America [482,483], and covers up to 80% of the biomass in tallgrass prairie [295,439,482,484]. Within the Great Plains, *A. gerardii* has been growing for over 10,000 years along prominent sharp rainfall gradients that range from semiarid to heavy rainfall [485,486]. Time and environmental heterogeneity has lent support for local

adaptation of *A. gerardii*, giving rise to distinct ecotypes (dry, mesic, and wet) [485,487–489]. Previous investigations have revealed that *A. gerardii* ecotypes vary in functional traits that influence microbially mediated processes [489,490]. Although there are studies investigating intraspecific [491] and interspecific (reviewed in [489]) plant responses to climate [492], the role of the root-associated bacterial communities in plant host adaptation in the natural system remains unclear.

Here, we (1) investigated the relative importance of the environment and ecotypic variation of *A. gerardii* on establishing the plant root-associated bacterial communities; and (2) compared the abilities of three regional *A. gerardii* ecotypes planted reciprocally, to recruit microbes in local and non-local environments. We hypothesized that *A. gerardii* ecotypes would perform better in rhizosphere microbial recruitment of unique microbes at the site closely matching their *"home"* environment, highlighting the effect of the plant genetic background or ecotype on rhizosphere community assembly.

2.3 Materials and Methods

2.3.1 Study sites, samples collection and processing

We sampled three *A. gerardii* reciprocal gardens in the summer of 2019 before the flowering of the grass. The gardens had been established in 2009 and continually maintained at the three sites: Hays, KS (H) at Kansas State University Agriculture Experimental Station (38°85'N, 99°34'W), Manhattan, KS (MHK) at USDA Plant Materials Facility (39°19'N, 96°58'W), and Carbondale, Illinois (C) at Southern Illinois University Agriculture Research Station (37°73'N, 89°17'W), giving us an excellent opportunity to study the interactive effects of local environment and hosts on the root-associated bacterial communities (Supplementary Table

1). Experimental details of the reciprocal garden experiment have been published in Galliart et al. [487]. Briefly, in 2009, seeds were collected from four populations, which jointly defined each of the three regional ecotypes. Morphological traits of the populations vary among ecotypes, but populations do not differ within ecotypes, thus confirming the regional nature of the ecotypes [440,443,493,494]. Seeds were collected from mixed grass prairie in Central Kansas (referred as CKS/Dry: CDB, REL, SAL, WEB), and tallgrass prairie in Eastern Kansas (EKS/Mesic: CAR, KON, TAL, TOW) and Southern Illinois savanna (SIL/Wet: 12MI, DES, FUL, WAL) (Acronyms of populations are listed in Supplementary Table 1) across the natural rainfall gradient with 580 mm/year, 871 mm/year and 1167 mm/year of mean annual precipitation, respectively. Selected populations originated from intact prairies within a 80 km radius of each reciprocal garden site [494]. Seeds representing the three ecotypes and twelve populations were germinated and grown in a greenhouse using potting mix (Metro-Mix 510). Established 3- to 4-months-old seedlings were then planted at each three reciprocal garden sites (size - 4 × 8 m), in which 12 plants (4 populations x 3 ecotypes) were planted in a complete randomized block design with 10 blocks (rows) for a total of 120 plants per site. Plants were planted 0.5 m apart along each row, and the soil around the plants was covered with the water-permeable landscape cloth for weed control. After almost 10 years after establishment in 2009, we sampled *A. gerardii* root-associated bacterial communities in the reciprocal gardens for this experiment.

To track microbial communities, we collected the top-most soil layer (top 15 cm) due to the highest microbial activity in this layer of soil. We collected a single core (15 cm deep x 1.25 cm diameter) as close as possible to each plant. The side of the plant was picked randomly to ensure sample heterogeneity. Each core represented an independent sample, i.e., cores were not pooled with other cores. Seven plants had not survived the transplanting or through the ten years

in the common gardens. However, the plant mortality differed in the Pearson's chi-squared test neither for ecotype (χ^2-squared = 4.44, df = 2, p-value = 0.109) nor population (χ^2-squared = 9.43, df = 12, p-value = 0.665), suggesting that mortality was random without predictable patterns across ecotypes or populations. In addition, we obtained 10 additional soil cores (15 cm deep x 1.25 cm diameter) randomly within each site to characterize the soil pH, texture and moisture content at the site level. All collected samples were sealed in ziplock bags on site, transported on ice, and stored at -20°C until processed.

We extracted total DNA from 0.150 g of roots and rhizosphere soil from all 353 samples using an Omega E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) as per the manufacturer's protocol with a slight modification. Roots were picked manually from the collected soil cores, shaken to remove non-adhering soil, and weighed for the DNA extractions. Any soil that remained attached to the roots was considered rhizosphere soil [495,496]. We mechanically lysed the cells on a Qiagen TissueLyser II (Qiagen, Hilden, Germany) using glass beads for 2 mins at 20 rev/s prior to any downstream DNA extraction steps. Even though the mechanical lysis was a step in the DNA extraction process, the grass roots were never fully homogenized in the process; therefore, our results represent mainly rhizosphere microorganisms and may have excluded many endorhizosphere organisms. The extracted DNA was eluted to 100 µL final volume. The DNA yield and concentration was measured using a Nanodrop and a Qubit™ dsDNA BR Assay Kit. Extracted DNA was sequenced (2 x 250 cycles) with the 16S rRNA V4 region amplified using the primers 515F and 806R at the Kansas State University Integrated Genomics Facility. Some of the acquired rhizosphere samples did not produce adequate DNA extracts for metabarcoding or failed to produce metabarcode sequencing data. In our downstream statistical analyses, we used Analysis of Variance (ANOVA) and its permutational analog (PERMANOVA), which are robust even for

heteroscedastic data and non-balanced designs [497], to characterize our datasets due to the uneven numbers of remaining replicates within each site, ecotype and population. Altogether, our data included a total of 284 *A. gerardii* rhizospheres across the three sites (Supplementary Table 2).

2.3.2 Soil chemistry and soil properties analyses

We performed soil %C, %N analysis on an aliquot of rhizosphere soil samples. For soil chemistry, the rhizosphere soil samples were homogenized from each soil core (total n = 360) through a 4-mm sieve to homogenize the soil and remove rocks and large pieces of roots and followed by handpicking small roots from each soil sample. Due to the abundance of root material in the sampled cores, the weight of some soil samples were not enough to accurately measure the soil chemistry. We did not observe the pattern across low weight samples and therefore removed them from following processing resulting in a total of 278 samples used for the soil chemistry. A 15 g subsample of sieved soil was then dried at 55°C for a week. Soil samples were then homogenized to a fine powder in a mixer mill (SPEX Instruments, Metuchen, NJ) and re-dried them at 55°C. Dried and ground soil (55 mg) were used for the %C and %N measurement using dry combustion followed by gas chromatography on a Thermo Scientific FlashSmart 2000 CN Soil Analyzer (Milan, Italy). To correct %C and %N data for outliers, we removed all the values that were ± two standard deviations from the mean within each site prior to the analysis. As a result, we removed 34 samples from %C (total samples for %C = 244; H = 79; C = 59; MHK = 106) and 30 samples from %N (total samples for %N =249; H = 83; C = 59; MHK = 106) (Supplementary Table 4). The C:N ratio was calculated manually using %C and %N values (total samples for C:N = 244). We used the ANOVA in R studio for the overall statistical analysis (with

site, ecotypes, populations nested within populations and blocks as factors) as well as the effect of ecotype at each site followed by pairwise comparisons using Kruskal-Wallis test [498].

The collected soil samples reserved for pH, moisture content and soil texture (total n = 30) were sent to Kansas State University Soil Testing Laboratory (www.agronomy.k-state.edu/services/soiltesting/). The fresh and dry soil weights were recorded to estimate the moisture content. The moisture content was calculated using $MC=(w-d)/w*100$ formula where w and d are the wet and dry weight of soil, respectively. 10 g of soil was used for the pH measurement using the saturation paste method using 1:1 soil and water ratio. 25 g of soil was used for soil texture where the ratios of sand/silt/clay were measured, and the soil textures were identified using the soil texture triangle. We used the ANOVA in R studio for the overall statistical analysis followed by pairwise comparisons using Kruskal-Wallis test (v 4.1.1) [498]

2.3.3 Sequence data processing and analyses

We used QIIME 2 v. 2021.4 [499] to process a total of 8,353,179 raw sequences, resulting in 5,628,302 bacterial sequences after quality control. We used QIIME 2 plugin cutadapt [500] to remove the primer sequences. Any sequences with ambiguous bases, with no primer, with greater than 0.1 error rate mismatch with primer or any mismatches to the sample-specific 12 bp molecular identifiers (MIDs) were discarded. Following initial quality control, we used DADA2 [501] with the same parameters across 2 different runs and truncated the reads to length where the 25th percentile of reads had a quality score below 15 (Forward 231 and Reverse 229). The first run included 24 samples and was used as a trial run for the project, and the rest of the project samples were sequenced when the quality of the samples was confirmed. Since the same primers were used for the first 24 samples, the quality control and primer removal using DADA2 were performed

separately. Samples then were merged together (total n = 284) and analyzed as one dataset. We used the pre-trained SILVA database (v. 138) in QIIME 2 for taxonomic assignment of the bacteria. Sequences were blinded to amplicon sequence variants (ASVs) and any unknown or unclassified ASVs were removed from downstream analysis. We rarefied the data set to 10,000 reads per sample (resulting in 2,104,416 high quality sequences) to minimize biases resulting from differences in sequencing depth among samples before estimating diversity indices and downstream analyses [502]. We used QIIME 2 for the sequence processing pipeline, whereas the subsequent statistical analyses for microbial richness, diversity and composition were performed using R studio [503].

We used ANOVA to test for main and interactive effects in observed richness (S_{Obs}), community (Shannon's H'), and phylogenetic (Faith's PD) diversity of the rhizosphere associated bacterial communities among the sites (Hays (H), Carbondale (C), Manhattan (MHK)), ecotypes (dry, mesic, wet), and populations (12MI, CAR, CDB, DES, FUL, KON, REL, SAL, TAL, TOW, WAL, WEB) nested within ecotype. Following overall ANOVA, we used pairwise comparisons using Kruskal-Wallis test to identify factors that were driving the significant effects. To identify the specific group driving the significance we ran Pairwise TukeyHSD with the adjustment for the multiple comparisons (R studio v 4.1.1) [498].

We used the vegan package [504] to estimate the pairwise Bray-Curtis distances to compare the bacterial communities among the different factors. We then used the ggplot2 package [505] to visualize these data using Non-Metric Multidimensional Scaling (NMDS) ordinations. We used a nonparametric PERMANOVA to determine whether bacterial communities differed compositionally among sites, ecotypes, and populations as well as their two- and three-way interactions. In this model, "population" was nested within "ecotype." Following the

PERMANOVA, we performed pairwise comparisons using the pairwise.adonis function. We also used betadispr function in the vegan package [504] to test whether the community dispersion differed between any significant groups. To determine if any sites, ecotypes, or ecotypes within each of the sites had Amplicon Sequence Variants (ASVs) that were disproportionately more abundant in one group than in another, we used indicator species analysis with the "multipatt()" function in R package "indispecies" (v. 1.7.12) [506].

2.3.4 Oligotyping analyses

We used minimum entropy decomposition (MED) [507] algorithm with default parameters to identify sequence variants among the 16S rRNA amplicon sequences. The MED partitions the sequences into discrete sequence groups by minimizing the total entropy in the dataset. We then concatenated the sequences assigned to a specific genus using the Silva (v. 138) reference database and used the supervised oligotyping method available in the oligotyping pipeline version 2.1 [508]. In this supervised oligotyping method, we used Shannon entropy, with a threshold value of minimum 0.2 and minimum substantive abundance threshold of 10, to obtain genus-level oligotypes. We selected Pseudomonas and Rhizobium oligotypes for further analysis because of their overall high relative abundance across the bacterial ASVs and generated oligotypes (Oligos: Pseudomonadales-Oligos (n=6); Rhizobiales-Oligos (n=5)).

2.4 Results and discussion

We analyzed a total of 5,628,302 (19,818 ± 4531 per sample) high quality sequences assigned to 1,441 ASVs (phyloseq v 1.42.0 [509], R ape [510]. Of the recovered reads, an average of 90% was annotated to the genus level using the Naivé Bayesian classifier [511]. Any reads

assigned to chloroplasts and mitochondria as well as any unknown or unclassified ASVs were removed from downstream analyses. The bacterial taxon assignments along with their counts in each sample are provided in Supplementary Table 3. Our data were dominated by the phylum Proteobacteria (45% of all sequences), followed by Actinobacteria (15%), Acidobacteria (12%) and Bacteroidetes (6%). A small proportion of sequences remained unclassified (0.29%). ASV relative abundances were dominated by Proteobacteria (28% of all ASVs), Actinobacteria (14%), Firmicutes (12%) and Bacteroidetes (9%). Similar to sequences, the proportion of unassigned ASVs was small (0.3%).

2.4.1 Differences in precipitation are associated with the distinct soil chemistry and texture parameters across sites

Our overall analysis (with site, ecotypes, populations nested within populations and blocks as factors) highlighted that site was the main driver of the soil chemistry parameters (ANOVA, pH: $F_{2,27} = 226.31$; $P < 0.001$; Moisture content: $F_{2,27} = 124.90$; $P < 0.001$) (Figure 2.1, Table 1, Supplementary Table 4). As expected, due to the precipitation gradient from east to west, we observed a reciprocal decrease in pH from Hays to Carbondale (H: 7.6 ± 0.11; MHK: 6.96 ± 0.21; C: 5.47 ± 0.29; C vs MHK: $P < 0.001$; C vs H: $P < 0.001$; H vs MHK: $P < 0.001$). We also observed differences in the soil texture across sites. Both Carbondale and Hays were identified as Silt Loam (C: Silt = 73.7%; Clay = 15.1%; Sand = 11.2%; H: Silt = 70.1%; Clay = 17.3%; Sand = 12.6%) whereas Manhattan had more sandy soil and was identified as Sandy Loam (MHK: Silt = 8.2%; Clay = 15.1%; Sand = 57.9%). Consistent with our observations on soil texture and pH, we further showed differences in the moisture content among the sites - Carbondale had the highest moisture content, followed by the Hays and Manhattan. Even though we expected Manhattan to have a

higher moisture content than Hays, water retention would not be optimal in the Manhattan sandy soil.

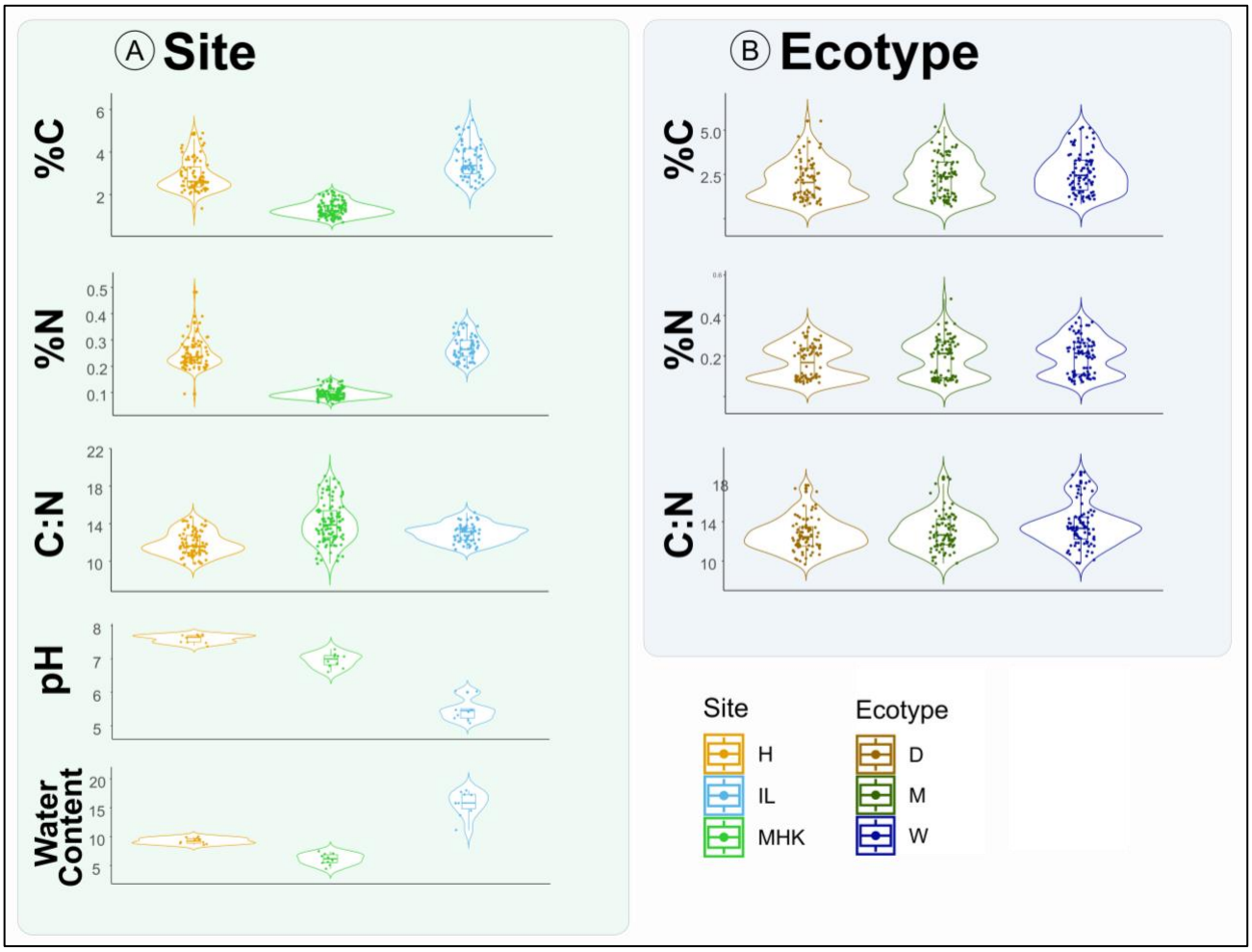

Figure 2.1 Soil chemistry across locations and ecotypes. (A) Soil chemistry and water content among three sites located across the precipitation gradient: Hays, Manhattan, and Carbondale. Soil pH, %C, %N, and the water content. (B) %C and %N concentration across dry, mesic, and wet ecotypes.

Next, we compared the carbon (%C) and nitrogen (%N) content as well as C:N ratio in the rhizosphere surrounding soil (ANOVA, %C: $F_{2,222} = 290.03$; $P < 0.001$; %N: $F_{2,225} = 495.31$; $P < 0.001$; C:N: $F_{2,221} = 51.36$; $P < 0.001$) (Figure 2.1, Table 2.1, Supplementary Table 4). We used pairwise comparison tests and showed that Carbondale was significantly higher in %C and %N

than Hays (%C - C: 3.57± 0.794; H: 2.89 ± 0.74; %N - C: 0.27 ± 0.05; H: 2.48 ± 0.06; C vs H: $P < 0.001$) and Manhattan (%C - MHK: 1.31 ± 0.34; %N - MHK: 0.10 ± 0.02; C vs MHK: $P < 0.001$), while Manhattan was also lower than Hays in %C (H vs MHK: $P < 0.001$) and %N (H vs MHK: $P < 0.001$). C:N ratio also differed across all locations, and was higher in Manhattan than in Carbondale (C:N - MHK: 14.13 ± 2.193; C: 13.01 ± 0.94; MHK vs C: $P < 0.001$) and Hays (C:N - H: 11.90 ± 1.125; C vs H: $P < 0.001$), while Carbondale was also higher then Hays (C vs H: $P < 0.001$). In contrast to the clear differences among the sites, we observed higher %C and C:N in wet compared to dry ecotype (ANOVA: %C: $F_{2,223} = 3.47$; $P = 0.03$; Dry vs Wet: $P = 0.028$; ANOVA: C:N: $F_{2,223} = 5.29$; $P = 0.005$; Dry vs Wet: $P = 0.011$), while %N was not statistically different among ecotypes across all locations (ANOVA: %N: $F_{2,247} = 2.55$; $P = 0.079$) (Figure 2.1, Table 2.1, Supplementary Table 4).

Table 2.1 Soil Chemistry ANOVA. Statistical analyses (ANOVA and pairwise test) reveal locations (C-Carbondale, H-Hays, MHK-Manhattan) had a strong influence on the soil chemistry and water content parameters.

Index	ANOVA						Pairwise test		
		Df	Sum Sq	Mean Sq	F-value	P-value	C	H	
pH	Location	2	23.88	11.94	226.31	7.50E-06	2.00E-16	-	H
	Residuals	27	1.43	0.05			2.90E-14	1.20E-06	MHK
Moisture content	Location	2	474.61	237.3	124.9	2.26E-14	6.30E-11	-	H
	Residuals	27	51.3	1.9			5.70E-15	2.20E-05	MHK
C%	Location	2	22542.1	11271.1	290.03	2.20E-16	7.70E-07	-	H
	Residuals	222	8627.3	38.9			2.00E-16	2.00E-16	MHK
N%	Location	2	1.53	0.81	495.31	2.20E-16	0.002	-	H
	Residuals	225	0.37	0.002			2.00E-16	2.20E-16	MHK
C/N	Location	2	229.07	114.53	51.37	2.20E-16	0.00037	-	H
	Residuals	221	491.14	2.22			1.14E-02	4.50E-11	MHK

Due to the extremely strong site effect, we believe that the differences among dry and wet ecotypes were driven by the sites, therefore, in order to focus exclusively on the effect of ecotype, we split our data set and analyzed them at individual sites. The *"homesite"* soil characteristics play an important role in plant adaptation to the environment and ecotypic divergence [485]. Therefore, the differences in the ecotypic physiology such as root length and litter deposit biomass that varied across ecotypes even when growing at the same site, could potentially result in differences of the microbial recruitment [486,489] In our analysis by site, we observed that the differences in %C were driven by the wet ecotype in Manhattan which was higher when compared to dry and mesic ecotypes (MHK: ANOVA %C: $F_{2,85} = 5.59$; P = 0.005; Wet vs Dry: P = 0.002; Wet vs Mesic: P = 0.002). In addition, we observed that %N and C:N were also significantly different across

ecotypes. Similar to %C, differences were driven by the higher values associated with wet ecotype (MHK: ANOVA %N: $F_{2,85} = 7.76$; $P < 0.001$; Wet vs Dry: $P = 0.003$; Wet vs Mesic: $P = 0.003$; C:N: $F_{2,84} = 6.60$; $P = 0.003$; Wet vs Dry: $P = 0.018$) (Figure 2.1, Table 1, Supplementary Table 4). As expected from the combination of higher water content, lower pH and soil texture factors, Carbondale was associated with the higher %C and %N parameters [512]. We surmise that the soil texture was one of the main factors that contributed to the significance of the wet ecotypic soil chemistry difference in Manhattan. The finer soils are associated with the higher stabilization of SOM and total %C storage [513], therefore we did not observe differences between ecotypes in the Silt Loam soil of Carbondale or Hays. In the sandy soil of Manhattan, the differences in the soil carbon deposit would become more distinct between ecotypes, and resulted in higher wet ecotypic carbon deposits due to the wet plant host having a higher overall biomass [513,514]. Additionally, although the root C:N strongly differed among the ecotypes across sites [515], our data provided no support for differences in the rhizosphere soil %N among the ecotypes, suggesting that the rhizosphere soils are resistant to the N rhizodeposits, and such changes in the rhizosphere soil may take longer than the decade that the experiment has been in place [516,517].

2.4.2 Site had a strong impact on the ecotypic root-associated bacterial communities' richness and diversity

Site was the main driver of the bacterial communities, with a strong effect on bacterial richness and diversity (ANOVA, S_{Obs}: $F_{2,245} = 7.13$; $P < 0.001$; Shannon's H' index: $F_{2,245} = 26.56$; $P < 0.001$; Faith's PD index: $F_{2,245} = 4.09$; $P = 0.018$) (Figure 2.2A). Pairwise tests showed that community richness (S_{Obs}) and Shannon's diversity were lower in Manhattan than in the other two sites (Table 2.2). On the other hand, the phylogenetic diversity (Faith's PD) in Carbondale was

marginally higher than in Hays and Manhattan (Table 2.2). Phylogenetic diversity analyses suggested that Manhattan was favorable for generalist microbes characterized by the lower diversity [452,454] due to the intermediate soil chemistry and moisture conditions as compared to Hays and Carbondale. The higher bacterial phylogenetic diversity we observed in this study could be highly attributed to the differences in amount of precipitation and edaphic properties among the sites. Apart from the main effects, we also observed some significant interaction terms in the overall model. Although, the significant interaction terms were contributes to the location and ecotype effects in community richness and phylogenetic diversity (ANOVA, S_{Obs}: $F_{10,245} = 2.83$; $P = 0.025$; Faith's PD index: $F_{10,245} = 2.72$; $P = 0.0304$), our pairwise comparison tests did not identify the significant combinations (Pairwise TukeyHSD, S_{Obs}: $P_{adj} > 0.283$; Faith's PD index: $P_{adj} > 0.280$).

Similar to the alpha-diversity, the bacterial communities clustered by site indicate that sites had strong effects on bacterial composition (PERMANOVA, $R^2 = 0.42$; $F_{2,245} = 102.53$; $P = 0.001$; Figure 2.2B). Our pairwise comparisons further corroborated that all sites had significantly different bacterial compositions (Pairwise Adonis, C vs H: $R^2 = 0.435$; $P = 0.001$; $P_{adj} = 0.003$; C vs MHK: $R^2 = 0.313$; $P = 0.001$; $P_{adj} = 0.003$; H vs MHK: $R^2 = 0.274$; $P = 0.001$; $P_{adj} = 0.003$). We also conducted a dispersion analysis, and observed that bacterial communities in Manhattan ($F_{2,279} = 3.899$; $P = 0.015$) were more heterogeneous than in Carbondale ($P = 0.020$). Similar to richness, the variation in bacterial community compositions among the sites were likely a result of precipitation-associated biotic and abiotic differences [518] as well as interspecific microbial competition [452,454]. For example, the mean precipitation in Carbondale is 1167 mm/yr, double that in Hays (580 mm/yr). This precipitation gradient as well as differences in the soil chemistry and texture among the three sites (Figure 2.1, Supplementary Table 4) would result in differences

in pH which is a key factor influencing bacterial diversity [519,520] (Table 2.2). Here, we showed that the site had a strong effect on bacterial diversity and composition. We next address if there are any bacterial populations that differed in relative abundance among the sites.

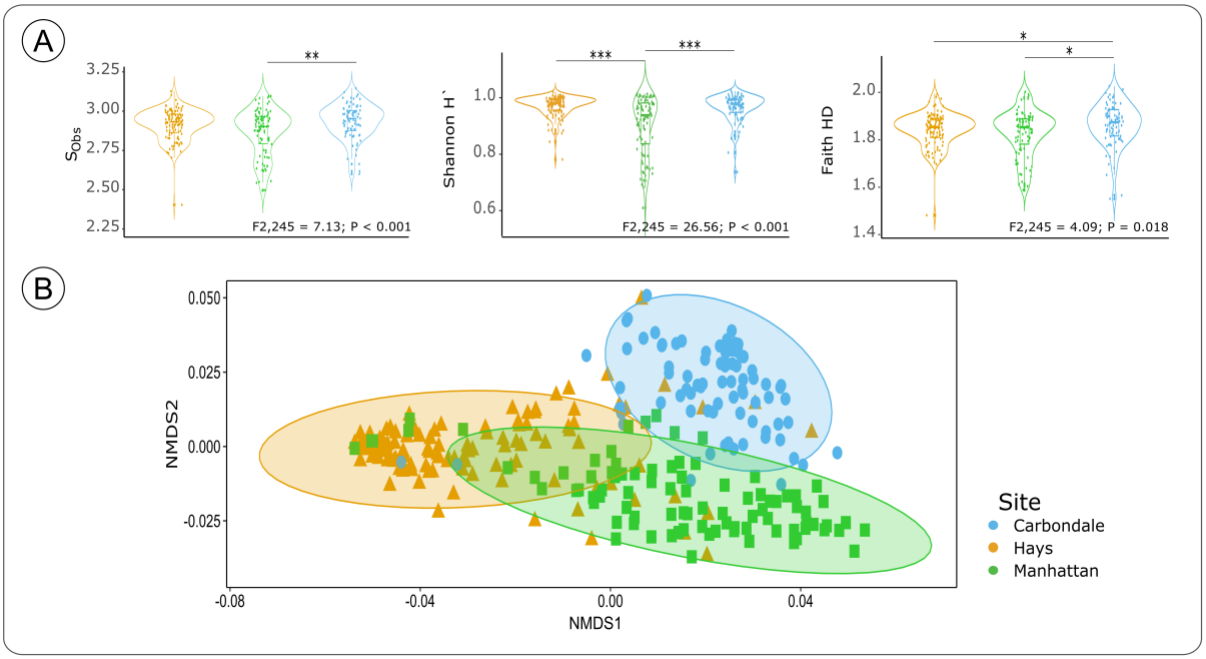

Figure 2.2 Bacterial alpha and community composition among three sites located across precipitation gradient. (A) Bacterial α-diversity indices among three sites located across precipitation gradient: Hays, Manhattan, and Carbondale. Species observed richness (S_{Obs}), Shannon's H index, and Faith-PD index. (B) Location impact on *Andropogon gerardii* rhizosphere bacterial composition. NMDS plot of *A. gerardii* rhizosphere communities associated with three site locations across the precipitation gradient: Hays, Manhattan, and Carbondale. NMDS ordinations were obtained from Bray–Curtis similarity matrix (P-values: * −0.05, ** −0.01, *** −<0.01).

Table 2.2 Alpha Diversity ANOVA. Statistical analyses (ANOVA and pairwise test) reveal locations (C-Carbondale, H-Hays, MHK-Manhattan) had a strong influence on the rhizobiome diversity and richness

Index	ANOVA			Pairwise test		
	chi-squared	F(2,245)	P-value	C	H	
SObs	10.42	7.13	0.005	H = 2.70; P = 0.1002	-	H
				H = 9.35; P= 6.70e-o3	H = 4.08; P = 0.0653	MHK
Shannon's H	25.171	26.56	3.42e-o6	H = 0.14; P = 0.7	-	H
				H = 16.63; P = 6.90e-o5	H = 21.00; P = 1.40e-o5	MHK
Faith's PD	6.7829	4.09	0.03366	H = 4.7; P = 0.044	-	H
				H = 5.47; P = 0.044	H = 0.30; P = 0.585	MHK

Index	ANOVA			Pairwise test		
	chi-squared	F(2,245)	P-value	Dry	Mesic	
SObs	3.46	3.46	0.033	H = 0.30; P = 0.585	-	Mesic
				H = 3.80; P = 0.078	H = 6.86; P = 0.026	Wet
Shannon's H	0.91	0.91	0.404	H = 1.15; P = 0.285	-	Mesic
				H = 1.12; P = 0.285	H = 5.40; P = 0.060	Wet
Faith's PD	2.51	2.51	0.108	H = 0.08; P = 0.779	-	Mesic
				H = 2.79; P = 0.095	H = 4.10; P = 0.130	Wet

Our indicator taxon (alpha=0.005) analyses identified 194 taxa that differed among the sites. The majority of the taxa were associated with the wettest and driest sites (Carbondale (n=181); Hays (n=151)), whereas fewer were associated with the mesic intermediate site (Manhattan (n=38)) (Supplementary Table 5), suggesting that Carbondale and Hays harbored higher number of habitat specialist microbes due to the prevailing environmental conditions [521,522]. Of our three common gardens, Manhattan had the most intermediate conditions for growth of *A. gerardii*, where plants were less likely to experience abiotic stress including water availability stress in Hays and waterlogged conditions in Carbondale. We further observed that the majority of soil bacterial indicator ASVs were assigned to the following five phyla - Acidobacteria (Total n=31; C = 21, H = 5, MHK = 5); Actinobacteria (Total n=66; C = 15, H = 47, MHK = 4); Planctomycetota (Total n=20; C = 13, H = 7, MHK = 0); and Proteobacteria (Total n=109; C = 53,

H = 41, MHK = 15); and Verrucomicrobia (Total n=23; C = 14, H = 9, MHK = 0) (Supplementary Table 5). It is unsurprising that these phyla dominated in our indicator taxon analysis since they are ubiquitous among rhizosphere associated bacterial communities [490,523–525]. However, these indicators were not evenly distributed across sites, suggesting that this pattern was due to the soil moisture affecting the bacterial distribution. Soil moisture can directly affect the soil microbial composition and functionality by differentiating drought tolerance among taxonomic and functional groups of microorganisms [526,527]. For example, limited soil moisture restricts the solute mobility and therefore decreases substrate supply to the soil microbes. Therefore, highly moisture-sensitive Gram-negative bacteria populations (Acidobacteria, Planctomycetes, Verrucomicrobia) were affected by the limited water, resulting in lower number indicators in Hays and disproportionately higher in Carbondale [528–530]. In line with this argument, we also observed that Gram-positive bacteria (Actinobacteria) were disproportionately more abundant in Hays than in Carbondale and Manhattan [531].

2.4.3 Ecotypes shaped the rhizosphere inhabiting bacterial richness and diversity

While there was a strong site effect on all alpha diversity metrics, a marginal ecotype effect was observed only in (S_{Obs}) richness (ANOVA, S_{Obs}: $F_{2,245} = 3.46$; $P = 0.033$). The pairwise comparisons among ecotypes indicated that the mesic ecotype host-root-associated bacterial communities had a higher observed richness (S_{Obs}) than the wet ecotype (Mesic vs Wet: $H = 6.86$, $P_{adj} = 0.026$). We observed no differences in bacterial diversity and phylogenetic diversity among host ecotypes (ANOVA, Shannon's H' index: $F_{2,245} = 0.91$; $P = 0.404$; Faith's PD index: $F_{2,245} = 2.51$; $P = 0.108$) (Figure 2.3A). It was not surprising that the ecotype effect was lower than the site effect. Plant ecotypic effects can often be suppressed by the local biotic and abiotic environmental

factors. For example, the environmental gradient defines the local microbial source pool available to the plant and therefore directly affects the source rhizosphere communities [468,532–534]. Taking into consideration the overwhelming effect of the local environment (site), we aimed to test if our ecotypes differed in bacterial richness (S_{Obs}) locally. To do this, we split our data set by sites, and performed separate ANOVAs for each site with ecotype, population, and their interaction as the explanatory factors. We used this model separately for Carbondale, Hays, and Manhattan, to focus on the effect of ecotypes at home and away from home sites. Following that, we observed that only in Hays ($F_{2,87} = 15.06$; $P < 0.001$), but not in Manhattan ($F_{2,87} = 0.10$; $P = 0.905$) or Carbondale ($F_{2,79} = 0.41$; $P = 0.668$), did the ecotypes differ. We further used pairwise tests and identified that the wet ecotype in Hays had significantly lower richness (S_{Obs}) compared to dry (Wet vs Dry: $P_{adj} < 0.001$) and mesic (Wet vs Mesic: $P_{adj} < 0.001$) ecotypes. These results highlight the potential differences in ecotype recruitment and structuring of the rhizosphere communities even when planted in a common environment. The overall effect of ecotype on bacterial community composition was also weaker than the site effect in the overall model (PERMANOVA, $R^2 = 0.01$; $F_{2,245} = 2.91$; $P = 0.012$). We used pairwise comparisons and showed that the difference among the ecotypic bacterial composition was driven by the wet (Wet vs Dry: $R^2 = 0.028$; $P_{adj} = 0.015$) and mesic ecotype (Wet vs Mesic: $R^2 = 0.038$; $P_{adj} = 0.015$; Figure 2.3B).

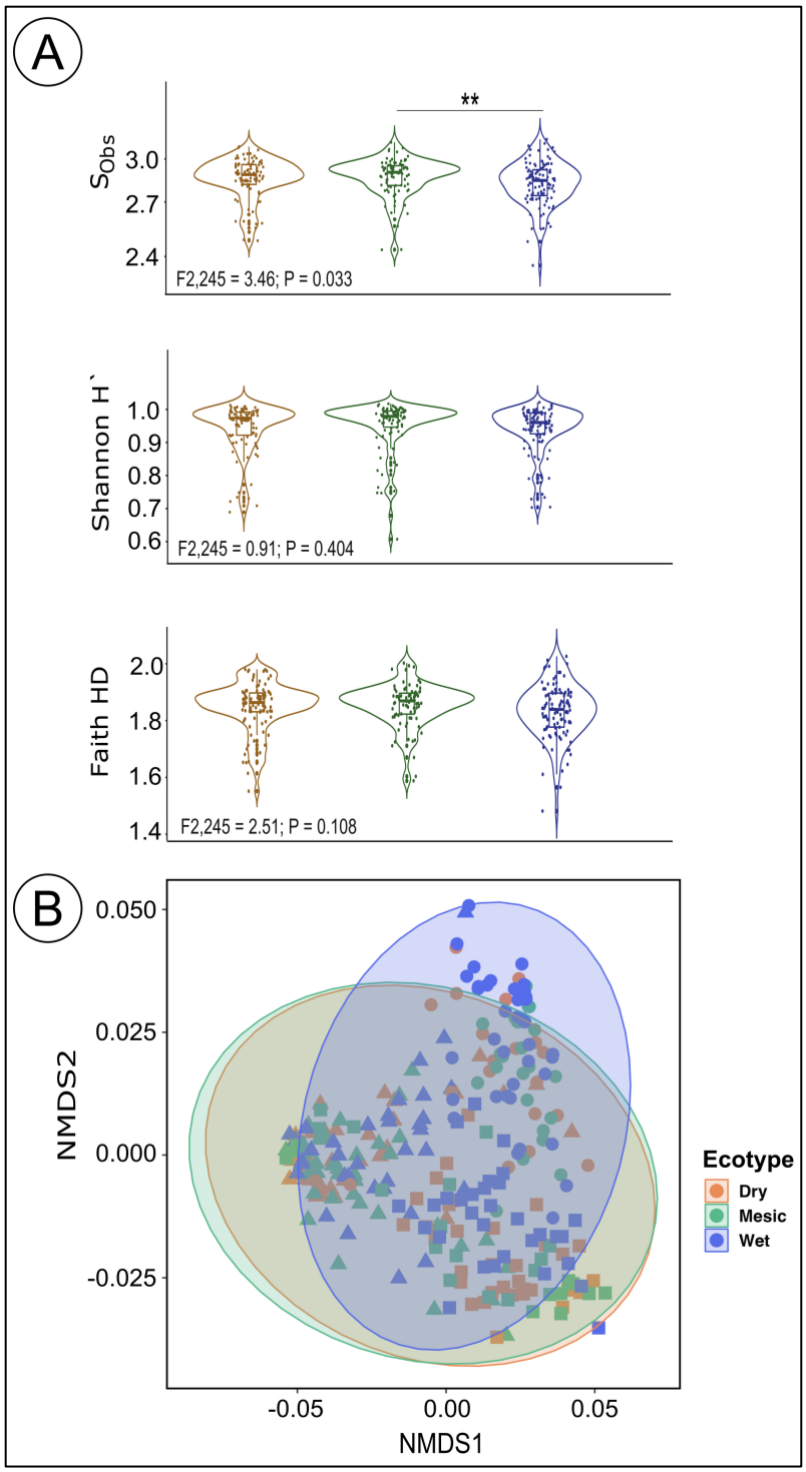

Figure 2.3 Bacterial alpha and community composition among three ecotypes. (A) Bacterial α-diversity indices among the dry, mesic, and wet ecotypes across all locations. Species observed richness (S_{Obs}), Shannon's H index, and Faith-PD index. (B) Ecotype impact on *Andropogon gerardii* rhizosphere bacterial composition. NMDS plot of *A. gerardii* rhizosphere communities associated with three ecotypes across three site locations. NMDS ordinations were obtained from Bray–Curtis similarity matrix (P-values: * −0.05, **−0.01, ***−<0.01).

When we considered the impact of ecotypes on the unique microbial populations at each site, interestingly, we noticed that dry and wet ecotypes harbored more unique taxa when they were planted at their "*home field*", except for mesic ecotype planted in Manhattan (C: Dry = 79, Mesic = 39, Wet = 127), (H: Dry = 121, Mesic = 71, Wet = 49) (MHK: Dry = 77, Mesic = 67, Wet = 72) (Figure 4.4C). Putting together our results on microbial community and indicator taxon analyses, we surmised that interaction between plant ecotypes and sites resulted in the wet and dry ecotypes harboring a higher number of habitat specialist microbes in Carbondale and Hays [521,522]. While the dry ecotype was well-suited to its native environment in Hays, the wet ecotype bacterial communities differed, suggesting that the wet ecotypic communities were not well-adapted to an environment (Hays) that is extremely different from its native location (Carbondale). Therefore, with a mismatch of plant host and root-associated bacterial communities h in Hays, the wet ecotype would not be able to get a "*home field advantage*", resulting in a higher number of generalists - abundant soil microbes that are good at colonizing plants [452,454,522,535,536]. Another limiting factor for the wet ecotype to recruit and retain native specialist microbes in Hays is driven by lower drought stress tolerance of the plant host [485]. Due to the physiological local adaptation to the wetter environment, wet ecotype might be poorly adapted to the drier environment of Hays - resulting in the lower photosynthetic rate [485]. We surmise that lesser volume of photosynthetic products exuded in the soil by the wet ecotype in Hays would limit the microbial taxa the wet ecotype could support in the drier environment [537]. In addition, the limited photosynthates exuded by the wet ecotype would then be metabolized by the more abundant [522] and faster colonizing generalists [538,539]. On the other hand, the dry ecotype in Hays produced lesser biomass but maintained higher photosynthetic rates, which potentially could result in continuous and steady supply of photosynthate in limited quantities

favoring slow growing microbial specialists [522]. Due to the general lower relative abundance of specialists in the samples, statistical analysis on ecotypic Shannon's diversity and Faith PD as well as indicator taxon might not be sensitive enough to show the impact of ecotypes on its associated root-associated bacterial communities [540–542].

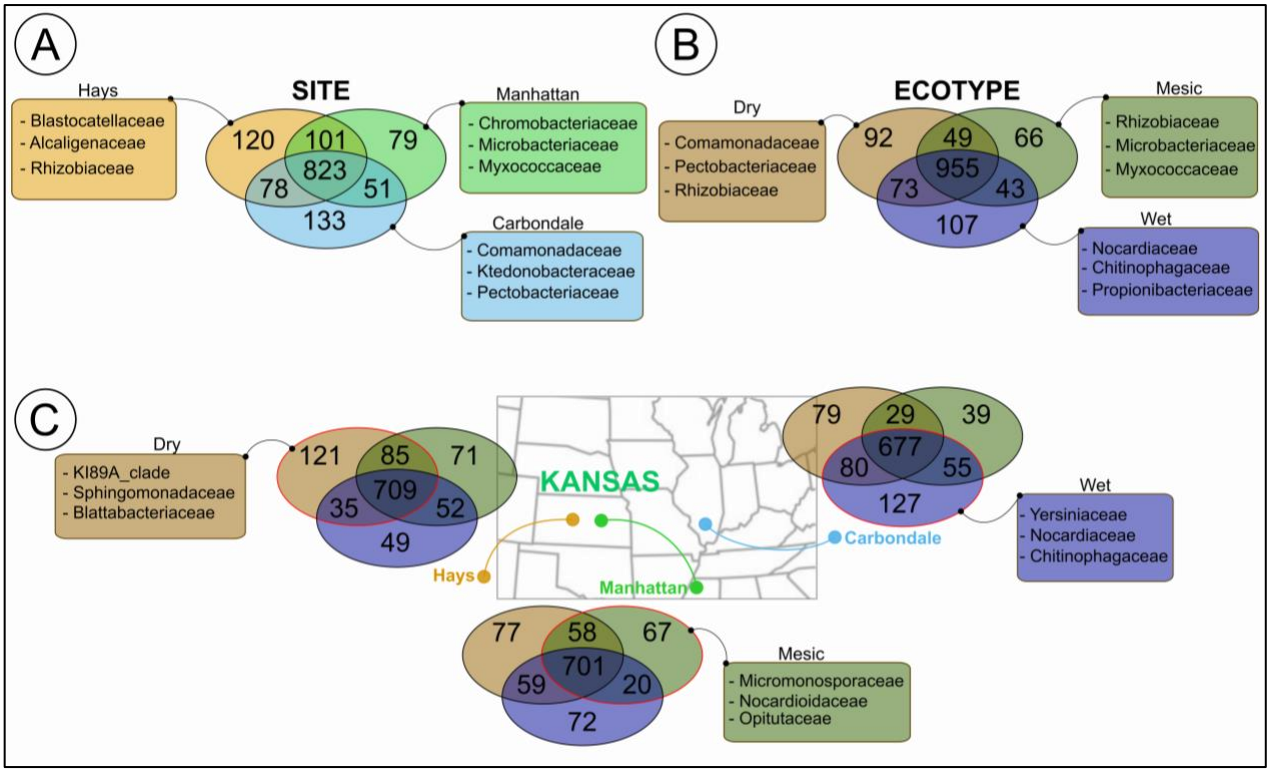

Figure 2.4 Venn diagrams representing the overlapping and unique ASVs (numbers in the circle) among (A) all locations, (B) all ecotypes, and (C) ecotypes in Carbondale, ecotypes in Hays, and ecotypes in Manhattan. The circle with a red edge represents the home ecotype for that location. Inserts represent the top three families. Partial information on unique ASVs is shown here, full information is in Supplementary Table 16. Higher numbers of unique ASVs suggest the "home-field advantage" of the *Andropogon gerardii* ecotypes.

In order to test this idea, we calculated how many microbial taxa were uniquely observed in each site, to provide insights into the environmental and functional selection of potential specialists microbial populations. We showed that Carbondale (133) harbored the highest number of unique microbial taxa, followed by Hays (120) and Manhattan (79) (Figure 2.4A,

Supplementary Table 6). We then looked at the unique taxa associated with ecotypes across all sites. Wet ecotype had the highest number of unique microbes (107), followed by Dry (92) and Mesic (66) (Figure 2.4B, Supplementary Table 6). To confirm for the independence of the unique microbe group assignments we additionally run the Pearson's chi square test for both site ($P > 0.05$) and ecotypes ($P > 0.05$). Our results highlighted that ecotypes gained a "*home field advantage*" when planted in their native environment and were able to match the plant host ecotypes to recruit and retain a higher number of unique microbial populations. On the other hand, the mesic ecotype was consistently associated with a lower number of unique microbial taxa. We hypothesize that the intermediate environment for *A. gerardii* in Manhattan resulted in harboring general microbial taxa that could be equally associated with all the ecotypes, however lacking the unique microbial drivers. Therefore, mesic ecotype was less adapted to recruit microbial specialists and was associated with a lower number of unique taxa across all the sites.

2.4.4 Matching of the plant host ecotype and root-associates bacterial communities was evident at the ecotype homesite

We used indicator taxon analysis to give insights into the interactive relationship between the ecotypic plant host and its root-associated bacterial communities (Supplementary Table 7). We identified numerous indicators that were significantly different among the ecotypes. We further showed that, considering individual sites, there was a strong effect of the ecotypes on the indicator taxa. Our study demonstrated that plant host ecotypes matched best with their root-associated bacterial communities, showing the highest number of indicator taxa, when the plant ecotypes were at their "*home field".*

In the indicator taxon analysis (alpha = 0.005) across all the sites, we identified 26 indicator taxa that differed among the ecotypes. Wet ecotype was associated with the highest number of indicators (n=20), followed by Mesic (n=6) and Dry (n=0) ecotypes (Supplementary Table 8). The majority of soil bacterial indicator ASVs were assigned to Acidobacteria (Total n=10; Dry = 0, Mesic = 2, Wet = 8) and Proteobacteria (Total n=10; Dry = 0, Mesic = 1, Wet = 9). Similarly, taking into consideration the strong effects of sites, we further focused our analysis by studying the ecotypic effects at different sites. We split our data set by sites, and performed separate PERMANOVAs for each site with ecotype, population, and their interaction as the explanatory factors. We used this model separately for Carbondale, Hays, and Manhattan, to focus on the effect of ecotypes at home and away from home sites. We observed that ecotypes differed at all sites (PERMANOVA, H: $F_{2,109} = 2.55$; P = 0.021, MHK: $F_{2,89} = 2.87$; P = 0.014, IL:$F_{2,81} = 2.55$; P = 0.015) (Figure 2.3). In our pairwise comparisons of community composition in Carbondale and Manhattan, wet ecotype only differed from mesic (C: Wet vs Mesic: $P_{adj} = 0.033$, MHK: Wet vs Mesic: $P_{adj} = 0.042$), while other ecotypic comparisons across all sites were not significant ($P_{adj} > 0.05$).

In Carbondale, we further observed that even though the ecotypic bacterial communities dispersion was significantly different ($F_{2,79} = 3.84$; P = 0.026), the dry ecotypic communities were more dispersed than those of mesic and wet ecotypes. In addition, we did not identify ecotype-associated indicator taxa in Carbondale among the ecotypes (n=0), highlighting that the differences in ecotypic recruitment might be on a smaller scale of unique and lower abundance taxa (Supplementary Table 8). In Manhattan, mesic ecotype was significantly more dispersed ($F_{2,87} = 10.72$; P = 0.01) when compared to dry and wet ecotypes. In Manhattan, we identified 11 indicator taxa - 4 indicators were associated with mesic (Actinobacteria=2; Proteobacteria=1;

Verrucomicrobia=2) and 7 indicators with wet ecotypes (Actinobacteria=3; Proteobacteria=3; Cyanobacteria=1). We observed that in Hays, wet ecotype differed in composition from dry and mesic ecotypes (Dry vs Wet: P=0.046, Mesic vs Wet: P=0.030), but no significant differences were observed after adjusting for multiple comparisons (P_{adj} =0.05). Interestingly, ecotypes did not differ in Hays (P_{adj} =0.05). Hays is near the edge of continuous distribution of where *A. gerardii* is commonly found [485], suggesting that the drier environment might have a strong impact on diversity of bacterial populations that can proliferate under the arid conditions [543,544]. Therefore, in Hays, we hypothesize that *A. gerardii* ecotypes 1) had a lower diversity of bacterial populations for recruitment; 2) experienced high abiotic stress from the drier conditions; and 3) had to compete for the recruitment of the smaller microbial specialist populations. Although our results suggest that all ecotypes had a more challenging survivorship in the harsher environment, will the dry and wet *A. gerardii* ecotypes be more resilient and successful in recruiting microbes due to their "*memory*" from surviving in a harsh "*home*" environment [471,545]? Will ecotypes get a "*home field advantage*" in the recruitment of microbes at their "*home*" locations?

To test that, we performed the indicator taxon analysis on ecotypes at each site (Supplementary Table 9). Dry ecotype recruited 64 indicator taxa ASVs in Hays, 43 in Carbondale and only 15 in Manhattan. Similarly, wet ecotype in Carbondale had the highest number of indicators (120), followed by Hays (76) and Manhattan (19). Mesic ecotype recruited 87 indicators in Carbondale, 51 in Hays and only 7 in Manhattan. The majority of the indicator taxa from dry ecotype were Proteobacteria (41), Actinobacteria (32), followed by Acidobacteria (12). On the other hand, wet ecotype had disproportionately higher relative abundance in Proteobacteria (69) and Actinobacteria (37), followed by Verrucomicrobia (19). Even though lower microbial recruitment in Manhattan could be generally due to lower number of indicators in Manhattan as

we discussed earlier, it is clear that both wet and dry ecotypes recruited more indicator taxa when they were growing in their home environments, suggesting how *"home field"* confer a physiological and adaptive advantage to the plant host and associated root-associated bacterial communities.

Since we observed differences in indicator taxa and unique ASVs across sites and ecotypes, we further conducted oligotyping analysis to inspect the concealed diversity within ASVs at a higher resolution. (Supplementary Table 10). We noticed that the relative abundance of ASVs and oligos associated with Pseudomonadales (12.9 ± 19.0%) and Rhizobiales (11.9 ± 3.6%) displayed opposite abundance patterns across the sites (Figure 2.5). Pseudomonadales had lower relative abundance of ASVs across samples from Hays and Carbondale compared to samples from Manhattan (Figure 2.5A). On the other hand, the relative abundance of Rhizobiales was similar throughout Hays and Carbondale and had a high degree of differences between samples in Manhattan (Figure 2.5B). Interestingly, we noticed dissimilarities among sites and ecotypes across both Pseudomonadales-Oligos and Rhizobiales-Oligos (Figure 2.5). Even though the relative abundance distribution patterns of oligos were obviously driven by site, we observed differences in ecotypic recruitment across ecotypes. Further work is necessary to provide more insights into recruitment patterns of strain-level Pseudomonadales and Rhizobiales populations by the plant host to understand the impact of bacterial populations on hosts' resilience to environmental stresses. However, our results suggested that precipitation-induced stress (drier in Hays and wetter in Carbondale) could result in a shift of plant host-bacterial communities interaction, changing the Pseudomonadales-Oligos and Rhizobiales-Oligos composition. For example, in Hays, Pseudomonadales-Oligos-GG were more prominent in the dry ecotype as compared to the wet ecotype, while Pseudomonadales-Oligos-AG were highly detected in mesic ecotype (Figure 2.5A).

On the other hand, in Manhattan, Pseudomonadales-Oligos-GA decreased in relative abundance in both dry and wet ecotypes in Manhattan, with an increase in relative abundance of Pseudomonadales-Oligos-AA. Similarly, in Carbondale, there was a higher relative abundance of Pseudomonadales-Oligos-AA in the wet ecotype as compared to dry ecotype (Figure 2.5A). We further noticed that in Hays, there was a higher relative abundance of Rhizobiales-Oligos-ATATC in dry ecotype as compared to wet ecotype. However, the relative abundance of Rhizobiales-Oligos-ATATC was higher in the wet ecotype as compared to dry ecotype in Manhattan. Interestingly, Rhizobiales-Oligos-TGATG was substantially higher in relative abundance in wet and mesic ecotypes as compared to dry ecotype in Carbondale (Figure 2.5B). The differences in the Pseudomonadales-Oligos and Rhizobiales-Oligos across the sites potentially highlight the tendency of plants to recruit microbial strains that could contribute to the plant hosts' ecotypic resilience [471,546]. The intraspecific genome content may be particularly important in understanding these host-microbe interactions. The intraspecific genome content may be particularly important in understanding these host-microbe interactions.

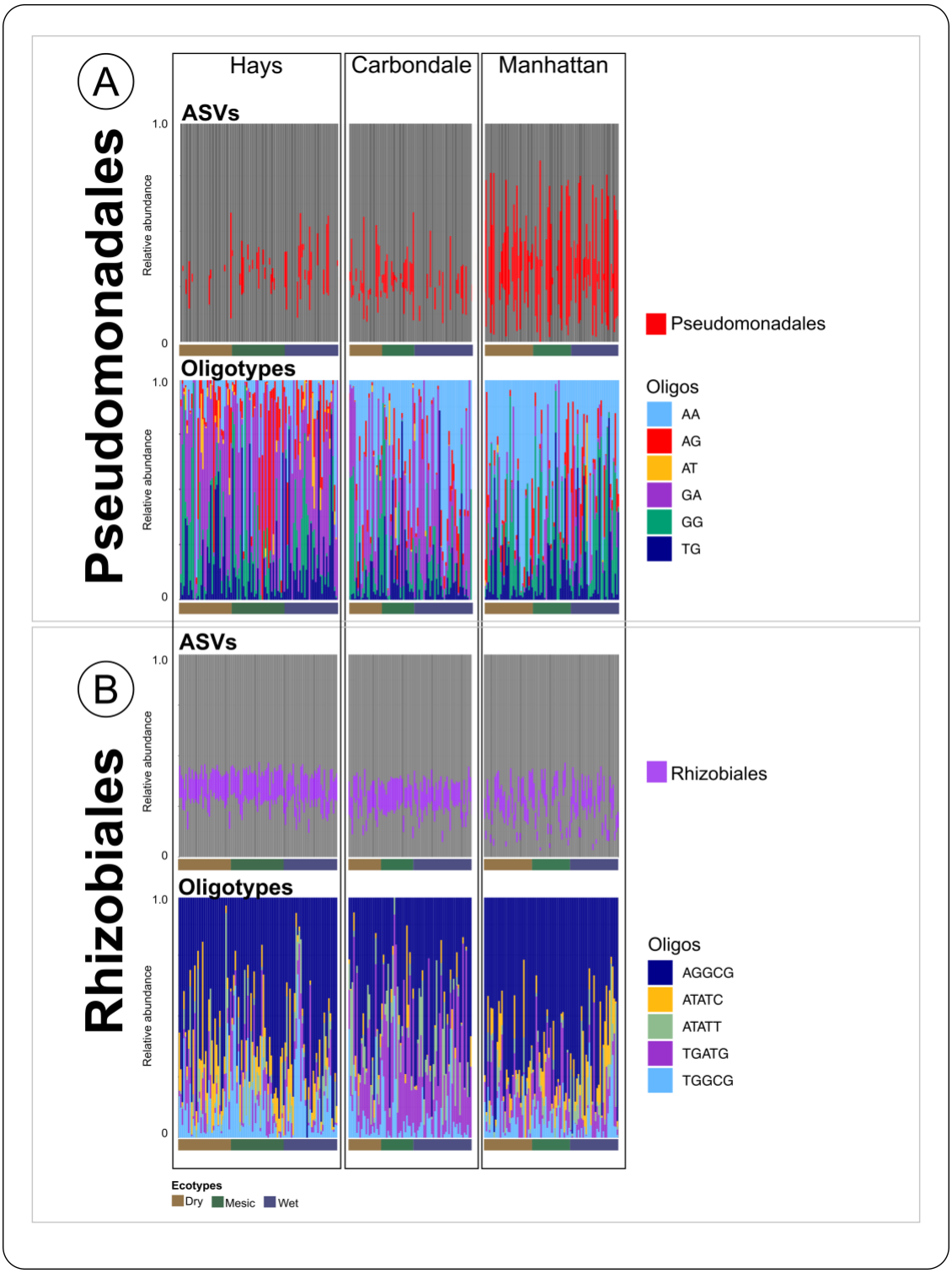

Figure 2.5 Relative abundance of each oligotype within the Pseudomonadales and Rhizobiales diversity for each sample among three sites located across the precipitation gradient. The proportion of the relative abundance of the (A) Pseudomonadales (red, top) and Pseudomonadales-Oligos (bottom) and (B) Rhizobiales (purple) and Rhizobiales-Oligos (bottom) suggest the impact of locations and ecotypes on rhizobiome strain-level composition.

2.5 Conclusion

Plant responses and adaptation to stress depend on a combination of environmental factors and plant genetics [451,452]. While the environment defines the soil-associated microbial pool, our results highlighted the plant-host's ability to recruit soil microbial communities under different environmental conditions. We showed that plant hosts were more successful in the recruitment of both the general and unique microbial populations when grown at their homesite [547]. Our observations also suggested the *"home field advantage"* and mutual association between plant and specific soil microbes [471,472,546]. From our study, we further propose that, at their home sites, ecotypes originating from these harsher environments and experiencing constant abiotic stresses, were better able to recruit specialist microbes with the potential stress relief functions [547]. We argue that in the study of plant host and root-associated bacterial communities, in line with the generalist and specialist hypothesis, plant host's resiliency under abiotic stress is more dependent on specialized microbes [452,454,522,535,536] rather than the core microbiome [465,548–550]. Our study suggests that in terms of the resistance to abiotic stress, the key factor is in less abundant and specialized microbes with specific functions rather than general common microbes which are abundant in soil. We further observed, in our study, the fragile relationships between plant hosts and associated specialized microbes. Although it might be challenging to tease apart the *"specialist vs generalists"* and *"home field advantag*e" concepts, we used oligotyping analysis and showed the ecotypic variations in recruitment of bacterial stains from the same genera. These observations further highlighted the complexity of these ecotype-host relationships. After growing in a common garden for over 10 years, we could still see the distinct microbial communities recruited by the ecotypes. Therefore, these differences across rhizosphere microbiomes recruited by ecotypes across all sites demonstrated that the plant host-bacterial interaction is much more exclusive and

fragile than we imagined. We believe that ecotypes rely on these relationships with microbes to overcome abiotic stress [551], and with the change in climate, these relationships might be threatened. Taking all these factors into consideration, there is a crucial gap in identifying specialist microbes, and understanding these relationships and communication signals with plant hosts, to predict the response of species to the changing climate change.

2.6 Acknowledgements and Funding

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2.7 Author Contributions

A.J., L.J. and S.T.M.L. designed the study. L.J. designed the reciprocal gardens experimental design, and A.J., L.J. and S.T.M.L. maintained sites. Sample collection was performed by S.S., E.H., M.G., A.J., S.T.M.L. and S.T., S.S., A.K., K.W. extracted DNA with

Nanodrop and Qubit quality analysis. A.K. and Q.R. performed 16S rRNA sequencing bioinformatic analysis. Q.R. and S.T.M.L generated oligotypes data. A.K. performed statistical analyses. A.K. and S.T.M.L wrote the manuscript, prepared figures, and supplementary files. A.J. and L.J. authors aided in manuscript and figure revisions. S.T.M.L., A.J. and L.J. acquired fundings for this study. All authors read, contributed to manuscript revision, and approved the submitted version.

2.8 Conflict of Interest

The authors disclose no conflicts of interest.

Chapter 3 - Interaction of plant-derived metabolites and rhizobiome functions enhances drought stress tolerance

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3.1 Abstract

3.1.1 Background

Plants have evolved alongside microbes, enabling plants to better cope with abiotic and biotic stresses. The interactions between plant roots and local soil microbes are critical for environmental adaptation and plant health. Plants actively regulate the microbial community composition in their rhizospheres to recruit specific microorganisms that enhance their fitness in the ecosystem they inhabit. This study builds on prior research suggesting that plants have a "*home field advantage*" in recruiting microbes unique to their "*home*" environment, reflecting mutual recognition and targeted recruitment of microbes.

3.1.2 Results

Using gene- and genome-centric approaches, we assessed the functional potential of root-associated microbes and profiled their host metabolites to uncover the metabolic outputs potentially regulating host–microbe interactions. Our results showed that plants adapted to drier environments experience less stress, producing fewer stress-related metabolites and impacting the recruitment of microbes with genes linked to stress relief pathways. In particular, plant-derived trimethyllysine is highly associated with microbial populations capable of improving nutrient uptake, producing plant growth-promoting compounds, and modulating stress responses.

3.1.3 Conclusion

This study highlights the critical interplay between host exudates and microbial substrate uptake as the primary mechanism of rhizosphere assembly. We demonstrate that plants actively produce metabolites to recruit microbial populations with the functional potential to enhance their

ability to thrive in stressful environments. This research provides insights into the mechanisms of plant-microbe communication, rhizosphere recruitment, and the complex interplay of plant-microbe interactions. Furthermore, this study highlights promising avenues for manipulating rhizosphere microbiomes to support conservation agriculture in the face of climate change.

3.1.4 Keywords

Microbiome, plant-host microbe interactions, shotgun metagenomics, metabolomics.

3.2 Background

The "*home-field advantage*" hypothesis in plant–soil microbe interactions posits that plants thrive better when they are growing in an environment containing microbial communities that have coevolved with or coadapted to over time [97,450,552–554]. Our previous work has shown that plant hosts perform better at recruiting microbes unique to their *"home"* environments, suggesting mutual recognition between the plant host and local soil microbes [555]. These results also suggest that plants originating from more stressful conditions, such as saline or drought-prone environments, selectively recruit specialized beneficial microbes for their functional potential to reduce host stress [556–559]. Understanding how local soil microbes confer functional advantages to plant hosts is essential for fully understanding the dynamics of interactions between plant hosts and microbes as well as their implications for plant health and resilience.

Numerous studies have demonstrated the effects of specific bacterial taxa on plant health and growth [560–564]. For example, the bacterial genera *Pseudomonas* and *Rhizobium* are known for their nitrogen-fixing abilities, which increase the nutrient availability of the host [561,565]. Additionally, *Bacillus* and *Burkholderia* can contribute to phosphorus solubilization, further

supporting plant growth and resilience [566–568]. While studies on specific taxa reveal valuable insights into plant–microbiome interactions, we suggest that rhizosphere recruitment is not based on the attraction of specific taxa but rather on the functional potential of microbes [569]. Additionally, while studies exploring system dynamics rely on the taxonomic assignment of sequencing data to evaluate this interaction, we know little about how rare, low-detected, or uncultured microbial taxa can impact plant hosts or other microbes.

Plant–soil microbe recognition and communication occur through complex nutrient and metabolite exchanges, where plant-released exudates can be utilized by rhizosphere microbial communities [56,570]. The rhizosphere is surrounded by a soil matrix in which plant roots continuously produce and secrete a diverse array of organic metabolites, which vary in chemical composition and are mostly controlled by host genetics [167], the host developmental stage [571], and many abiotic and biotic factors [43,56,569,572–574]. Plant metabolites also serve as nutrient sources and signaling molecules to facilitate the repelling of pathogens and the recruitment of beneficial soil microbes. Thus, host nutrient acquisition, pathogen resistance, and overall health and performance are enhanced [56,575]. The rhizosphere microbiome (rhizobiome) consistently differs from the soil microbiome, primarily because of the selective influence of plant root exudates [569,576]. Some metabolites selectively affect the growth of specific microbes and differentiate between beneficial and pathogenic microorganisms [47,372]. For example, plants can exude compounds for various purposes: aromatic organic acids (nicotinic, shikimic, salicylic, cinnamic, and indole-3-acetic) as energy sources for specific microbial populations; signaling molecules (phytohormones, specialized metabolites, volatile organic compounds, and peptides); or *"cry for help"* compounds such as terpenes in response to biotic stress, attracting beneficial rhizosphere microbes [47,56,112,577,578]. This complex plant host–microbe interaction can result

in variations in microbial composition even among individuals of the same plant species. While many studies have used the local adaptation of plant ecotypes to further our understanding of plant–rhizobiome interactions [169,573,579,580], little information exists on the impact of ecotypic differences in plant-produced metabolic profiles on the rhizosphere microbial community and functions.

Abiotic factors can drive shifts in plant-associated microbial communities and functions. For example, sulfate-reducing bacteria (SRB) are abundant in waterlogged soils because of their anaerobic respiration abilities [581,582], whereas other bacteria, such as Actinobacteria, dominate drought-tolerant communities [583–585]. The plant-host adaptation to local environments likely also results in coadapted microorganisms, which also undergo reciprocal evolutionary changes that select for specific associations [586–588]. Like their plant host, microbes face environmental stresses, resulting in microbial populations with specific genetic or functional potential to thrive under unfavorable conditions [589–592]. This may provide plant hosts with complementary benefits, and mutual adaptation can enhance the resilience and productivity of both plants and their associated microbial communities [593,594]. This dynamic process influences the genomes, behaviors, and ecological roles of hosts and microbes, fostering interdependent and often specialized relationships that account for plant–microbiota interaction diversity, specificity, and stability [595,596]. Although this is a crucial aspect of the plant–host–microbe interaction, information on the chemical exchange between the plant host and its associated microbes remains limited.

This study focuses on the interaction between plant host exudates and associated rhizobiome functional capabilities within the framework of the plant as a holobiont—a complex, integrated system composed of the plant host and its microbial communities. We aim to deepen

the understanding of the mechanisms underlying plant–microbe and microbe–microbe recognition, communication, and recruitment. Building on our previous work, we (1) investigated the functional potential of the recruited rhizosphere microbes through comprehensive gene-centric and genome-centric analyses of rhizosphere communities and (2) adopted a holistic approach for studying plant holobiont systems by integrating microbial data with plant-host metabolic profiles, attempting to provide a comprehensive view of the complex interplay between local microbial communities and the plant host. We hypothesized that plants adapted to drier environments would experience less stress, resulting in fewer stress-related metabolites, which in turn would impact the recruitment of microbes with the functional potential to provide host stress relief. Our work improves the understanding of the factors that influence plant host rhizobiome recruitment and provides insight into host ecotype responses in the face of a changing climate.

3.3 Results and Discussion

In this study, we used both community gene-centric and genome-resolved metagenomics, paired with host metabolomics, to elucidate how plant ecotypic characteristics and differences in mean annual precipitation (MAP) influence plant rhizobiome interactions in the dominant tallgrass prairie grass *Andropogon gerardii* (Figure 3.1).

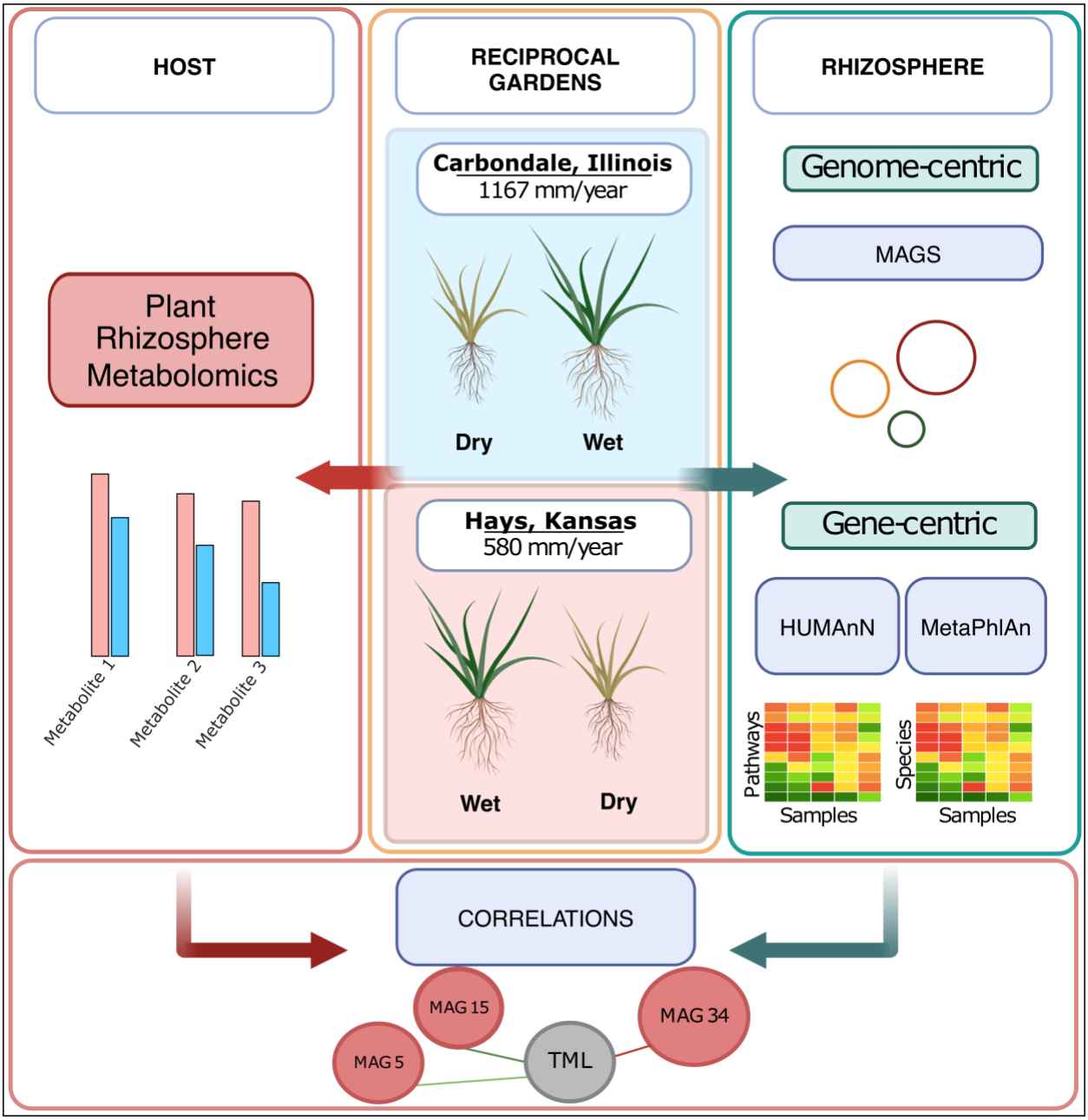

Figure 3.1 The schematics of the experimental design highlighting the flow of the project. Briefly, we collected rhizosphere samples from reciprocal gardens in Carbondale, Illinois, and Hays, Kansas, growing Dry and Wet regional *A. gerardii* ecotypes. We conducted shotgun metagenome sequencing to evaluate the microbial functional potential of these rhizosphere samples using gene- and genome-centric approaches. First, we performed gene-centric taxonomic community profiling (MetaPhlAn) and metabolic pathways profiling (HUMAnN). Next, we assembled 34 non-redundant metagenome-assembled genomes (MAGs) and performed genome-centric profiling of the MAGs. To assess the host metabolic output, we performed plant rhizosphere metabolites profiling. Finally, we conducted a correlation analysis to identify key omics variables across the datasets.

3.3.1 Distinct metabolic profiles between the Dry and Wet ecotypes reflect the Dry ecotype's adaptation to the "home" environment

We detected 1,191 plant metabolites across all 24 rhizosphere samples from Hays and Carbondale (Figure 3.2, Supplementary Table 12). Overall, metabolites were classified as amino acids (17.72%), others (13.85%), phenolic acid (12.68%), lipids (12.68%), alkaloids (9.49%), organic acids (8.73%), flavonoids (6.88%), terpenoids (5.46%), nucleotide and derivatives (5.04%), lignans and coumarins (4.70%), quinones (0.92%) and tannins (<0.01%) (Figure 3.2A, Supplementary Table 12).

We observed subtle metabolome differences between the Dry and Wet *A. gerardii* ecotypes (Figure 3.2B, Supplementary Table 12). In the Dry ecotype, the predominant metabolite classes were alkaloids (18.18%), amino acids (18.19%), and lipids (18.11%). Compared with the Dry ecotype, the Wet ecotype had a greater proportion of alkaloids (21.73%) and amino acids (23.43%), and a lower proportion of lipids (14.73%) (Figure 3.2B, Supplementary Table 12). The metabolites associated with the Dry ecotype included lupane-20(29)-en-3-on-28-oic and 4-hydroxybenzoic acid 4-(6-O-sulfo)glucopyranoside compounds, which have potential antimicrobial and antioxidant properties [597,598], and cis-abienol - antimicrobial metabolite [599] that can enhance plant stress tolerance and defense mechanisms.

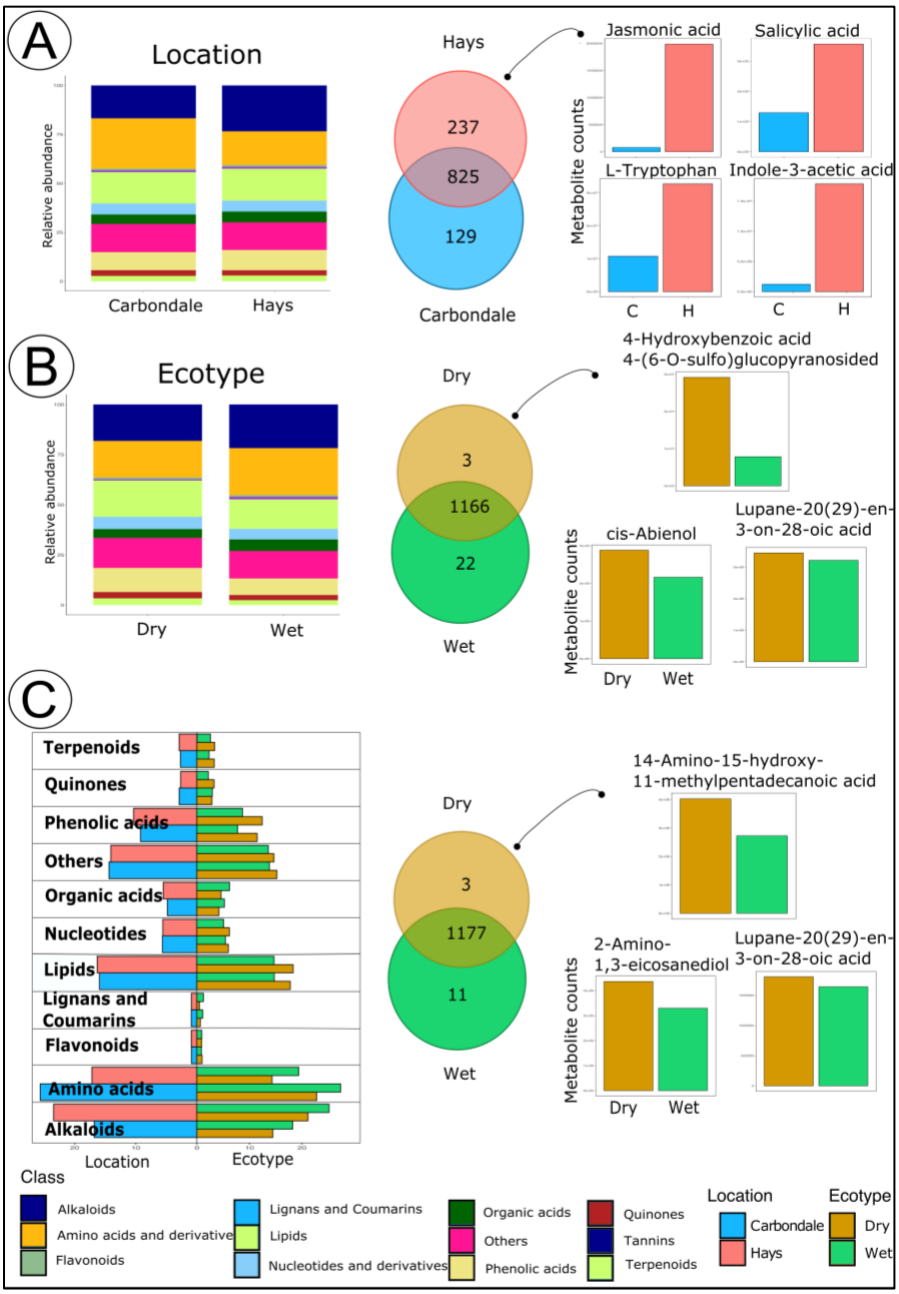

Figure 3.2 Plant hosts displayed different metabolic profiles across ecotypes and reciprocal garden locations. (A) The relative abundance of the plant host metabolites within Hays and Carbondale. The Venn diagrams with the number of metabolites significantly different (not overlapped; $p<0.05$) across two locations and bar plots with four metabolites (shortlisted metabolites) significantly higher in Hays ($p<0.05$). (B) The relative abundance of the plant host metabolites within Dry and Wet ecotypes. The Venn diagram with the number of metabolites significantly different across two ecotypes and three metabolites that were significantly higher in the Dry ecotype. (C) The two-sided bar plot represents the split of the metabolic classes across two ecotypes between the two locations. The Venn diagram with the number of metabolites significantly different between the two ecotypes in Hays and the three metabolites that were significantly higher in the Dry ecotype in Hays.

The 366 metabolites that varied between locations (ANOVA, Hays: n=237; Carbondale: n=129) (Figure 3.2A, Supplementary Table 12) provided further insights into the ecotypic host–rhizobiome interactions that may enhance plant resilience in drier regions. We identified a diverse array of metabolites that were differentially more abundant in the drier Hays site than in the wetter Carbondale site. These metabolites were associated with plant-microbe communication (e.g., indole and indole-3-acetic acid (IAA)), as well as compounds involved in plant immune responses, such as jasmonic acid (JA) and salicylic acid (SA), L-Tryptophan. Only 25 metabolites differed between the *A. gerardii* ecotypes (Dry: n=3; Wet: n=22) (Figure 3.2C, Supplementary Table 12). In pairwise comparisons between the Dry and Wet ecotypes in Hays, we identified 14 metabolites that differed between them (Figure 3.2C). Only three of these (14-amino-15-hydroxy-11-methylpentadecanoic acid, 2-amino-1,3-eicosanediol, and lupane-20(29)-en-3-on-28-oic acid) were elevated in the Dry ecotype (Supplementary Table 12). Notably, lupane-20(29)-en-3-on-28-oic acid and 14-Amino-15-hydroxy-11-methylpentadecanoic acid can exhibit antimicrobial properties [600], a finding that is consistent with the observed antimicrobial metabolites in our overall ecotype analysis across the two sites. 2-amino-1,3-eicosanediol is involved in the synthesis of eicosanedioic acid (EDA), which can function as a building block for the construction of complex lipids essential for resilience in drier environments [601].

Our metabolic profiling results indicated that ecotypes adapted to their environment in response to the drier conditions. We further demonstrated that Dry and Wet ecotypes may respond differently in the context of plant–microbe interactions, even within the same location. This crucial interaction between the ecotypes and their associated rhizobiome communities may be essential to understanding plant host resilience to drought. As a result, we asked, *which microbial pathways*

could be crucial in the Dry ecotype–microbe interactions and could enhance the plant host resilience in a drier environment.

3.3.2 Microbial communities differ in functional potential and their impact on the plant host

After obtaining insights from the plant metabolic profiles, we focused on profiling the rhizosphere microbial diversity and pathways within the entire community to determine whether any specific taxa (Supplementary Table 13) or gene functions (Supplementary Table 14) were associated with the ecotypes or locations. Shotgun sequencing of the samples (n=24) resulted in approximately 1.8 × 10^9 sequences with an average of ~ 77 × 10^6 ± 12 × 10^6 reads per metagenome (Supplementary Table 15). We used MetaPhlAn and identified that of 2,538 bacterial taxa (Supplementary Table 13), but only 1,557 were classified at the genus level. We further showed that the rhizosphere microbial communities differed between the locations and ecotypes (Figure 3.3A). With the insights from plant metabolome and rhizobiome composition differences [555], we focused on elucidating ecotypic rhizobiome differences in Hays, with a specific question: what microbes and pathways might contribute to the "*home-field advantage*" hypothesis and enhance Dry ecotype resilience in the drier environment in Hays [97,552,554]? In Hays, only four microbes differed between the Dry and Wet ecotypes (Figure 3.3B, ANOVA: $p < 0.001$; Hays: Wet vs Dry: $p<0.025$). Notably, we observed a greater abundance of *Kribbella speibonae* in the Dry ecotype in this drier site, suggesting its importance in enhancing host growth in the drier environment. *Kribbella speibonae* can catalyze the conversion of 3-phosphonatooxypyruvate to phosphoserine, which is crucial for the biosynthesis of amino acids, including serine, cysteine, and glycine, all vital for plant growth and development [602–604]. In contrast, the Wet ecotype had a

greater abundance of *Kribbella* sp. VKM Ac 2572 (n=2) (Figure 3.3B). The observed differences in the abundance of these bacteria, even at the genus level, likely indicate potential variations in their functional and ecological roles. This aligns with our observation that rhizosphere recruitment is driven not by the attraction of specific taxa but by the functional potential of the recruited microbes [569,605,606]

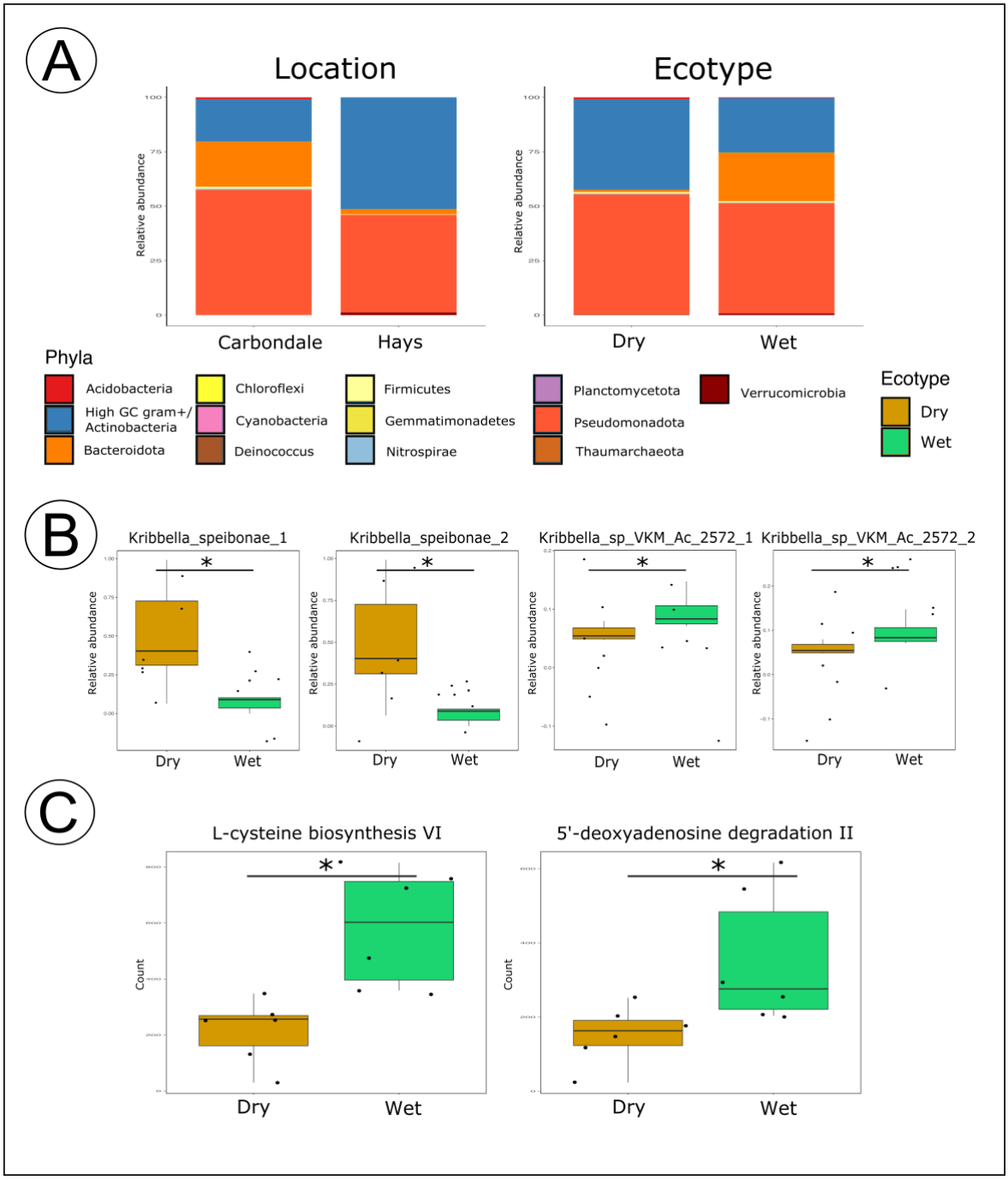

Figure 3.3 The gene-centric profiling of potential bacteria taxa (MetaPhlAn) and metabolic pathways (HUMAnN) corroborated the *"home-field advantage"* hypothesis, showing the Dry ecotype displaying resilience in the drier environment of Hays. (A) Stacked bars with the relative abundance of the taxonomic phyla within two locations and two ecotypes. (B) The abundance of four *Kribbella* species significantly differed between the Dry and Wet ecotypes in Hays. (C) Two metabolic pathways thant significantly differed between the Dry and Wet ecotypes in Hays, with the higher counts associated with the Wet ecotype. Significant p-values marked by asterisks * represent $p < 0.05$.

We next performed a functional analysis (HUMAnN) of metabolic characteristics potentially contributing to the overall functioning of the rhizobiome across locations and ecotypes (Supplementary Table 14). Although we identified a total of 548 pathways across the metagenome samples (Figure 3.3C), only two pathways, 5'-deoxyadenosine degradation II and L-cysteine biosynthesis VI (from L-methionine), differed significantly (ANOVA: $p < 0.013$; Hays: Wet vs Dry: $p<0.026$) between the Dry and Wet ecotypes and both were highly associated with the Wet ecotype (Figure 3.3C). The predominance of L-cysteine biosynthesis in the Wet ecotype-associated microbes corroborated our *"home-field advantage"* hypothesis that the Wet ecotype would not be as efficient in recruiting organisms that aid in environmental resilience as its Dry ecotypic counterparts. Microbe-associated L-cysteine biosynthesis facilitates the L-cysteine synthesis from L-methionine in a four-step process, in which the first two steps occur in the methionine cycle. Moreover, the latter two are part of the transsulfuration pathway. L-cysteine synthesized by Wet ecotype-associated microbes could act as an antioxidant and be crucial in the production of glutathione, an essential molecule for mitigating oxidative stress in its associated plant host [607–609]. More interestingly, the first step of the L-cysteine biosynthesis pathways results in the production of S-adenosyl-L-methionine (SAM), a critical methyl donor in many biochemical reactions. Radical SAM enzymes convert SAM into a toxic byproduct, - 5'-deoxyadenosine. The differentially higher abundance of the 5'-deoxyadenosine degradation pathway in the Wet ecotype suggested the upregulation of various stress-related biochemical pathways [610]. These pathways might include the synthesis of *"stress ethylene,"* potentially triggered by environmental challenges such as drought and/or salinity stress in Hays. Our findings on microbial SAM-stress-associated pathway interactions suggest the potential exchange between

the Wet-ecotypic plant host and its rhizobiome under a drier, non-native environment (Hays). This resulted in increased plant host stress signals and the corresponding byproducts. The production of plant-host stress-associated products might have attracted or activated pathways in the rhizosphere microorganisms [611–614], enhancing their potential to alleviate stress.

3.3.3 Distinguishable microbial populations and pathways were associated with ecotype and precipitation

While community-level metagenome analyses provided insights into which microbial pathways might influence plant–rhizobiome interactions, we also aimed to better understand which microbial populations and functions could impact the plant host resilience. To this end, we resolved a total of 34 non-redundant MAGs in our genome-centric metagenomic analysis (Figure 3.4, Supplementary Table 16). These MAGs were assigned to the following phyla: Patescibacteria (6), Pseudomonadota (5), Desulfobacterota (2), Nitrospirota (2), Verrucomicrobiota (2), Bacteroidota (2), High GC Gram+ (2), Acidobacteriota (1), Planctomycetota (1) all archaea were assigned to Thermoproteota (7), and four MAGs remained unassigned (Figure 3.4A). We observed that MAP was the main driver of the differences in rhizobiome microbial composition [615–617] (Figure 3.4B). Among the 34 resolved MAGs, we detected higher abundances of the bacterial phyla Actinobacteria (2), Acidobacteria (1), and Desulfobacterota (2) in the drier Hays location. While we anticipated that the drier conditions in Hays would favor taxa such as Actinobacteria and Acidobacteria, we were surprised to detect Desulfobacterota in this drier site, as Desulfobacterota typically thrives in water-saturated environments [618,619]. This finding suggests that the potential plant host selection of rhizobiome microbes may be more driven by the microbial functional potential than their simple taxon affinities. In this vein, the determining

factors could include microbial functions that sustain plant hosts' activity and/or mechanisms that permit the host to thrive in unfavorable environments. In the wetter Carbondale location, we observed high detection of MAGs annotated to bacterial phyla Pseudomonadota (4), Patescibacteria (4), Bacteroidota (2), and archaeal phylum Nitrospirota. Similar to Hays, the presence of these microbial groups aligns with their ecological roles in nitrification and organic matter decomposition in wetter soil environments.

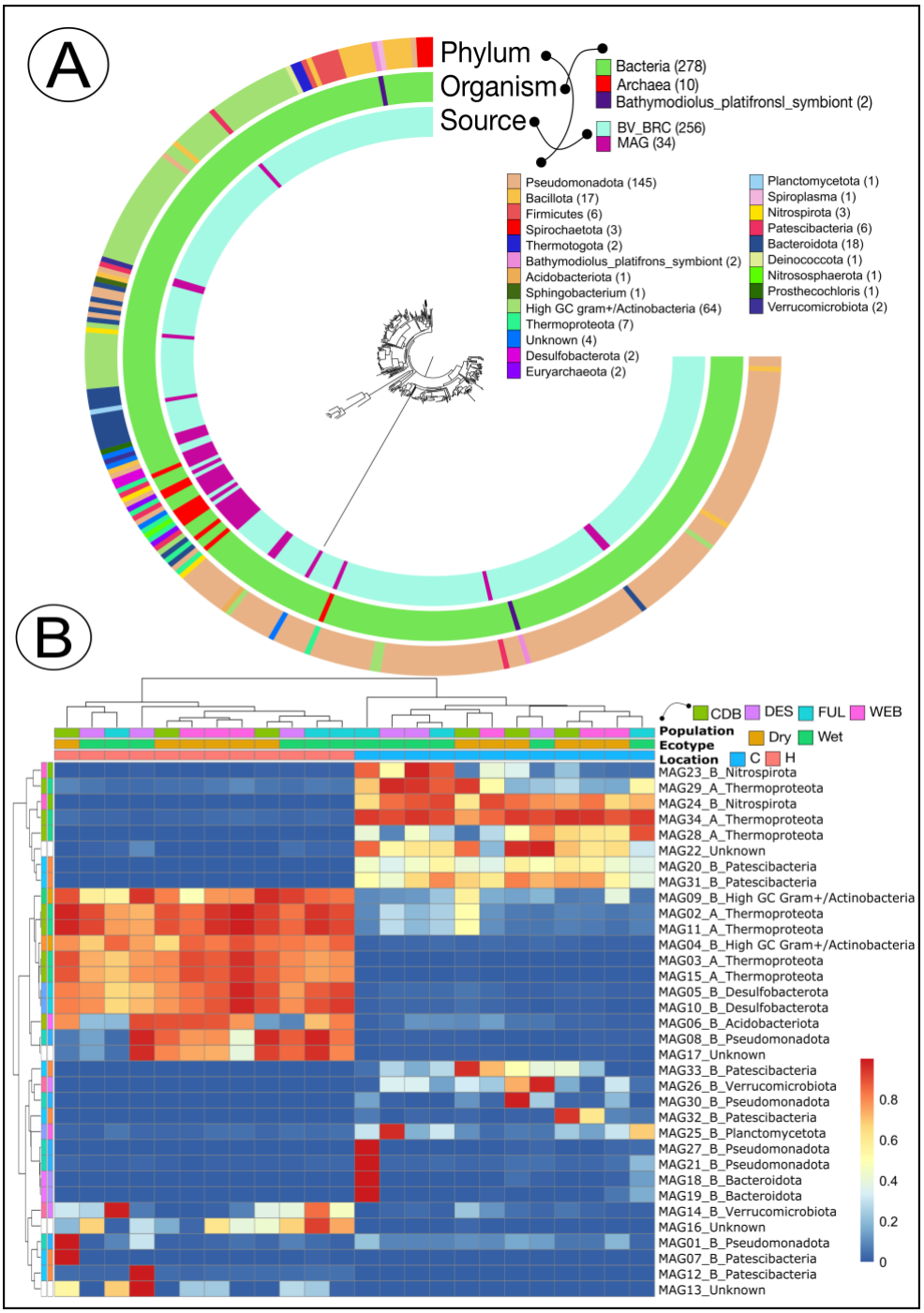

Figure 3.4 The phylogenomic placement of the MAGs and the detection of MAGS across collected rhizosphere samples. (A) Phylogenomic tree based on alignment of six bacterial ribosomal genes: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. The tree included 34 MAGs and 11 unique closest strains to identify relationships and the confirmation of the identity of MAGs. (B) Clustered heatmap with rows corresponding to 34 MAGs and columns corresponding to rhizosphere samples. The heatmap was clustered by rows and columns to observe the highest detection similarity within samples. Precipitation was the main driver of changes in the detection of the MAGs.

We next investigated the functional potential of these 34 MAGs to understand 1) how the MAGs that were more commonly detected in Hays may have adapted to the drier conditions and 2) the associated microbe–microbe and host–microbe communication functions in these microbial populations.

Microbial survival in the environment. Our previous work hypothesized that specific microbial populations could provide beneficial functions to the plant host under stressful environments [555]. This dependency on the microbial function arises from the unfavorable environmental conditions that the plant host might experience [167,367,592,620,621]. As such, the plant-host stress signaling molecules and microbial functional requirements in the dry Hays environment might differ from those in wetter Carbondale. Therefore, the specialization of the microbial functional potential and their ability to survive in stressful environments go hand in hand [583–585,606,622], with bacterial survival relying heavily on their ability to sense and respond to environmental changes [623]. Here, we observed several mechanisms that microbial populations highly detected in Hays might utilize to survive in the drier environment (Figure 3.5, Supplementary Table 17). For example, MAGs 1, 27, and 30 (all assigned to phylum Pseudomonadota) had all necessary EnvZ and OmpR regulon genes used for regulating the expression of outer membrane porins in response to osmotic stress [624,625]. In addition, these MAGs also had an active BarA-UvrY(SirA) two-component regulatory system involved in bacterial sensing and responding to changes in their environment. Other highly detected microbial populations in this study, including MAGs 8 (Pseudomonadota), 15 (Thermoproteota), 17 (unassigned), 27 (Pseudomonadota), and 30 (Pseudomonadota) had the genomic potential to produce both (GlrK and GlrR) proteins of the GlrKR two-component system responsible for regulating gene expression in response to environmental stimuli [626,627]. The survival capacity

of these microbial populations is the first of many requirements for enhanced plant host resilience to abiotic stress due to plant-microbe interactions. With the identification of numerous microbial survival mechanisms in our resolved MAGs, we next investigated the functional potential of host-microbe and microbe-microbe communication capabilities.

Figure 3.5 Genes/subsystems (subset) presence (red) and absence (gray) in selected MAGs. Rows correspond to the subset of genes/subsystems related to microbial mechanisms highly detected in Hays potentially unitized to survive in the drier environment, and columns correspond to the MAGs these genes/subsystems were observed in.

Host-microbe and microbe-microbe signaling. To provide a direct beneficial function to the plant host, soil bacteria must become a member of the plant-host rhizosphere. We considered multiple levels of interactions when describing the possibility of plant-microbe communication. We explored the genomic potentials of the MAGs in their ability to perceive and interpret plant-host signals, their physical capacity to navigate toward these signals, rhizoplane biofilm formation, and their ability to bypass the plant immune responses to integrate successfully into the rhizosphere or endophyte community. First, we investigated the chemotaxis signaling, which is critical in host signal recognition (Figure 3.5, Supplementary Table 17). This process begins with detecting the signals by transmembrane chemotaxis receptors, specifically methyl-accepting chemotaxis proteins (MCPs) [628,629]. We observed that MAGs 1 (Pseudomonadota), 14 (Verrucomicrobiota), 16 (unassigned), 17 (unassigned), and 27 (Pseudomonadota) possessed all the necessary genes for CheA histidine kinase and the coupling protein CheW, which are required to form a ternary complex with MCPs for effective signal transduction [630,631]. This interaction forms a two-component system with CheY, where phosphorylated CheY (observed in MAGs 1 (Pseudomonadota), 2 (Thermoproteota), 8 (Pseudomonadota), 16 (unassigned), and 17 (unassigned)) activates motor proteins responsible for bacterial motility [632,633]. Additionally, we observed that MAGs 1 (Pseudomonadota) and 9 (High GC gram+) contained genes necessary for catabolizing the plant-exuded myo-inositol (inositol), suggesting their potential roles in root colonization [634–636].

Our analysis revealed that MAGs 1, 27, and 30, all assigned to the phylum Pseudomonadota, possessed the necessary genes to produce the flagellum-specific ATP synthase FliI enzyme, which is vital for the assembly and function of bacterial flagella [637,638] (Figure 3.5, Supplementary Table 17). This functionality is essential for motility, chemotaxis, adherence,

and colonization. Additionally, we observed that MAGs 1 and 30 had a variety of genes (MAG 1: *chpA, flgH, flgI, flgG, fliP, fliG, fliM, fleQ, fleN, flil, pilT, pilM, pilG* and *pilH*; MAG 30: *flgB, flgH, flgC, flgG, fliP, flgE, filG, fliM, fliA, ppdD*, and *rfe*) potentially involved in the formation, regulation, and functioning of the bacterial flagellum [637,639–641].

We observed that MAGs 9 (High GC Gram+) and 27 (Pseudomonadota) could produce a long regulatory protein with the LuxR domain, which is commonly involved in bacterial quorum sensing and stress response systems (Figure 3.5, Supplementary Table 17). Additionally, MAG 27 (Pseudomonadota) also had the potential to produce acyl-homoserine lactones (AHLs), critical signaling molecules for N-butanoyl-L-homoserine lactone-binding transcriptional activation [642] (Figure 3.5, Supplementary Table 17). In contrast, MAG 9 (High GC Gram+) did not have the potential to produce AHL signaling molecules because of the absence of a LuxI-encoding gene, suggesting that LuxR solos in MAG 9 (High GC Gram+) might be involved in recognizing environmental stimuli and sensing host-derived signals [642,643].

Additionally, we observed that MAGs 1 (Pseudomonadota) and 30 (Pseudomonadota) had a genomic potential to produce four essential products necessary for biofilm formation: PGA outer membrane secretin PgaA, PGA synthesis deacetylase PgaB, Biofilm PGA synthesis N-glycosyltransferase PgaC, and PGA synthesis auxiliary protein PgaD [591,644] (Figure 3.5, Supplementary Table 17). Since bacterial biofilms are multicellular communities formed by certain bacterial species to thrive in hostile environments, effective cell-to-cell communication through quorum sensing is essential for biofilm formation and maturation.

We then explored the potential for the MAGs to attach to the host roots [605,645,646]. We observed that MAG 1 (Pseudomonadota) could produce the outer membrane porin F (OprF) (Figure 3.5, Supplementary Table 17), which can function as a root adhesion protein and play an

essential role in root attachment [647]. Additionally, MAGs 9 (High GC Gram+), 27 (Pseudomonadota), and 30 (Pseudomonadota) had various genes (MAG 30: hslO, amn, recA, ruvB, dacC; MAG 27: recA and ruvB; MAG 9: recA) that facilitate the attachment process [648]. We also observed that MAG 30 (Pseudomonadota) possessed the genes pta and ompA (Figure 3.5, Supplementary Table 17), which are associated with virulence and the invasion of host cells, thus contributing to intracellular colonization [649,650].

Taken together, our gene- and genome-centric metagenome analyses corroborated with each other and with our previous hypothesis on the importance of "*home-field advantage*" in enhancing plant host resilience under drier environmental conditions. Microbial populations at both the community and genome levels displayed potential functions that could enable microbial survival and enhance microbe–microbe–plant interactions under the drier environmental conditions in Hays. After analyzing the host and microbial functions separately, we next asked which microbial functions could have the greatest impact on the plant host.

3.3.4 Ecotype–microbial interactions resulted in enhanced plant host resilience under drier conditions

In this study, our gene-centric analyses revealed that plant-host stress-associated metabolites could enhance the recruitment of specific microorganisms, whereas genome-centric analyses identified the microbial population functions and plant–host interaction. With these insights, we next investigated the interactions between the *A. gerardii* ecotypes and their associated rhizobiome functions to determine which resultant interactions could enhance plant host drought resilience. Our DIABLO network analysis (cutoff=0.83) revealed that MAG 34 (Thermoproteota) was negatively correlated with the host-derived peptides cyclo-(Gly-Phe) and trimethyllysine

(TML) and positively correlated with 3-amino-2-naphthoic acid, DL-tryptophan, and 3,5-dihydro-2H-furo[3,2-C]quinolin-4-one (Figure 3.6A). Furthermore, five MAGs (MAG 3 - Thermoproteota; MAG 4 - Actinobacteriota; MAG 5 - Desulfobacterota; MAG 10 - Desulfobacterota; and MAG 15 - Thermoproteota) were positively correlated with trimethyllysine (TML; Figure 3.6A). Interestingly, these five MAGs were detected more frequently in Hays than in Carbondale (Figure 3.6B, Figure 3.6B), and were also detected at higher levels in association with Dry ecotype in Hays. TML is important in plant metabolism, serving as a precursor to carnitine, a compound crucial for various metabolic and physiological functions [651,652]. Similar to mammals and fungi, plant carnitine production begins with the breakdown of lysine-containing proteins, which then ungergo lysosomal or proteasomal protein degradation via a four-step pathway [651,653]. Carnitine is essential for transporting fatty acids into the mitochondria for β-oxidation, which produces energy essential for various metabolic processes, particularly under abiotic stress [651]. In addition, one of the most common forms of carnitine - L-carnitine, serves multiple functions in plants, including detoxification, antioxidant activity, regulation of oxidative stress, lipid metabolism, stress signaling, and responses to oxidative damage during drought and high salinity [651,652,654]. Under drought stress, TML production and its conversion to carnitine could support the plant's ability to manage oxidative stress and maintain cellular functions during water limitation. Despite the critical and diverse roles of TML in plants, our understanding of the role of TML is limited. We hypothesize that the lower TML levels produced by the Dry ecotype in Hays demonstrate the lower stress levels experienced by the Dry ecotype in its native (drier) environment. These findings support our two key hypotheses: 1) Dry ecotype in its native Hays environment experiences lower stress levels, leading to reduced production of stress-related metabolites, which aligns with the *"home-field advantage*" hypothesis; 2) the positive association

between the five MAGs and the host-produced TML suggest that these MAGs may contribute to alleviating plant stress or potentially provide a priming effect for the plant host. Although the lower TML levels were associated with the Dry ecotype in our network analyses, these differences were not statistically significant (Kruskal–Wallis: chi-square = 1.64, df = 1, p = 0.200) (Figure 3.6C). We observed that this variability was driven by a population effect where the Dry populations (CDB and WEB) had significantly lower levels of TML compared to the Wet (DES and FUL) populations (Kruskal–Wallis: chi-square = 8.44, df = 3, p = 0.038) (Figure 3.6D). Although the effect of population genetic diversity on metabolic profiling has been documented by others [655], this was the first instance in our analyses where it became evident [555]. These observations highlight the complexity of metabolic responses within different populations and suggest that factors such as local adaptation or genetic diversity might significantly influence the production of metabolites like TML. This underlines the importance of considering even finer population-specific resolution when studying host metabolic traits. With insights from the plant ecotypes, we next asked *what could be the interaction between the plant host and these 5 MAGs that would result in higher production of TML by the Dry ecotype in a drier environment (Hays)*?

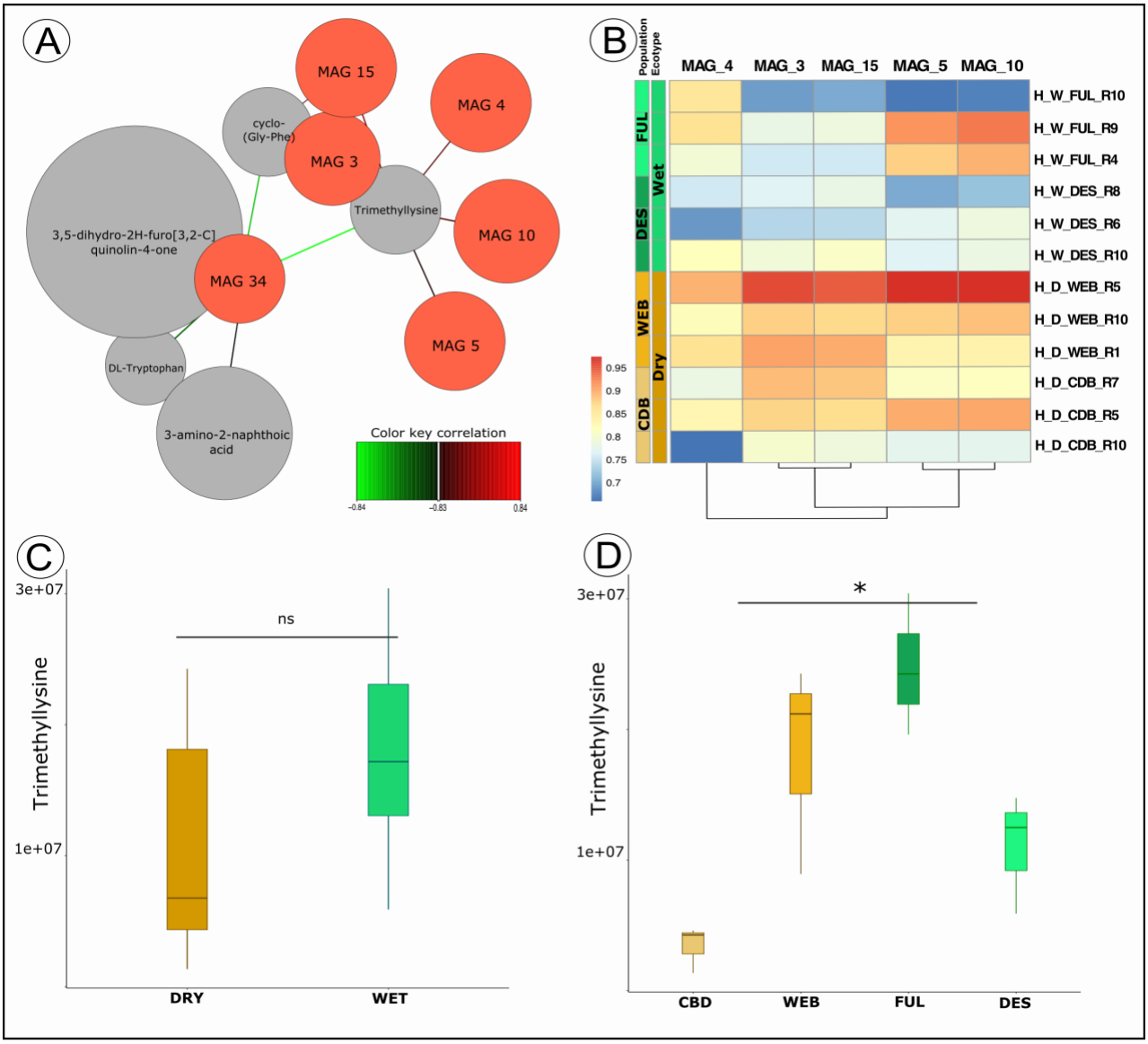

Figure 3.6 Plant host-derived metabolite, lower trimethyllysine (TML) positively correlated with five MAGs and was highly associated with Dry ecotype populations in Hays. (A) Network analysis of plant–host metabolite (gray) couples with MAGs detection levels (red). A correlation cutoff of 0.83 was used to visualize only strong associations, with (Green) lines indicating negative correlations and (Red) lines marking a positive correlation of MAGs with the host metabolites. (B) The detection heatmap with rows corresponding to rhizosphere samples collected in Hays and columns corresponding to the five MAGs of interest. (C) The detection of the TML among Dry and Wet ecotypes in Hays. Not significant p-value marked by "ns." (D) The detection of TML among Dry and Wet populations in Hays. The p value marked asterisks * represent $p < 0.05$.

Through our DIABLO analysis, we aimed to gain deeper insights into how the five MAGs associated with dry site interacted with plant-derived TML, particularly how these interactions might enhance the dry ecotype resilience in drier conditions such as those in Hays. We suggest that the relationships among TML, plant performance, and microbial activity is complex and neither simple nor unidirectional. The positive correlation between lower TML and the five MAGs suggests that they play a vital role in alleviating stress, thus enabling the host to better cope with stressful conditions in Hays. Consistently, the ability of these MAGs to thrive in the drier, more stressful environment in Hays might facilitate a healthier rhizosphere through several indirect mechanisms, including 1) improved nutrient availability and mobilization, 2) the production of plant growth-promoting compounds, and 3) the modulation of stress responses.

One avenue that may result in positive plant outcomes through TML–microbe interactions could be attributed to the ability of our resolved MAGs to use TML as a nutrient source to synthesize compounds critical for microbial stress tolerance and energy metabolism [651,652,656]. The resolved MAGs could synthesize carnitine from TML to stabilize their cell structures, manage oxidative stress, and maintain energy production under drier conditions in Hays [657]. For example, we observed that MAGs 4 (High GC Gram+), 5 (Desulfobacterota), and 10 (Desulfobacterota) could produce L-carnitine dehydratase/bile acid-inducible protein F, an enzyme that dehydrates L-carnitine to form other metabolites, which, in turn, would potentially make them available to the plant in different nutrient forms (Figure 3.7). We observed that all five MAGs possessed a diverse array of enzymes that catalyze the conversion of various substrates into ammonia: MAG 3 (-monophosphatase; phosphatidate cytidylyltransferase; probable serine/threonine-protein kinase pknK; serine/threonine-protein kinase PknB, PknD, PknE and MAG 5 and 10 - Inositol-1-monophosphatase) that may promote plant root development,

enhancing root depth and spread, as reported in [444,515] (Figure 3.7). We also surmised that the five MAGs could interact with plant host-derived TML, and enhance the plant drought tolerance through the production of stress z-related enzymes (MAG 3 - AqpZ, MAG 4 - AqpZ, ABC transporter, ATP-binding protein and permease protein, glycerol uptake facilitator protein and transporter OpuD, Cold shock protein of CSP family; MAG 5 - AqpZ, cold shock protein of CSP family, ribosomal arrest protein RaiA / cold shock protein of CSP family; MAG 10 - cold shock protein of CSP family, ribosomal arrest protein RaiA / cold shock protein of CSP family) and antioxidants (MAG 3 - manganese catalase; MAG 4 - Manganese catalase, Hydroxyacylglutathione hydrolase; 16S rRNA (uracil(1498)-N(3))-methyltransferase; MAG 5 - uncharacterized monothiol glutaredoxin ycf64-like, N-methylhydantoinase A and B, stress proteins YciF and YciE, glutathione synthetase, glutathione reductase, glutathione peroxidase and thioredoxin peroxidase, glutathione S-transferase family protein, glutamate--cysteine ligase, gamma-glutamyltranspeptidase and glutathione hydrolase, catalase KatE; MAG 10 - hhydroxyacylglutathione hydrolase, glutathione reductase, glutathione peroxidase and thioredoxin peroxidase, glutathione S-transferase; MAG15 - superoxide dismutase [Mn], Manganese catalase) (Figure 3.7). The production of microbe-derived antioxidants from these MAGs could reduce oxidative damage in the plant host under the drier and more stressful conditions in Hays, thus improving plant growth and productivity.

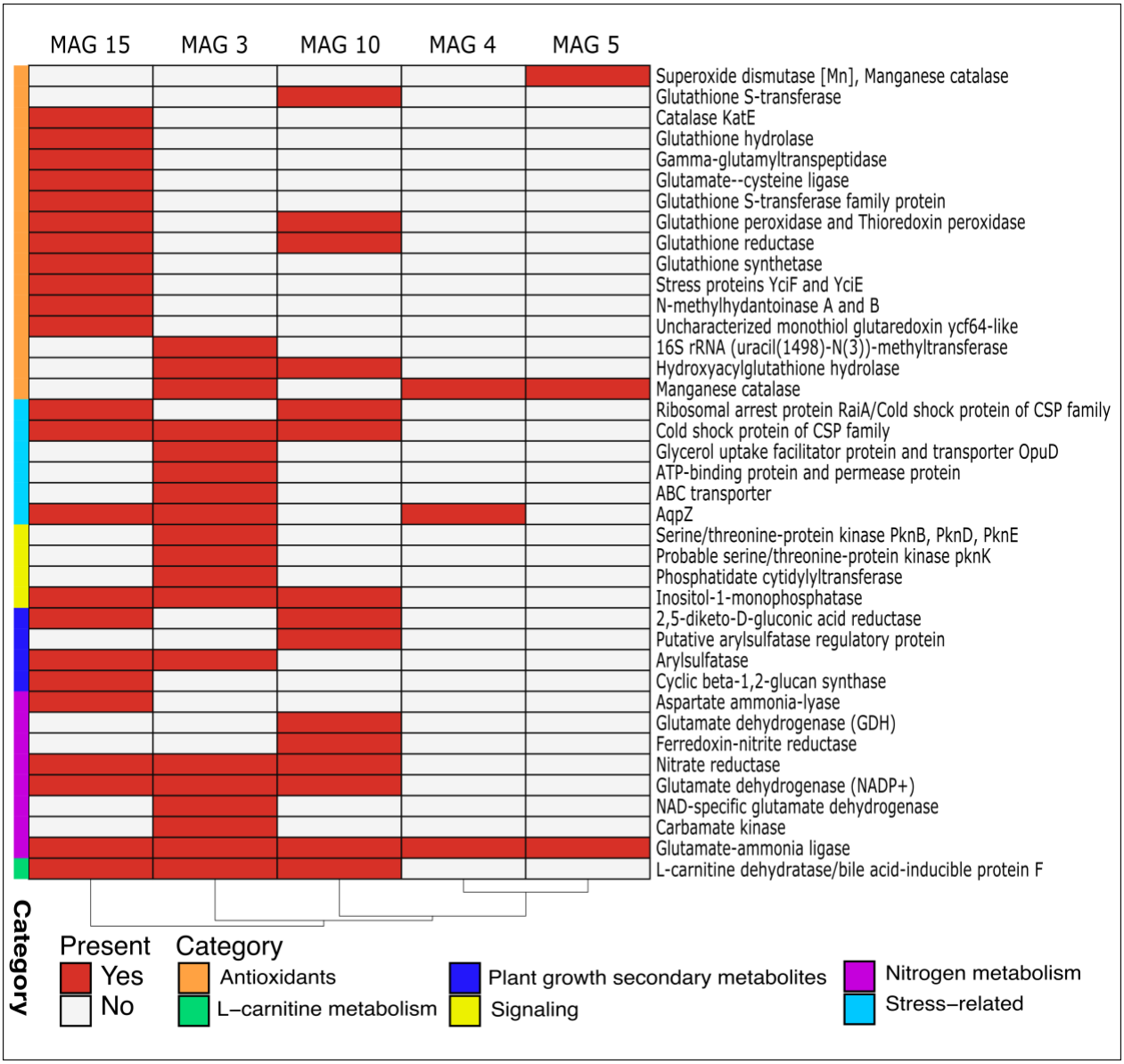

Figure 3.7 Genes/subsystems (subset) presence (red) and absence (gray) in the MAGs-of-interest. Rows correspond to the subset of genes/subsystems related to microbial mechanisms detected in five MAGs (columns) of interest, potentially promoting plant host survival and environmental performance.

Additionally, TML is a precursor to trimethylamine N-oxide (TMAO) in the human system [658,659]. Some research is starting to uncover the potential of TMAO in terrestrial plant systems, and its role in stress mitigation suggests a huge potential in supporting plants in marginal environments. While we did not find direct evidence of this conversion in the five MAGs of

interest, we detected trimethylamine-N-oxide reductase (TorZ) and trimethylamine-N-oxide reductase and associated c-type cytochrome (TorY) in MAG 27, suggesting its capacity to metabolize TML [660–662]. TMAO is increasingly recognized for its potential role in improving plant stress tolerance, especially in challenging environmental conditions, e.g., drought and heat [663,664]. The microbial TMAO, and in our study, specifically MAG 27, could act as osmoprotectants. Microbial populations can metabolize TMAO to improve their resilience to osmotic stress [663–665], allowing them to maintain metabolic activity around plant roots under dry conditions such as those in Hays. Although known only in microbial populations, it is conceivable that TMAO could stabilize proteins and cellular structures in plant cells as well [663,665,666]. TMAO's protein and enzyme stabilizing effects might also extend to antioxidant enzymes in the plant host [663,665], protecting the plant ecotypes from oxidative stress associated with environmental stresses in Hays and helping them maintain cellular integrity and physiological functions under drier conditions.

In summary, the interactions between plant-derived TML, ecotypic microbial populations, and TML derivatives strongly suggest that there exists a deliberate plant control of the associated microbes through plant-produced metabolites. We understand that the interaction between the plant-derived TML and associated microbial constituents extends beyond these few MAGs present in our study. However, our results set the platform to motivate new lines of investigation as they provide a rational, mechanistic basis to offer deeper insights into complex plant–microbe interactions. These findings further support the "*home-field advantage*" hypothesis, showing that the plants actively produce metabolites to recruit local microbial populations. These functional capabilities ultimately contribute to the ability of the host to thrive in stressful environments. The variability in the TML production among plant ecotypes and populations further highlights the

complexity of plants metabolic responses shaped by genetic diversity and the plant's local adaptations. Further research on TML and related processes is needed. Our current research deepens our understanding of the factors that drive rhizobiome recruitment and offers insights into how rhizobiome communities contribute to ecotype responses to climate change.

3.4 Conclusions

This study highlights the intricate interplay between plant-derived metabolites and rhizobiome community functions to enhance *A. gerardii* drought tolerance. Our data indicate that the interactions between plant host-derived trimethyllysine (TML) and the microbial capacity for chemotaxis, biofilm formation, and root attachment are more characteristic mechanisms of plant stress resilience than previously appreciated.

Our findings underscore how the functional potential of the rhizobiome communities is influenced by plant metabolites. Our work expands on the "*home-field advantage*" hypothesis, demonstrating that plants adapted to drier environments recruit microbial populations enriched in pathways that mitigate abiotic stress. Specifically, our data show that the plant-derived metabolite TML is correlated with microbial populations that enhance plant resilience through nutrient cycling, antioxidant production, and stress-signaling modulation.

This study and our methodological framework, which combines host metabolomics with microbial gene-centric and genome-centric analyses, provide a blueprint for future studies exploring plant–rhizobiome interactions. By linking host metabolomics to microbial genomic potential, we are able to systematically identify microbial pathways that are critical for stress tolerance. Our approach can also be applied to other plant systems to investigate stress–response mechanisms or to engineer microbiomes to enhance agricultural productivity. This study advocates

for a shift from taxonomic focus to functional rhizobiome characterization. Future work should explore how microbial metabolites may contribute to host resilience and expand our understanding of metabolite-mediated microbial recruitment.

While our study identified important host metabolites and microbial functions, some questions remain unanswered, specifically the signaling pathways through which TML mediates host–rhizobiome interactions. Future work should integrate metatranscriptomics to capture the real-time dynamics of these interactions. In summary, our work demonstrates that plant metabolite-microbial function interactions constitute a cornerstone of rhizosphere assembly and plant resilience to environmental drought stress. By leveraging these insights, we can design microbiome-based solutions to improve plant performance under climate change, paving the way for successful conservation efforts and sustainable agricultural practices.

3.5 Methods

The detailed sampling methods are described in Kazarina et al. [555] and are briefly summarized here. Following our previous work [555], we asked what plant-associated and microbial-associated functions in the plant–host rhizobiome interaction contribute to the "*home-field advantage*" and could potentially enhance *Andropogon gerardii* drought stress resilience.

3.5.1 Selection of rhizosphere samples and shotgun metagenomics

We selected 24 *A. gerardii* rhizosphere samples to gain deeper insights into microbial functions that could potentially enhance plant resilience and responses to microbial interactions during drought stress (Supplementary Table 11). Samples were chosen partially based on the results of Kazarina et al. [555] to better understand the host-microbe interactions and consisted of samples from two reciprocal gardens located in two precipitation extremes, (H - Hays, Kansas

(580 mm/year) and C - Carbonate, Illinois (1,167 mm/year), planted with two regional ecotypes (Dry and Wet) and 4 populations (Dry: CDB, WEB; Wet: DES, FUL) (2 locations x 2 ecotypes x 2 population x 3 samples) (Supplementary Table 11). We extracted total gDNA from 0.150 g of roots and rhizosphere soil from all 24 samples using the E.Z.N.A Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA), following the manufacturer protocol with slight modifications. Roots were shaken to remove non-adhering soil and weighed for the DNA extractions. Any soil that remained attached to the roots was considered rhizosphere soil [198,667]. We mechanically lysed the samples on a Qiagen TissueLyser II (Qiagen, Hilden, Germany) using glass beads, for 10 mins at 20 rev/s prior to any downstream DNA extraction steps. The mechanical lysis never fully homogenized the roots; therefore, our samples represented largely rhizosphere microorganisms, potentially excluding many endorhizosphere organisms. The extracted gDNA was eluted to 100 µL final volume. The DNA yield and concentration were measured using a Nanodrop and a Qubit™ dsDNA BR Assay Kit. Extracted DNA was kept at -20°C until shotgun metagenome sequencing. Extracted gDNA was used to construct DNA libraries using Illumina's Nextera XT Index Kit v2 kit. The products were visualized on an Agilent Tapestation 4200 and size-selected using the BluePippin. The final library pool of 24 samples was quantified using the Kapa Biosystems qPCR protocol and sequenced on the Illumina NovaSeq S1 flow cell chip in a 2 × 150 bp paired-end sequencing run.

3.5.2 Bioinformatic workflow of gene-centric metagenomic analyses

We used a biobakery meta-omics analysis environment [668] to taxonomically and functionally profile the microbial communities in the metagenome samples. Taxonomic profiling was performed via MetaPhlAn v.4.1.1 [669] with the CHOCOPhlAn v3.0.14 [670] database. For

functional profiling, we used HUMAnN3 v.3.9 [670] pipeline with MetaPhlAn v.4.1.1, Bowtie2 version 2.2.6 [671], DIAMOND v 2.0.15 [672], and MinPath v.1.5 [673]. The HUMAnN3 pipeline was performed with the default parameters.

3.5.3 Bioinformatic workflow of MAGs assembly and genome-centric metagenomic analysis

We used automated metagenomics bioinformatics workflows implemented by the programs 'anvi-run-workflow' [674] in anvi'o [675,676]. Anvio's workflows implement bioinformatic tasks, including short-read quality filtering, assembly, gene calling, functional annotation, hidden Markov model search [677], metagenomic read-recruitment, metagenomic binning [678], pangenomics, and phylogenomics. Workflows use Snakemake [679], and a tutorial can be found at the URL https://merenlab.org/2018/07/09/anvio-snakemake-workflows/.

We used 'iu-filer-quality-minoche' to process the short metagenomic reads, implemented in illumina-utils v2.11 [680], and removed low-quality reads according to the criteria outlined in Minoche et al. [681]. We used MEGAHIT v1.2.9 [682] to co-assemble quality-filtered short reads into longer contiguous sequences (contigs). Due to the high sample heterogeneity, we split the metagenomes into 4 even experimental groups based on location and plant ecotypes (location-ecotypes: H-Dry, H-Wet, C-Dry, and C-Wet) for the co-assembly. We used the following strategies to process contigs: (1) 'anvi-gen-contigs-database' on contigs to compute k-mer frequencies, and identify open reading frames (ORFs) using Prodigal v2.6.3 [683]; (2) 'anvi-run-hmms' to identify sets of bacterial [684] and archaeal [685] single-copy core genes using HMMER v.3.2.1 [686]; (3) 'anvi-run-ncbi-cogs' to annotate ORFs with functions from the NCBI's Clusters

of Orthologous Groups (COGs) [687], and (4) ‘anvi-run-kegg-kofams’ to annotate ORFs from KOfam HMM databases of KEGG orthologs [688,689].

We recruited metagenomic short reads to contigs using Bowtie2 v2.3.5 [671], and converted the resulting SAM files to BAM files using samtools v1.9 [690]. We profiled the BAM files using ‘anvi-profile’ with a minimum contig length of 1000 bp to eliminate shorter sequences and minimize noise. Each metagenome was then profiled to store contig coverages into single anvi’o profile databases, and we used ‘anvi-merge’ to combine all profiles into an anvi’o merged profile for downstream visualization, binning, and statistical analyses. We used ‘anvi-cluster-contigs’ to group contigs into initial bins using CONCOCT v1.1.0 [691], and used ‘anvi-refine’ to manually curate the bins based on tetranucleotide frequency and different coverage across the samples. We marked bins that were more than 70% complete and less than 10% redundant as metagenome-assembled genomes (MAGs). We used the “detection metric” to assess the presence of MAGs in a given sample, and considered a MAG as detected in a metagenome if the detection value was > 0.25, which is an appropriate cutoff to eliminate false-positive signals in read recruitment results, for its genome.

3.5.4 Phylogenomic analysis and functional potential of MAGs

We uploaded and annotated MAGs on the Bacteria and Viral Bioinformatics Resource Center (BV-BRC) platform [692]. We used the similar genomes finder function in BV-BRC to download 11 similar genomes for each MAG (a total of 34 MAGs and 324 similar genomes). We used a collection of six bacterial ribosomal gene alignment to construct the phylogenetic tree: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. We explored these sequences across all the assembled draft genomes and similar genomes using

anvi'o program 'anvi-get-sequences-for-hmm-hits', and aligned the sequences of each gene using MUSCLE v3.8. [693]. We then used IQ-TREE v1.6.12 [694] with the 'WAG' general matrix model [695] and 1,000 ultrafast bootstrap to compute maximum likelihood phylogenetic trees for each alignment. Finally, we used anvi'o v7.1 program 'anvi-interactive' program to visualize the constructed phylogenetic trees and associated metadata. Following the confirmation of identity, we utilized Subsystems genes [696] and pathway functions in the KEGG database [689], to compare the potential functions between MAGs.

3.5.5 Metabolomic profiling of the plant host rhizosphere

Sample preparation. The samples selected for plant host metabolite profiling were collected during early Fall 2023 within one week of each sampling time (C - September 03, 2023, H - September 13, 2023). Roots were picked from the selected samples and shaken to remove any soil; the soil that remained on the roots was considered rhizospheric soil. Rhizosphere soil was separated from the roots, and a 20 g soil sample was aliquoted into a 50 mL centrifuge tube. We added 25 mL of deionized water to each of the 50 mL tubes and mixed the contents by shaking for 3 hours at room temperature. The samples were then centrifuged for 5 min at 8000 rpm. The supernatant was transferred to the new 50 mL tube and stored at -80°C until metabolomic analysis (MetWare® Bio, MA, USA).

Sample extraction and chromatography–mass spectrometry. An aliquot of each sample (9 mL) was placed at 4°C overnight, and then vacuum freeze-dried. After freeze-drying, 70% methanolic internal standard extract was added at a ratio of 30 times the concentration. The samples were vortexed for 15 mins, and sonicated in an ice water bath for 10 mins. The extracts

were centrifuged at 12,000 rpm at 4°C for 3 mins, and filtered using a 0.22 μm filter before liquid chromatography and mass spectrometry. Ultra-performance liquid chromatography (UPLC) (ExionLC™ AD) and tandem mass spectrometry (MS/MS) (Applied Biosystems QTRAP 6500) were used for data acquisition. Liquid phase condition consisted of (1): liquid phase chromatographic column: Agilent SB-C18 1.8 μm, 2.1 mm × 100 mm; (2) Mobile phase: A phase—ultrapure water (0.1% formic acid added), B phase—acetonitrile (0.1% formic acid added); (3) elution gradient: the proportion of B phase was 5%, within 9.00 min, the proportion of B phase increased linearly to 95% and remained at 95% for 1.00 min, 10.00–11.10 mins, the proportion of B phase decreased to 5% and balanced at 5% up to 14.00 mins; (4) flow rate: 0.35 mL/min; (5) column temperature: 40°C; and (6) injection volume: 2 μL.

Linear Ion TRAP Mass Spectrometer (Q TRAP) (AB6500 Q TRAP UPLC/MS/MS system) was used to obtain both mass spectrum scans using both Linear Ion TRAP Mass Spectrometer modes. Operating parameters of ESI source were as follows: ion source, turbine spray; source temperature 550°C; Ion spray voltage (IS) 5500 V (positive ion mode) / -4500 V (negative ion mode); The ion source gas I (GSI), gas II (GSII) and curtain gas (CUR) were set to 50, 60 and 25.0 psi respectively, and the collision-induced ionization parameter was set to high. The multiple reaction monitoring (MRM) mode was used for the QQQ scan and collision gas (nitrogen) was set to medium. DP and CE of each MRM ion pair were completed by further DP and CE optimization, and a specific set of MRM ion pairs was monitored based on the eluted metabolites at each period.

Secondary metabolites were identified based on their secondary spectrum information. Signals from isotopes, and repeated signals containing cations such as K+, Na+, and NH_4^+ were excluded during the qualitative analyses. Metabolites were quantified by triple quadrupole mass

spectrometry with multiple reaction monitoring (MRM). In MRM mode, the first quadrupole screened precursor ions specifically for the target compound, excluding ions of other molecular weights. After ionization induced by the impact chamber, the precursor ion was fragmented, and a characteristic fragment ion was selected through the third quadrupole and excluded the interference of other non-target ions.

Data preprocessing. Missing values in the acquired raw data were filled using 1/5 of the minimum value of each metabolite, and then the Coefficient of Variation (CV) value of the quality control (QC) sample was calculated, and the metabolites with a CV value less than 0.3 were removed from the further analysis. The CV signifies the relationship between the standard deviation and the mean in the original dataset and was used as an indicator of data dispersion. The Empirical Cumulative Distribution Function (ECDF) was used to analyze the frequency of compound CVs falling below a specified reference value. A composite QC specimen was prepared from the mixture of extracts from all samples to assess the reproducibility of the entire metabolomics workflow. Throughout data acquisition, a single quality control sample was systematically incorporated for every 10 test samples.

The mass spectrum data was processed using Analyst 1.6.3 (AB Sciex, Framingham, Massachusetts, USA). The MRM metabolite multi-peak detection diagram depicting the compounds identified within the sample was generated. Each peak in the mass spectrum is color-coded to signify the detection of a specific metabolite. The characteristic ions of each compound were selected by triple quadrupole, and their signal intensities (CPS - Counts Per Second) were measured. Subsequently, the mass spectrometry data were analyzed through MultiQuant software, facilitating the integration and correction of chromatographic peaks. The area of each

chromatographic peak represents the relative abundance of the corresponding compound. The correction of mass spectrum peaks for each metabolite across various samples was conducted by considering retention time and peak distribution data to ensure the precision of both qualitative and quantitative analyses.

We used the vegan package [504] in R Studio to estimate the pairwise Bray–Curtis distances to compare the relative metabolic content of metabolites among the different samples. We then used the ggplot2 package [505] to visualize these data using non-metric multidimensional scaling (NMDS) ordinations.

3.5.6 Statistical analysis

We (1) first identified key omics variables for the integration process; and then (2) maximized the common and correlated information between the datasets, and finally (3) visualized the results to identify relevant microbial metagenomics and host metabolomics correlations. We used Analysis of Variance (ANOVA) to test for main and interactive effects among groups. Following the overall ANOVA, we used pairwise comparisons using Pairwise Wilcoxon Tests with the false discovery rate (FDR) method to correct for multiple comparisons to identify factors that were driving the significant effects. P-values of less than 0.05 were considered statistically significant. Statistical analyses were performed in RStudio [697]. We used the program - Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) to identify the key correlations between host metabolomics and metagenomic datasets (MAGs, MetaPhlAn, and HUMAnN) [698–701]

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3.7 Author Contributions

A.J., L.J., and S.T.M.L. designed the study. L.J. designed the reciprocal gardens experimental design, and A.J., L.J., E.H., and S.T.M.L. maintained the sites. A.K., S.S., S.P., and S.T.M.L performed sample collection. S.P., A.K., B.A., L.R., and S.S. extracted DNA and performed quality analysis with Nanodrop and Qubit for the metagenomics analysis. A.K., B.A., and L.R. prepared the samples and processed the data for the metabolomics analysis. A.K. and S.T.M.L. performed the gene-, genomic-centric, and metabolomics data analyses. A.K. and S.T.M.L wrote the manuscript, and prepared the figures, and files. A.J. and L.J. aided in manuscript and figure revisions. S.T.M.L., A.J., and L.J. acquired funding for this study. All the authors have read, contributed to the manuscript revision, and approved the submitted version.

3.8 Declarations of Interests

The authors declare that they have no competing interests.

3.9 Funding

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Chapter 4 - Native rhizobiome immune priming mechanism increases plant host stress resilience

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4.1 Abstract

The plant rhizosphere harbors a diverse range of microbial species, which play integral roles in regulating plant-host stress. Although evidence exists that the plant host selectively recruits microbes from soil, there is limited knowledge available on the functional potential of the recruited microbes and the mechanisms they use to communicate with the host and fulfill their roles. Previously, our field study using reciprocal gardens revealed that Dry (Hays, Kansas, 580 mm rainfall/year) and Wet (Carbondale, Illinois, 1167 mm rainfall/year) *Andropogon gerardii* ecotypes perform better at recruiting beneficial microbes when grown at *"home"* environments, supporting the *"home-field advantage"* hypothesis. Here, we used culturomics to single cell culture microbes from Hays and Carbondale rhizosphere soils and assembled 320 (Dry: 159 and Wet: 161) draft genomes of these microbes. We evaluated communication strategies and beneficial functional potentials of the obtained microbes and analyzed microbial metabolites. Here, we show that Dry ecotypic microbes produced high levels of phospholipid - Lyso-phosphatidylethanolamine (LPE), which can potentially enhance the plant host's response to drought-related stress. Our study provides a better understanding of the complex mechanisms of plant host-microbe interactions, and how those interactions may enhance the plant host's resilience to environmental stresses.

4.1.2 Keywords

Culturomics, plant-microbe interactions, functional potential, metabolomics, SynCom, RNAseq, metabolite challenge

4.3 Introduction

There is an increasing interest in using microbial communities to improve plant productivity and enhance plant resistance to abiotic and biotic stresses. One of the approaches is the manipulation of the plant rhizosphere through inoculation with microbes, ranging from one species [702] to the assembly of synthetic microbial communities (SynComs). SynCom is a microbial community designed by mixing a limited number of selected strains, aiming to mimic the natural community function by maintaining critical characteristics and demonstrating a benefit to the host plant [353,357,703]. Members of SynComs are often selected based on the following criteria: 1) microbes that are compatible plant mutualists [327,704]; 2) microbes that stably and robustly colonize the plant host [705–707]; 3) microbes with specific beneficial functions [330]. While studies have made significant progress in assembling plant-associated SynComs, several critical knowledge gaps remain because of the complex interplay between various biotic and abiotic factors. One pressing matter is the understanding of the collective metabolic outputs resulting from microbe-microbe and plant-microbe interactions, which are crucial for engineering SynComs that promote plant growth and resilience.

Plant rhizobiome establishment is not random but follows assembly rules [49,50,708,709], which in most part may be governed by the host [49,50] and products from interacting microbes [130,357,710,711]. Soil microbes are also crucial in shaping plant host immune responses, forming dynamic interactions that influence plant health and resilience [710,712]. Beneficial microbes can colonize plant roots, and enhance the plant's immune system by priming it for more robust responses to pathogens and/or environmental stressors [713–715]. These microbial populations can trigger systemic resistance, activating defense pathways that can make the entire plant more resilient to stress or resistant to many diseases [710,715,716]. Additionally, rhizosphere microbes

can suppress harmful pathogens by outcompeting them for resources as well as by producing antimicrobial compounds [717,718]. Hormones, enzymes, and secondary metabolites produced by soil microorganisms can interact with plants to trigger immune signaling pathways, and significantly enhance plant immune responses [719,720]. For example, some metabolites can mimic plant hormones such as jasmonic acid, salicylic acid, and auxin which are important in plant defense, activating acquired resistance [721–723]. This activation, in turn, prepares the plant host to respond more swiftly and effectively to pathogens and environmental stresses [713–715]. The symbiotic relationship between the plant host and its rhizobiome not only boosts the plant's immune system but also facilitates host nutrient uptake improving its growth [724–726].

Soil microbial lipids, such as fatty acids, phospholipids, lipopolysaccharides, and other lipid-derived compounds, are increasingly recognized for their role in influencing plant host immune responses [727–729]. These microbial lipids interact with the plant receptors, trigger immune responses, and enhance the host's resilience under abiotic and/or biotic stresses [730–733]. For example, microbial lipids can be recognized by plant pattern recognition receptors to initiate defense mechanisms like the production of antimicrobial compounds and reinforcement of cell walls [734,735]. While microbial lipids may activate plant defenses against pathogens, it is poorly understood how microbial lipids may influence plant immune responses to drought stress. Drought conditions can alter the composition and activity of soil microbial communities, potentially affecting the production and availability of microbial lipids [736–738]. However, the ways in which these changes influence plant immune signaling and overall resilience to drought are not well-documented. The interactions between microbial lipids and plant stress hormones, such as abscisic acid (ABA), which governs drought responses, are particularly underexplored.

Addressing this gap could reveal new strategies for enhancing plant drought tolerance through microbial interventions.

Previous studies on the SynCom assembly mostly focus on stochastic assembly or selection of compatible microbial populations with inadequate attention to microbial functions, their metabolites, and the multidirectional system of community interactions. We suggest that we can exploit the success of native rhizobiome and metabolic mutualisms among the microbial populations to design robust probiotic SynComs. In a reciprocal gardens study [739], we observed that *Andropogon gerardii* regional ecotypes perform better in their "home" environments. Thus, we hypothesize that the dry ecotype *A. gerardii* would have specific rhizosphere microbial populations that may enhance its ecotypic resilience to a drier environment [494,740,741]. Here, we took advantage of the microbial assemblage differences among the plant host ecotypes [170,739,741], and aimed to provide insight into the stress-associated functional potential of the dry ecotype rhizosphere microbial community. In this study, we asked the following questions - *What are the crucial functional differences among the microbial members in the A. gerardii dry and wet ecotype rhizobiomes that could contribute to improving the host stress tolerance? What are the microbial functions that are necessary for survival, communication, and stress regulation during and through abiotic stress? How does the host interact with the associated rhizosphere microbiomes?* This study combines culturomics and multi 'omics approaches to propose metabolic pathways of interest in ecotype-derived microbial populations that could enhance plant resilience to abiotic stress. We observed that *Acinetobacter*-derived Lyso-phosphatidylethanolamine (LPE) enhanced the plant host's resilience to abiotic stress. Our findings mechanistically link *Acinetobacter*-derived LPE, and other microbial metabolites to provide insights into microbial LPE's role as a potential foundation in forming a robust SynCom.

4.4 Materials and Methods

Figure 4.1 illustrates the methods for sample collection, rhizobiome microbial member isolation and culturing, and identification of functional potentials and metabolites of microbial members. In this study, we focused on elucidating the microbial functions and mechanisms that might serve as a robust microbial SynCom, enhancing the growth of the plant host.

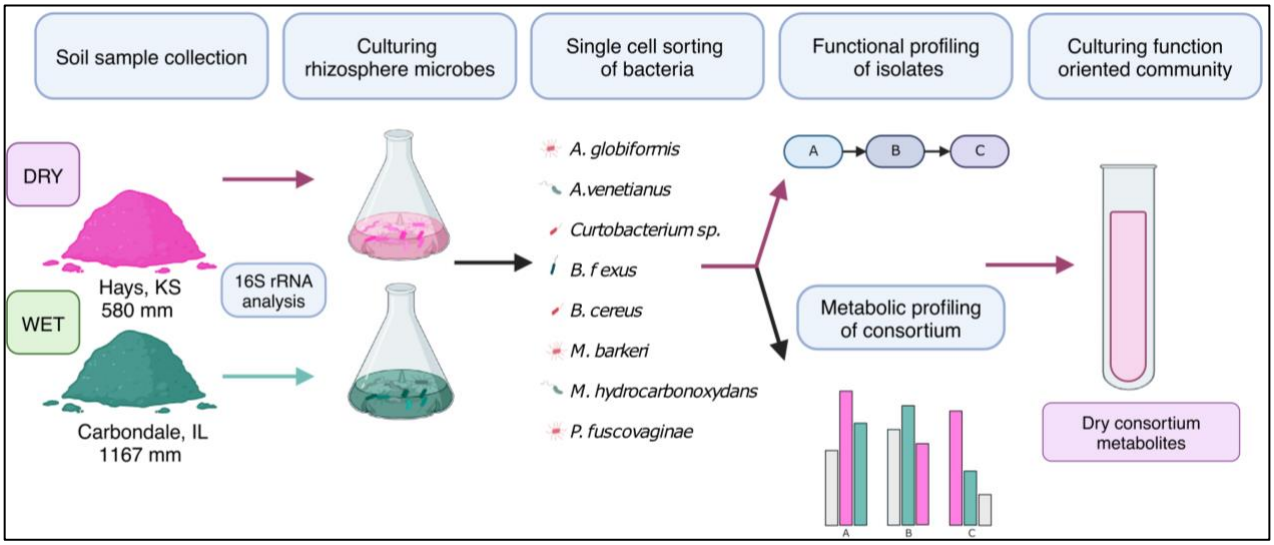

Figure 4.1 The schematics of the experimental design highlighting the flow of the project. Briefly, we used rhizosphere soil from Dry and Wet regional *A. gerardii* ecotypes to culture associated bacterial communities. We then performed single cell sorting of the microbial populations, followed by functional profiling of isolates, and metabolic profiling of the resultant consortium, to unveil functional mechanisms of microbes.

4.4.1 Culturing of microbes

To prepare and assemble an ecotype-associated SynCom, we collected three soil cores (1.25 cm in diameter to a depth of 15 cm) at the base of six *A. gerardii* plants representing regional populations in June 2021. Soil cores were collected from three western (Western Kansas) and three eastern (Southern Illinois) *A. gerardii* populations (Supplementary Table 18) and were pooled to represent the microbial consortium associated with the dry and wet ecotypes, respectively. This

soil was used to culture dry and wet microbial communities. The dry and wet ecotype soils (50 mg) were added to 5 ml of R2A broth [742], incubated overnight at 30°C and shaken at 150 rpm in culture tubes. The cells were diluted using phosphate-buffered saline (PBS, pH 7) and ultrapure water (cells: PBS: water 1:1:8), to increase the probability of obtaining diverse microbial populations.

4.4.2 Metabarcode sequencing of soil and microbial consortium

We characterized the microbial communities associated with the microbial consortium and the soil that was used to prepare the consortium. The environmental soil DNA and the microbial DNA from the consortium were extracted using the E.Z.N.A. Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol with slight modifications. Environmental DNA was extracted from an aliquot of 0.25 g of manually homogenized soil samples in triplicates (n=6). To extract the microbial DNA (five replicates per soil type (n=10)), 20 - 25 mL of the consortium was aliquoted into 2 mL centrifuge tubes, and centrifuged for 10 mins at 8000 rpm at 4°C. The supernatants were decanted and discarded, and 1000 µL PBS buffer was added to one of the 2 mL centrifuge tubes to resuspend the pellet. The resuspension was transferred to the next tube to acquire one pooled 2 mL centrifuge tube per consortium. This 2 mL tube was centrifuged again for 10 mins at 8000 rpm at 4 °C to pellet the cells, and the supernatant was discarded prior to the DNA extraction as per manufacturer's protocol. Both environmental and microbial DNA were eluted to a 100 µL final volume. The DNA yield and concentration were measured using a Nanodrop and a Qubit dsDNA BR Assay Kit. Eluted DNA samples (soil (n=6) and consortia (n=10)) were submitted to sequencing in separate runs (2 x 250 cycles). The 16S rRNA V4 amplicons were sequenced at the Kansas State University Integrated Genomics Facility

using the Illumina MiSeq platform with barcoded 515F and 806R [515F-GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGGTWTCTAAT] primers (Illumina, San Diego, CA, USA).

QIIME 2 v2021.4 [743] was used for raw sequence data processing. The QIIME 2 plugin cutadapt [744] was used to remove the primer sequences. For initial quality control, any sequences with ambiguous bases, no primer, greater than 0.1 error rate mismatch with primer or any mismatches to the sample-specific 12 bp molecular identifiers (MIDs) were discarded. Following the initial quality control, we used the DADA2 pipeline with the same parameters across all five runs. We truncated the reads to a length where the twenty-fifth percentile of reads had a quality score below 15 (Forward 210 bp and Reverse 180 bp). Since the same primers were used for every sample set, the quality control and primer removal using DADA2 were performed separately to extract Amplicon Sequence Variants (ASVs) (Callahan et al., 2016). Samples were then merged (total n = 232) and analyzed as one data set. Pre-trained SILVA database (v.138) was used for the taxonomic assignment of the bacteria [745]. We rarefied the data to 10,000 reads per sample to minimize biases resulting from differences in sequencing depth among samples before estimating diversity indices and downstream analyses [746] (Supplementary Table 19A). QIIME 2 was used for all following sequence processing, whereas the subsequent statistical analyses for microbial composition were performed in R studio [747]. We used the vegan package [504] to estimate the pairwise Bray–Curtis distances to compare the bacterial communities among the different factors. The data were visualized with ggplot2 package [505] using non-metric multidimensional scaling (NMDS) ordinations. We used a non-parametric PERMANOVA to determine whether bacterial communities differed compositionally among soils and consortium sample types and ecotypes as well as their two- and three-way interactions.

4.4.3 Oligotyping analysis

We used minimum entropy decomposition (MED) [675] with default parameters to identify sequence variants among the 16S rRNA amplicon sequences in the microbial consortium samples (Supplementary Table 19B). The MED partitions the sequences into discrete sequence groups by minimizing the total entropy in the data set. We then concatenated the sequences assigned to a specific genus using the SILVA (v. 138) reference database, and used the supervised oligotyping method available in the oligotyping pipeline version 2.1 [265]. In this supervised oligotyping, we used Shannon entropy with a threshold value of minimum 0.2 and minimum substantive abundance threshold of 10 to obtain genus-level oligotypes. We focused on oligotypes of *Pseudomonas* and *Acinetobacter* for this analysis because of their potential beneficial effects on the plant-host interaction.

4.4.4 Single cell microbial isolates

We single cell sorted individual bacterial cells and performed full genome sequencing of the microbial isolates to evaluate the functional potential of the individual microbial members in the consortium. Single cell isolates were sorted into 96-well plates using the Prospector® system (Isolation Bio, CA) (Supplementary Table 20A). To produce an adequate amount of the bacterial biomass for sequencing, we regrew each isolate in nutrient-limited R2A broth for 4-6 days, and Isolates with no or very limited growth after 6 days were re-inoculated into nutrient-rich LB broth for the same duration. We used sterile 12-inch bamboo skewers to inoculate isolates in the 3 mL culture tubes. After 4-6 days, samples were aliquoted into three equal portions - gDNA extraction, short-term archive, and long-term 50% glycerol stock archive. Both archives were stored at -80℃.

We used the One-Tube Bacterial Genomic DNA Extraction Kit (Bio Basic, cat. BS8403) to extract gDNA as per the manufacturer's instructions, and the extracted gDNA was stored at -20℃ until sequencing. We used Nanodrop® and Qubit® to determine the quality and concentration of the extracted DNA for each isolate. Microbial isolates with less than 5 ng/μL of DNA were re-grown to reach the adequate DNA concentration for sequencing. We consolidated all samples into 96-well plates, with approximately 60 μL of extracted DNA in each well, with three blank wells for control on each plate. Samples were submitted in two batches and sequenced in two separate runs (Supplementary Table 20B).

Illumina DNA sequencing libraries were prepared using Low Volume Nextera protocol [748,749]. Whole genome sequencing was performed on the Illumina HiSeq X platform to obtain ~50 X coverage using 2x150 bp paired-end reads (Supplementary Table 20B). We performed de novo genome assembly and annotation with the default parameters through the Bacterial and Viral Bioinformatics Resource Center (BV_BRC) v3.31.12 [692]. Specifically, we used TrimGalore version 0.6.10 (Krueger et al. 2021) to trim the reads, and used Unicycler ver 0.4.8 [750] to assemble the bacterial genomes. We then polished the resulting contigs through 2 iterations of Pilon ver 1.24 [751]. We filtered and annotated contigs with ≥ 300 bp and ≥ 5 X coverage using RAST tool kit [752]. We called assembled genomes as draft microbial genomes if they met the following criteria: completeness > 70 %, contamination < 10 % and microbial DNA size > 1.5 Mbp. All downstream analyses were only performed on the draft genomes.

We uploaded and annotated the draft genomes in the Bacteria and Viral Bioinformatics Resource Center (BV-BRC) [692]. We identified similar genomes as our draft genomes in BV-BRC, and built phylogenomic trees using 11 similar strains to identify close relationships and approximate identity. Following identity confirmation, we annotated each draft genome using

Subsystems [696] and KEGG [689], and compared the functional potentials between the Dry and Wet ecotypic draft genomes.

4.4.5 Phylogenomic analysis

We build a phylogenomic tree using similar genomes to identify close relationships and approximate the identity of the draft genomes. We used the similar genomes finder function in BV_BRC to download 11 similar genomes for each of the non-redundant draft genomes (a total of 320 draft genomes and 231 similar genomes) (Supplementary Table 20C). We used a collection of six bacterial ribosomal gene alignments for constructing the phylogenetic tree: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. We explored the sequences of these genes across all the assembled draft genomes and similar genomes using anvi'o program 'anvi-get-sequences-for-hmm-hits', and manually aligned the sequences of each gene using MUSCLE v3.8. 1551. We then used IQ-TREE v1.6.12 87 [694] with the 'WAG' general matrix model [695] and 1,000 ultrafast bootstrap to compute maximum likelihood phylogenetic trees for each alignment. Finally, we used anvi'o v7.1 'anvi-interactive' program to visualize the constructed phylogenetic trees and associated metadata [678].

4.4.6 Microbial metabolomics

We obtained a comprehensive profile of the microbial metabolites from the soil, as well as combined single cell isolates (Dry and Wet) to help us identify potential targets for microbial synthetic community assembly. We prepared the soil samples in triplicate to ensure adequate volume for the analysis. After the overnight incubation, soil samples were centrifuged for 5 mins at 8000 rpm, and the supernatant (~15 mL) was transferred to new 50 mL tubes, and kept in -80℃

until further processing. We prepared the combined single cell isolates by combining the single cell isolates that passed the "draft genome" selection criteria. Dry (n = 159) and Wet (n = 161) single cell isolates were grown individually for 6 days in 1 mL of R2A broth, shaking at 150 rpm at room temperature. We then pooled 10 uL of each isolate into a single culture tube with 3 mL of R2A media. Each combined isolate sample was prepared in triplicates to ensure adequate volume for the metabolites analyses. Combined single cell isolate samples were then placed into the incubator for an additional 3 days at 150 rpm at room temperature. Following that, we aliquoted 50 mL from the triplicates and centrifuged for five minutes at 8,000 rpm. We then transferred the supernatant into new 50 mL tubes and kept it in -80℃ until further processing. Both soil and single cell isolate samples were shipped overnight on dry ice to Metware Biotechnology Inc. (https://www.metwarebio.com/). A detailed description of the metabolic data generation and processing is available in the Supplementary Methods (metabolic data generation and processing).

4.5 Results and Discussion

This study (Figure 4.1) was motivated by the observations of (1) differences in environmental conditions that shaped local bacterial populations, which were the primary drivers of ecotypic rhizosphere microbial communities. (2) plant ecotypes more efficiently recruit native microbes, suggesting a *"home field advantage";* (3) ecotypic variations in the recruitment of oligotypes from the same bacterial genera; (4) Dry *A. gerardii* ecotypes showed greater physiological and genetic resilience to drought-induced stress in drier environment [444,739]. As such, hypothesizing that microbes in the dry ecotype would enhance the plant host resilience under drought stress, we took the opportunity to decipher the membership, functions, and metabolites of the members in the Dry ecotypic rhizobiome.

4.5.1 Culturomics of ecotypic rhizobiome revealed the abundance of the plant-growth promoting bacteria

We analyzed 16S rRNA amplicons of the Dry and Wet ecotype-associated rhizobiome cultures. A total of 929,229 (71,950 ± 24,969) high-quality sequences were assigned to 570 ASVs (soil samples: 545 ASVs; cultured samples: 111 ASVs) (Supplementary Table 19A). Of the recovered reads, 88% were annotated to a genus using the Naivé Bayesian classifier and the RDP database (Wang et al., 2007). We removed any reads assigned to chloroplasts and mitochondria as well as any unknown or unclassified ASVs from downstream analyses. Our data were dominated by the phylum Pseudomonadota (33% of all sequences), followed by High GC gram+ (15%), and Bacteroidota (9.65%), and Firmicutes (6%). A small portion of the sequences were assigned to archaea (1.05%). The proportion of unassigned ASVs was small (0.35%). Our overall analysis of the microbial communities across two sample types (collected bulk soil samples and cultured samples) highlighted that the sample types differed in their community composition (PERMANOVA, $R^2 = 0.48$; $F_{1,15} = 25.03$; $p = 0.001$, Figure 4.2A,B). We further conducted oligotyping analysis for the consortia, focusing on the genera *Acinetobacter* and *Pseudomonas* because of their wide range of plant interactions that can affect plant biomass. We identified 12 *Pseudomonas* oligotypes that differed between Dry and Wet consortia (Figure 4.2C, Supplementary Table 21B), suggesting fine resolution differences between Dry and Wet ecotypic associated cultures. Interestingly, in contrast to *Pseudomonas*, we observed that most of the samples were dominated by one single *Acinetobacter* oligotype (Figure 4.2D, Supplementary Table 19B).

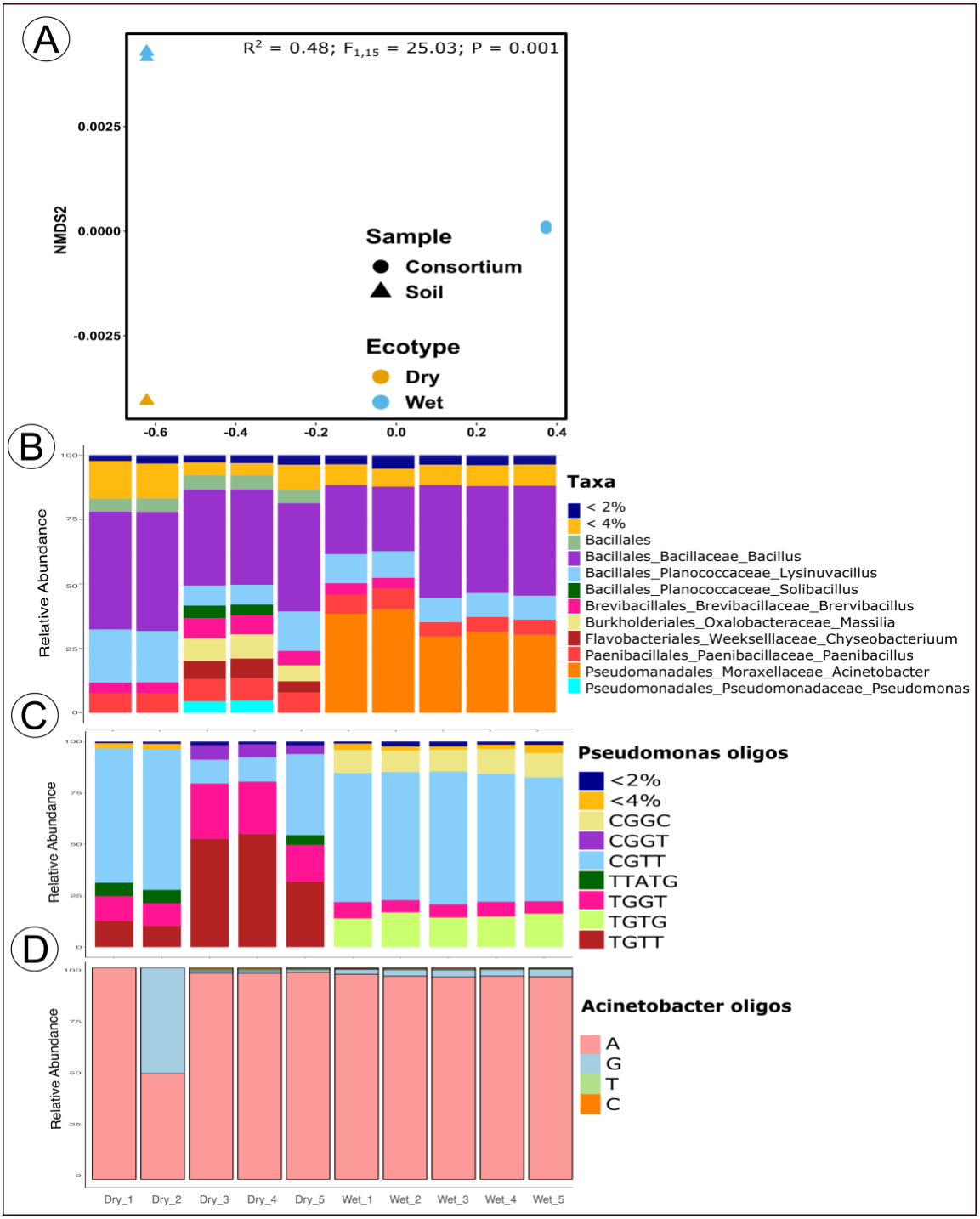

Figure 4.2 The effect of the sample type and ecotype on the bacterial community composition. (A) Sample type has the highest impact on the bacterial community composition. NMDS plot of microbial communities associated with rhizosphere soils from Dry and Wet regional *A. gerardii* ecotypes, and microbial consortia that were prepared from the ecotypic soils. NMDS ordinations were obtained from Bray–Curtis similarity matrix. (B) Relative abundance of taxa within Dry and Wet consortium samples. Relative abundance of each oligotype in (C) *Pseudomonas* and (D) *Acinetobacter* diversity for each consortium sample.

With the suggestion that fine resolution differences might contribute to the reason Dry ecotypic associated cultures could enhance plant host drought resilience, we performed culturomics on the ecotypic associated microbial cultures [445]. We isolated 584 (Dry: 273 and Wet: 311) bacterial cells through the single cell sorting, and successfully regrew 559 isolates using R2A and LB liquid media. The first genome sequencing run contained 487 samples (Dry: 222 and Wet 265) grown on the R2A media. The second run contained 119 (Dry: 58 and Wet: 61) bacteria isolates grown on the LB media (Supplementary Table 3A). From these data, we assembled a total of 320 (Dry: 159 and Wet 161) draft genomes (Figure 4.3). Four draft genomes from both Dry and Wet inocula were not assigned to any taxa. Most common Dry isolates were assigned to *Arthrobacter globiformis* (47), *Acinetobacter venetianus* (20), *Chryseobacterium sp (42), and Microbacterium barkeri* (16). Wet isolates were dominated by *Acinetobacter venetianus* (93), *Bacillus cereus* (30) and *Microbacterium barkeri* (6) (Supplementary Table 20B). Following the successful assembly of the dry consortium draft genomes, we examined the genomic information to determine what potential microbial functions could be associated with the enhanced resilience of the dry ecotype in drier environments.

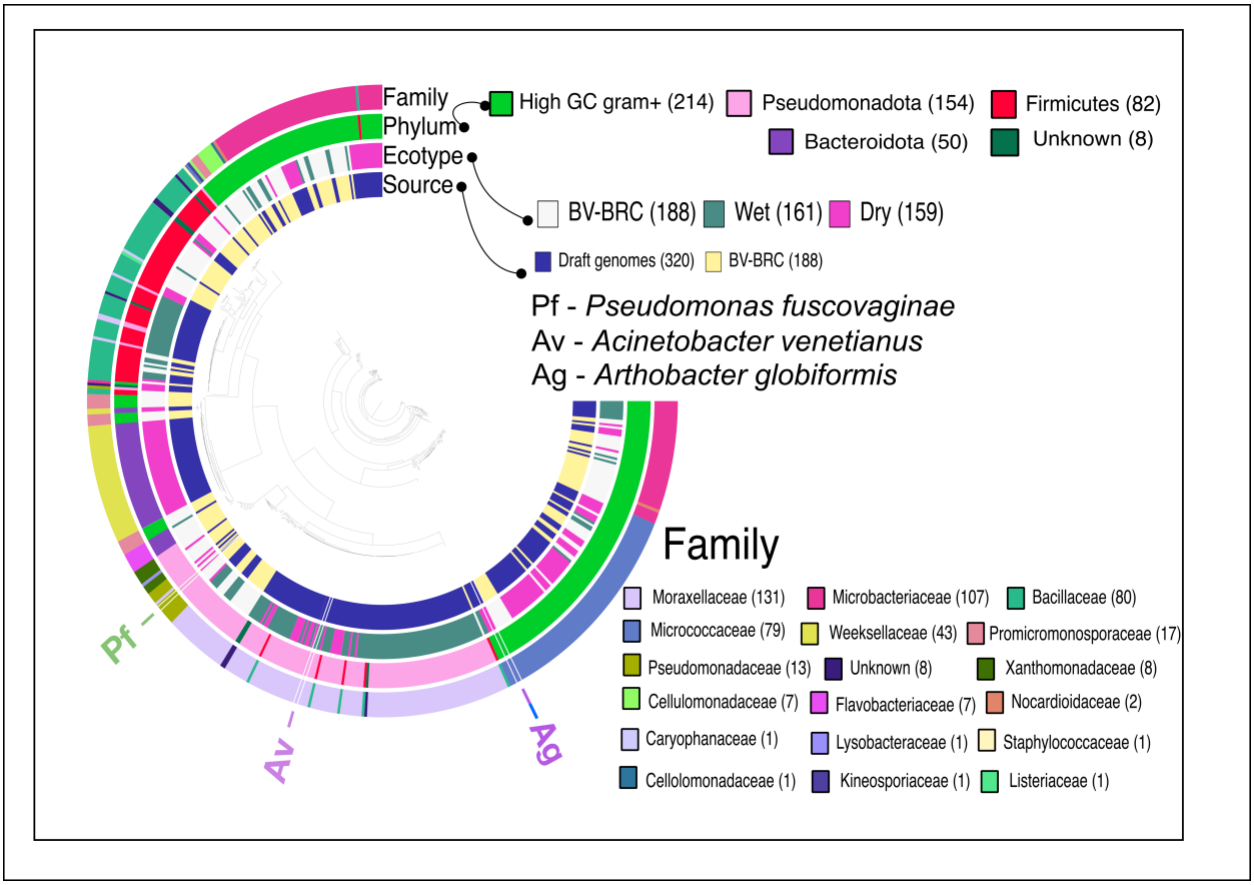

Figure 4.3 Phylogenomic tree based on alignment of six bacterial ribosomal genes: Ribosomal_L1, Ribosomal_L2, Ribosomal_L3, Ribosomal_L4, Ribosomal_L5, Ribosomal_L6. The tree included 320 microbial isolates and 11 unique similar strains to identify close relationships and identity of isolates. Additional data layers, i.e. source, ecotype, phylum, family and the position of *Pseudomonas fuscovaginae, Acinetobacter venetianus* and *Arthrobacter globiformis* are incorporated in the figure according to the key

4.5.2 Dry ecotypic bacterial isolates showed high number of microbial associated functions that confer benefits to plant host

Our findings on the bacterial diversity among the ecotype associated cultures as well as microbial identity from the culturomics, prompted us to further investigate the functional potential of these isolates. We asked: What microbial functions do the ecotype associated bacterial isolates possess that were necessary for abiotic stress resistance? Do the ecotype associated bacterial isolates possess functions that can convey benefits to the associated plant host?

Among both Dry and Wet isolates, we observed numerous genomic functional potentials to address osmotic stress. We observed that all 159 Dry isolates and 162 Wet isolates possessed glycine, serine and threonine metabolism pathways, with the potential to produce ectoine (Table 1). Three Dry isolates annotated to *Arthrobacter* sp. and one Wet isolate annotated to *Cellulomonas* sp. possessed potential pathways for L-ectoine production, which may help the bacterial cells to counteract the osmotic stress by stabilizing the internal water content and preventing protein denaturation under dry conditions in Western Kansas [753]. Further, 21 (*Acinetobacter venetianus*; n=19 and *Pseudomonas fuscovaginae*; n=2) bacterial isolates from the dry ecotype, and 98 (*Acinetobacter venetianus*; n=91, *Acinetobacter indicus*; n=4, *Bacillus cereus; n=1,* and unknown; n=2) wet bacterial isolates had osmolarity sensory histidine kinase EnvZ system. We also observed two-component system response regulator OmpR in 20 (*Acinetobacter venetianus*; n=19 and *P. fuscovaginae*; n=1) bacterial isolates from the dry ecotype and 98 (*Acinetobacter venetianus*; n=91, *Acinetobacter indicus*; n=4, *Bacillus cereus; n=1,* and unknown; n=2) in wet bacterial isolates. While EnvZ detects the changes in the environmental conditions, OmpR proteins activate bacterial genes responsible for promoting the cell response to osmotic stress through regulation of flagellar motility, biofilm formation, and host colonization [754,755]. Combined, this suggests that these ecotype-associated bacterial populations possess numerous mechanisms to respond to osmotic stress. Interestingly, we observed two additional regulatory proteins associated with the osmotic stress regulation in one *P. fuscovaginae* isolate from dry ecotypes - a transcriptional regulatory protein PhoP and sensor histidine kinase PhoQ. Both proteins are additional censoring proteins for the low PO_4^{3-} [756,757] and Mg^{2+} [758] levels in the environment, associated with bacterial virulence, and their metabolic utilization of plant-derived carbon sources [590]. Here, we surmise that *P. fuscovaginae* may experience lower

availability Mg^{2+} and PO_4^{3-} availability in drier soil conditions in Western Kansas, compared to more mesic environment in Southern Illinois [759,760]. Lower soil moisture in Western Kansas reduces nutrient availability to plant hosts, creating conditions that favor the survivability of *P. fuscovaginae* and similar microbial populations capable of withstanding abiotic stress caused by drier environment

Table 4.1 Functional potential of the Dry and Wet consortium isolates. The values represent the number of isolates possessing all the necessary genes to perform the potential plant-host beneficial function. Abbreviation keys are in the Appendix A

		Ai	*Av*	*As*	*Ag*	*Ar*	*Bc*	*Bf*	*Cel*	*Chr*	*Cur*	*Ean*	*Eac*	*La*	*Lf*	*Ld*	*Mb*	*Mh*	*Mp*	*Pf*	Unk	Total
Overall	**Dry**	0	19	0	47	4	6	3	0	43	1	0	1	1	0	1	16	3	7	4	3	**159**
	Wet	4	93	2	4	1	30	1	2	0	4	1	0	0	1	0	7	4	3	0	4	**161**
L-Ectoine	**Dry**	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**
	Wet	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	**1**
EnvZ	**Dry**	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	**21**
	Wet	4	91	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	**98**
OmpR	**Dry**	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	**20**
	Wet	4	91	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	**98**
ACC deaminase	**Dry**	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	**10**
	Wet	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**
AHLs	**Dry**	0	0	0	1	1	3	1	0	0	0	0	0	0	0	0	0	0	0	0	1	**7**
	Wet	0	0	0	0	0	27	1	0	0	0	0	0	0	0	0	1	0	0	0	1	**30**
pgaABCD	**Dry**	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	**20**
	Wet	0	87	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	2	**91**
QQ	**Dry**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	**3**
	Wet	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
IPDC	**Dry**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	**3**
	Wet	0	73	0	0	0	21	1	0	0	0	0	0	0	0	0	0	0	2	0	0	**97**
NIT	**Dry**	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	1	0	5	0	0	**15**
	Wet	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	**2**
Plant-induced NIT	**Dry**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	**2**
	Wet	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
NHase	**Dry**	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	**18**
	Wet	0	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	**39**
IGPS	**Dry**	0	15	0	39	1	5	3	0	34	1	0	1	1	0	1	13	2	5	3	3	**127**
	Wet	2	82	1	3	1	21	1	2	0	3	0	0	0	1	0	4	3	3	0	3	**130**

While the regulation of host osmotic stress could be a by-product of the microbial survival in the environment, we observed bacterial genomic functional potentials to directly regulate plant-host stress (Table 1). Similar to the glycine, serine and threonine metabolism pathway, we observed that many isolates (Dry; n=76, Wet; n=66) had the potential to utilize propanoate metabolism pathway at some capacity. However, only 10 Dry isolates (annotated to *Arthrobacter* sp. (n=2), *Microbacterium paraoxydans* (n=7), and *Arthrobacter globiformis* (n=1)), and 2 Wet

isolates (annotated to *Arthrobacter globiformis*) possessed all the necessary genes to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase which can hydrolyze 1-Aminocyclopropane-1-carboxylic acid (ACC) into ammonia and α-ketobutyrate [761–763]. Alas, these data suggest that our isolates are potentially able to regulate the plant host stress through ACC via the regulation of the plant host immune responses to microbial colonization, and modulating plant developmental processes [763] (Table 1). Based on the observed bacterial genomic functions, we hypothesize that these isolates may lower plant host's *"stress ethylene"* levels, resulting in longer roots and confer stress tolerance that maximizes plant survival during adverse conditions [654,761–763].

Next, we explored multiple mechanisms of host-microbe and microbe-microbe communications in Dry and Wet isolates (Table 1). Given the importance of biofilm on microbe-microbe interactions [764,765], we focused our analyses on microbial biofilm formation. Biofilms are syntrophic microbial communities that form for various purposes, such as protection against drought-induced unfavorable conditions [765]. We observed that 7 Dry isolates and 27 Wet isolates had all the necessary genes to produce N-acyl homoserine lactone hydrolase - an enzyme important in the production of N-acyl homoserine lactones (AHLs). The 7 Dry isolates were annotated to *Arthrobacter globiformis* (n=1), *Arthrobacter* sp. (n=1), *Bacillus cereus* (n=3), *Bacillus flexus* (n=1), and unknown (n=1), whereas the 30 Wet isolates were assigned to *Bacillus cereus* (n=27), *Bacillus flexus* (n=1), *Microbacterium barkeri* (n=1) and unknown (n=1). These 34 isolates were able to produce AHLs, which is a part of the quorum sensing (QS) and biofilm formation mechanisms, and can act as autoinducers to arrange the synchronized activity within bacterial communities [766]. We surmised that the QS mechanisms in our bacterial isolates would regulate bacterial behavior, such as metabolic rate, propagation and virulence, and are essential for establishing microbe-microbe and root-microbe associations [767,768]. Furthermore, the potential

production of AHLs by these isolates might contribute positively to the plant host-microbe interaction by inducing the growth-promoting effects and plant's resistance to phytopathogens as well as inducing cell wall reinforcement [715,769–771]. We also observed that 20 Dry isolates annotated to *Acinetobacter venetianus* (n=18) and *Pseudomonas fuscovaginae* (n=2), and 91 Wet isolates annotated to *Acinetobacter venetianus* (n=87), *Bacillus cereus* (n=1), *Microbacterium paraoxydans* (n=1), and unknown (n=2) possessed all necessary genes for the complete pgaABCD operon (Biofilm PGA outer membrane secretin PgaA, Biofilm PGA synthesis deacetylase PgaB, Biofilm PGA synthesis N-glycosyltransferase PgaC and, Biofilm PGA synthesis auxiliary protein PgaD) which are essential for the biofilm formation [591,644]. Interestingly, *Pseudomonas fuscovaginae* isolates (n=3) from dry ecotype rhizospheres had the functional potential to produce Acyl-homoserine lactone acylase PvdQ which can act as a quorum-quenching (QQ) - potentially preventing the biofilm formation, decreasing virulence, and reducing the infection of the potential plant pathogens [772,773].

We further observed that all isolates (Dry:159 and Wet:161) were able to utilize tryptophan metabolism pathway at some capacity with the potential to produce indole-3-acetic acid (IAA) [774] (Table 1). Some isolates possessed multiple mechanisms in the tryptophan metabolism pathway. For example, 3 Dry isolates that were annotated to *Pseudomonas fuscovagina*e (n=3), and 97 Wet isolates that were assigned to *Acinetobacter venetianus* (n=73), *Bacillus cereus* (n=21), *Bacillus flexus* (n=1), and Microbacterium paraoxydans (n=2) were able to produce aminotransferase and indole-3-pyruvate decarboxylase (IPDC) enzymes, which could convert tryptophan to IAA [775,776]. Further, 15 Dry isolates (*Arthrobacter globiformis*; n=9, *Microbacterium paraoxydans*; n=5, *Microbacterium barkeri*; n=1), and 2 Wet isolates (*Microbacterium paraoxydans*; n=2) were able to produce the enzyme nitrilase, which can directly

convert indole-3-acetonitrile (IAN) to IAA [777,778]. Interestingly, *Pseudomonas fuscovaginae* isolates (n=2) isolated exclusively from dry sites had the potential to produce plant-induced nitrilase, hydrolyses beta-cyano-L-alanine. Finally, 18 Dry isolates annotated to *Acinetobacter venetianus* (n=16) and *Pseudomonas fuscovaginae* isolates (n=2), and a high number (n=39) of Wet isolates annotated to *Acinetobacter venetianus* (n=36) and unknown (n=3) had the potential to produce nitrile hydratase which is necessary for the conversion of IAN to IAA with the indole-3-acetamide (IAM) as an intermediate step [779]. Similar to the tryptophan metabolism pathway, many Dry (78) and Wet (66) isolates had a potential to use phenylalanine, tyrosine and tryptophan biosynthesis pathways to produce IAA [780,781]. A large number of Dry (n=126) isolates (*Arthrobacter globiformis*; n=39, *Pseudomonas fuscovaginae*; n=3, *Microbacterium barkeri*; n=13, *Acinetobacter venetianus*; n=15, unknown; n=3, *Arthrobacter sp*; n=1, *Bacillus cereus*; n=5, *Chryseobacterium* sp.; n=34, *Microbacterium hydrocarbonoxydans*; n=2, *Microbacterium paraoxydans*; n=5, *Bacillus flexus*; n=3; *Curtobacterium* sp.; n=1, *Leifsonia aquatica*; n=1, *Lysobacter defluvii*; n=1, *Exiguobacterium acetylicum*; n=1) and Wet isolates (n=130) (Agromyces subbeticus; n=1; *Microbacterium barkeri*; n=4, *Curtobacterium* sp.; n=3, *Bacillus cereus*; n=21, *Arthrobacter globiformis*; n=3, *Bacillus flexus*; n=1, *Lysinibacillus fusiformis*; n=1, *Acinetobacter venetianus*; n=82, *Acinetobacter indicus*; n=2, *Microbacterium paraoxydans*; n=3, *Arthrobacter* sp.; n=1, *Microbacterium hydrocarbonoxydans*; n=3, *Cellulomonas* sp.; n=2, unknown; n=3) were able to produce indole-3-glycerol phosphate synthase, which can act as a branch point to synthesize IAA utilizing tryptophan-independent pathway [782].

Taked together, isolates from both dry and wet ecotypes possessed multiple gene functions that are essential for the bacterial populations to enhance microbial stress tolerance, as well as microbe-microbe and plant-microbe interaction. The abundant functional redundancies within the

microbial populations highlighted the complexity and challenge for assembling robust SynComs. Even more intriguing was the more numerous potential functions of Dry ecotype associated isolates to resist drought-associated abiotic stress and lesser nutrient availability, as well as the potential to regulate host-osmotic stress. With these results on potential functions of the isolates, we next investigate microbial products from the Dry ecotype associated isolates to provide further insights into crucial microbial metabolites that could enhance dry *A. gerardii* ecotype resilience under drier environmental conditions.

4.5.3 High abundance of antibacterial, communication and plant-host growth promoting microbial metabolites in Dry ecotypic isolates

We detected 2012 microbial metabolites from our cultured microbial consortia. The metabolites were distributed across 23 primary and 61 secondary classifications (Figure 4.4A, Supplementary Table 21A) across both the Dry and Wet ecotype associated cultures. A majority of the metabolites were classified as small peptides (n=699), whereas others were classified as heterocyclic compounds (n=202), benzene and substituted derivatives (n=195), and organic acids and/or their derivatives (n=139). Although we generated these data for both Dry and Wet ecotype cultures, in this study, we were primarily interested in gaining insights into how rhizosphere microbial populations could improve the dry ecotype resilience towards abiotic stress. Our previous work indicated that dry *A. gerardii* ecotype performed better in drier environments because of a *"home-field advantage"* in associating with microbial populations. Alas, we speculated that Dry ecotype associated microbes produce metabolites that could 1) enhance plant host-microbe communication, and 2) convey benefits to the dry *A. gerardii* ecotype. As such, we focused on microbial metabolites that from the Dry ecotype isolates.

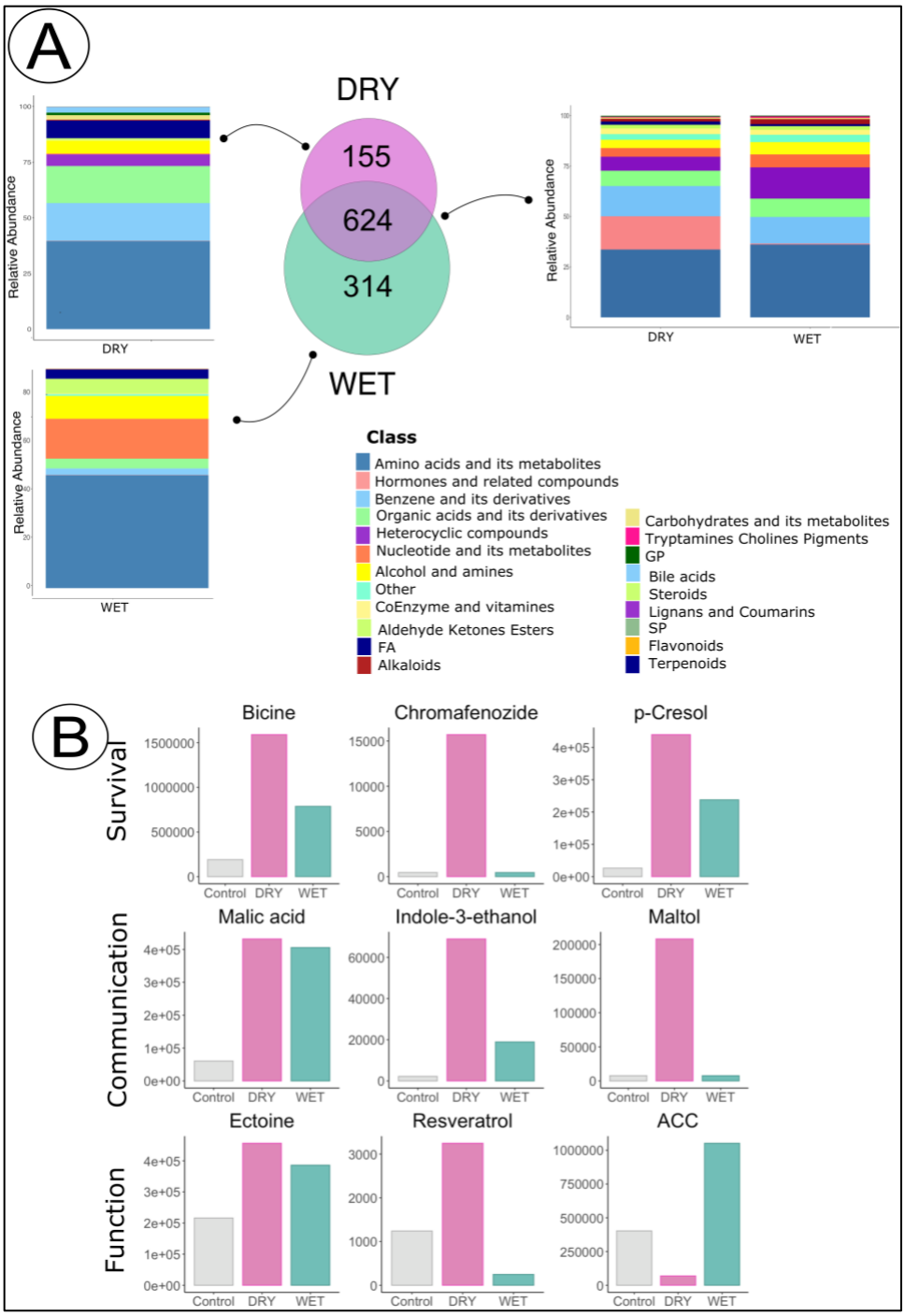

Figure 4.4 Metabolic profiling of the Dry and Wet consortiums. (A) Distribution of microbial metabolites (n=1093) found predominantly in the Dry and Wet ecotype consortiums, but not R2A media control. Venn diagram shows the number of shared and unique metabolites across consortia, and stacked bars show the relative abundance of metabolite classes across those groups. (B) The abundance of metabolites of interests detected across Dry and Wet consortia as well as R2A media control. Metabolites of interest represent critical functions in Dry consortium: survival, communication and function.

We identified 346 metabolites associated predominantly with Dry isolates (Figure 4.4B, Supplementary Table 21B). While majority of the metabolites were associated with basic bacterial cell metabolism or unknown metabolic function, we identified 116 compounds that have the potential for the following crucial functions: 1) bacterial survival in the dry environment; 2) bacterial ability to communicate with the host or other bacteria; and, 3) bacterial provision of beneficial functions to the host and/or other bacteria.

We identified 13 compounds that were associated with promoting microbial metabolism under various environments (Deoxycholic acid, Bicine, 3-Sulfocatechol, 3-Hydroxycinnamic acid and Allantoin) [783] (Figure 4.4C, Supplementary Table 21B). We also observed a high number (n=35) microbial metabolites with antibiotic, antifungal, pesticidal, and herbicidal features (Tritoqualine, p-Cresol, Chromafenozide, Mycophenolic acid). Interestingly, we also observed two metabolites (1-(4-Hydroxy-3-methoxyphenyl)-3-decanone/6-gingerol and EDTA) that could be involved in inhibition of the biofilm formation. The ability of the Dry isolates to produce these metabolites might enhance the survival of the dry ecotype bacterial populations in the drier environment in Hays [784–786].

Besides survival, we also identified various groups of signaling molecules (n = 11) that can facilitate plant-microbe or microbe-microbe chemical communication (Figure 4.4D). These groups included microbial volatile organic compounds (mVOCs) (3-(Methylthio)-1-propanol, Benzyl alcohol, N-(2-Phenylethyl)indomethacin Amide and SPH(d17:1)), allelopathic agents (Pipercyclo Butanamide-A (rel) and 3-Hydroxy-2-methyl-4H-pyran-4-one (Maltol)) as well as secondary metabolites (Inositol 1,3,4-trisphosphate). Combined, these indicate the presence of two or multi-directional communication within and among the bacterial populations as well as with the plant host [787–789]. Interestingly, we observed a higher concentration of the malic acid associated with

the Dry isolates, which is associated with chemotaxis of beneficial bacteria to the plant rhizosphere [112,577]. In addition, we observed a high concentration of 13 indole related compounds in the Dry isolates metabolites including indole 3-lactic acid, indole 3-ethanol, indole-3-phosphate, methoxy indole acetic acid and indole-3-carboxaldehyde (Figure 4.4D). Our results highlighted the ability of the Dry isolates to utilize tryptophan-dependent and -independent pathways, and the production of IAA molecules as a communication signal [774,790]. We surmised that during the plant growth and development, the two-way communication between the dry plant ecotype and its associated rhizobiome is crucial in facilitating the exchange of the metabolic resources, regulation of the plant hormone system, and enhancement of stress tolerance mechanisms [791].

We further found 41 compounds that could provide functional benefits (osmoregulation, lipid and cell wall synthesis, growth promotion and regulation, nitrogen intake, oxidative stress regulation) to the plant host or inter-microbial interactions (Figure 4.4E, Supplementary Table 21B). For example, we found Tetraethylene-glycol, L-Palmitoylcarnitine, Resveratrol metabolites predominantly associated with Dry isolates. These metabolites might be involved in enhancing bacterial and/or plant-host regulation of salt and osmotic stress in the drier environment in Hays [792–794]. Interestingly, we also observed higher Ectoine concentrations in Dry isolates' metabolites than in Wet isolates, suggesting the active utilization of the glycine, serine and threonine metabolic pathways in Dry isolates. Accumulation of ectoine which could potentially mitigate the osmotic stress in and lower it [753]. The lower osmotic stress would thus provide an advantage, and enhance the overall resilience of the host and associated microbes in the dry soil environments [592]. In addition, we observed that Dry isolates had a less 1-Aminocyclopropane-1-carboxylic acid (ACC) than the Wet isolates, suggesting that the ACC deaminase was utilized by the dry ecotypic bacteria. The ACC deaminase utilization may impact the plant host, resulting

in reduced levels of stress-induced ethylene, facilitating root development and enhancing overall resilience of the plant-host to the drier conditions [592,654,761–763].

Taken together, both Dry and Wet bacterial isolates had the potential to positively impact the growth and resilience of the plant-host as well as other microbes through exchange of interaction signals and metabolic products. However, the metabolic profile of the Dry isolates had a distinct potential to provide microbial products to enhance stress tolerance and sustain plant growth in the drier environmental conditions. These findings highlighted that Dry isolates could potentially provide the adaptive strategies for the plant to thrive under water-limited conditions.

4.5.4 Dry cultured bacterial metabolite showed potential of plant-host immune system priming

Inquisitively, we observed high levels of phospholipid Lyso-phosphatidylethanolamine (LPE) (17:1/0:0) in the metabolite profiles of the Dry isolates (Figure 4.5A). Combing through the metabolic pathways of the 159 Dry cultured isolates, we found that 17 isolates (17 - *Acinetobacter venetianus* VE-C3, 1 - *Lysobacter defluvii* IMMIB APB-9 DSM 18482) that could potentially produce Phospholipase A(2) (PLA2), utilizing the Glycerophospholipid metabolism pathway to produce LPE17:1 (Figure 4.5B,C). Phospholipids have a range of biological functions including the main structural component of the cell membrane; as a secondary messengers to regulate the plant growth and development; and in maintaining the cellular responses to change environmental in conditions [795,796]. Phospholipids are also critical in plant-microbe interaction because they stimulate the defense signaling system especially in pathogen pattern recognition. While there is a limited data available on LPE17:1, another plant-host produced similar phospholipid, LPE18:0, is commonly used in agriculture to delay plant senescence while simultaneously accelerating fruit

ripening [797–799]. LPE18:0 can also increase the ROS production in plant cells [800], and interestingly, have a priming effect on the plant immune system which in turn, promotes basal resistance against pathogens [801]. While ROS production is a normal byproduct of bioenergetic processes and sometimes used as a signaling molecule to start the cascade of the plant immune response, high levels of ROS can result in cell death [802,803]. Therefore, whether ROS would serve as signaling molecules or result in oxidative damage depends on the delicate equilibrium between ROS production, and their scavenging [801,804]. LPE18:0-treated plants increased intracellular H_2O_2, favoring the plant immunity over the release of ROS as an immediate defense response [801]. Although LPE17:1 and LPE18:0 differ slightly structurally (Figure 4.5C), we hypothesized that microbial LPE17:1 would similarly have a priming effect on the plant immune system, and therefore have a positive effect on the plant biomass production under abiotic stress conditions.

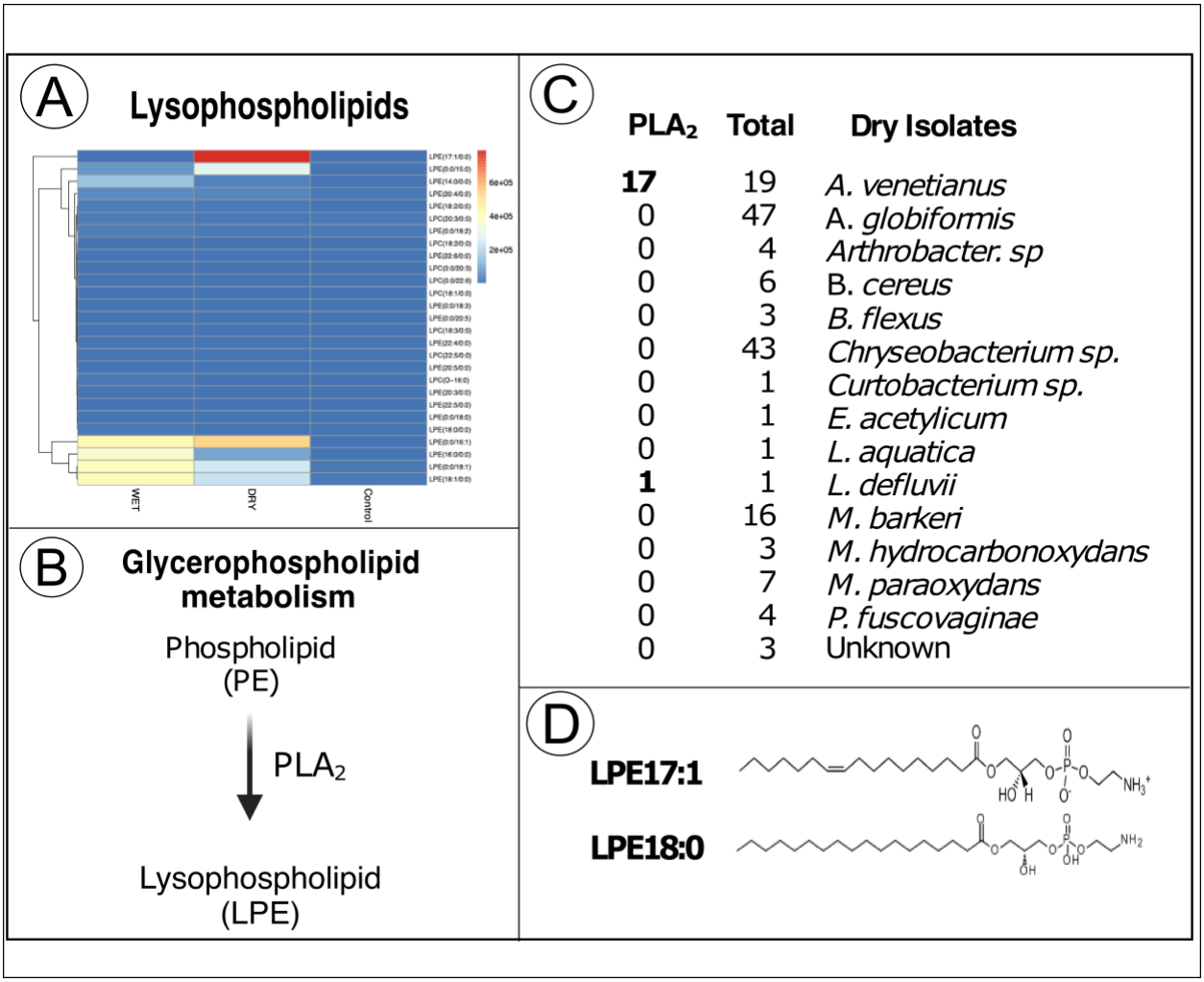

Figure 4.5 LPE17:1 and LPE18:0. (A) Heat map of lyso-phospholipid (Lyso-phosphatidylcholines (LPC) & Lyso-phosphatidylethanolamines (LPE)) metabolites detected across Wet and Dry consortiums. Uninoculated R2A media used for the microbial consortium growth was used as a control. (B) The schematics showing the glycerophospholipid metabolism pathway converting PE to LPE. Phospholipase A2 (PLA2) is the enzyme that removes a fatty acid group, and carries out partial hydrolysis of PE. (C) PLA2 enzyme observed across all Dry consortium isolates (D) The structure of the LPE17:1 and LPE18:0.

4.6 Conclusion

The ongoing global warming and the increasing frequency and duration of drought events create the challenge of maintaining adequate yields in agricultural production systems and putting conservation efforts on the natural landscapes at risk. Improving and developing new management

strategies for the sustainable and eco-friendly approaches to improve plant resilience to environmental stressors are needed. The development of microbial SynComs that focus on the ability of plants to recruit beneficial and functional microbes is receiving increasing attention. Although numerous studies have focused on building SynComs, differences in environmental conditions, plant-host genetic backgrounds, and complex microbe-microbe interactions present challenges in assembling stable SynComs. Combination of these factors indicate that system-specific, yet holistic approaches are needed to successfully assemble microbial communities. We suggest that SynCom assembly should focus on the functions of the community rather than the sum of the features of its individual members. Here, we used full genome sequencing of microbial isolates to assess their functional potential that could benefit the host or other microbes. We then used metabolome analyses of the community to identify the realized beneficial functions, and to pinpoint microbial isolates that possessed the genomic potential for production of these metabolites. We observed LPE17:1 as one of the most abundant metabolites produced by the Dry ecotype isolates. While the exact LPE17:1 function remain unknown, we hypothesize that it could prime the plant-host immune system. Using full genome sequencing, we identified that *Acinetobacter venetianus VE-C3* has the necessary enzyme Phospholipase A(2) to produce LPE17:1 using the glycerophospholipid pathway. While the interactions between *Acinteobacter*-derived LPE17:1 and the plant host, involving in the regulation of stress responsive genes during drought events still need to be elucidated, we suggest that LPE17:1 produced by *Acinetobacter venetianus VE-C3* could be a starting point for assembly of a plant-driven functional SynCom. Further identification of components of the signaling crosstalk between plant hosts and its associated microbes will allow development of novel SynComs to combat the biotic and abiotic stresses.

4.7 Acknowledgements

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4.7 Author Contributions

A.J., S.T.M.L. and A.K. designed the study. A.J., S.T.M.L, E.H., L.J. and AK collected the soil for the microbial consortium. S.S., A.K. and N.R. prepared microbial consortium and extracted DNA with Nanodrop and performed Qubit quality analysis. A.K performed 16S rRNA bioinformatics analysis. Q.R performed oligotyping analysis. A.K., B.A., L.R., H.W., L.H., grew and harvested, extracted DNA with Nanodrop, performed Qubit quality analysis and prepared samples for metabolic analysis of the microbial consortium. A.A., and E.A. adapted Illumina DNA Low Volume Nextera protocol for the full genome sequencing of the single cell isolates. Q.R., S.T.M.L., A.K., and S.T.M.L. performed bioinformatics analysis of single cell sequencing data. B.V., D.T., S.T.M.L., A.K, B.A. and L.R. performed data analysis of the single cells and metabolites. A.K. and S.T.M.L. preferred bioinformatics and analyzed RNAseq data. A.K. and S.T.M.L wrote the manuscript, prepared figures, and supplementary files. A.J. aided in manuscript and figures re-visions. S.T.M.L., L.J. and A.J. acquired funding for this study. All authors read, contributed to manuscript revision, and approved the submitted version.

4.8 Competing Interests

The authors declare no competing interests.

Chapter 5 - Discussion and Conclusion

In this dissertation, I explored the roles of plant-microbe interactions in the local adaptation process of plants and their stress resilience. Specifically, I explored how host genetic background and environmental factors influence rhizosphere microbiome recruitment and functional potential using various techniques, including broad metabarcoding community analysis, multi-omics, and culturomics. In this final chapter, I synthesize the key findings of my research, highlighting how they contribute to closing critical knowledge gaps in the field of microbial ecology. I also outline promising new directions for future research based on questions that emerged from this work.

As discussed in the introductory chapter, many research gaps and unanswered questions surround the complex plant holobiont system. Technological advancements in research tools continue to deepen our understanding of plant-microbe interactions, uncovering new layers of complexity within these relationships [19,805,806]. A central challenge in plant ecology today is understanding the mechanisms that govern these interactions, particularly within the rhizosphere [629,807–809]. Some key questions include: What are the primary driving forces behind microbial recruitment to the rhizosphere? Is microbial recruitment taxon- or function-oriented? What molecular and ecological mechanisms do the host plant and microbes use to shape holobiont interactions? What mechanisms do microbes use to influence plant health, growth, and stress resilience? And how do interactions among microbial members impact the overall structure and function of the holobiont system? Across the three data chapters of this dissertation, I have contributed to addressing several of these questions.

In the first chapter, I investigated the relative importance of local soil and plant genetic background on the rhizosphere community assembly. One of the key findings of my study was that locally adapted ecotypes exhibited a *"home-field advantage,"* recruiting unique local

microbes when growing in their *"home*" environments. Interestingly, ecotype-specific microbial recruitment was most pronounced in the more extreme environments, such as those characterized by consistent drought or water saturation, suggesting a potential co-adaptation between host plants and locally adapted microbial communities [810–812]. Additionally, the results indicated that ecotype-mediated microbial recruitment was not necessarily taxon-specific but may instead be shaped by the functional potential of the microbial community members [556–559,812,813]. Unfortunately, the functional interpretation based on 16S rRNA amplicon sequencing used in this data chapter is inherently limited and largely speculative, relying on assumptions drawn from reference databases and findings from other studies with different experimental conditions or even systems [814,815]. Therefore, while the observed patterns suggest possible functional selection by the host, validating these hypotheses requires more targeted approaches [816].

Despite this limitation, broad community analyses remain a valuable exploratory tool for addressing foundational ecological questions [280,281]. For example, future research could focus on the temporal dynamics in the rhizosphere community. Such temporal data could reveal whether ecotype-specific microbial recruitment strengthens or weakens over time, offering insights into the robustness, stability, and temporal dynamics of plant-microbe associations [817]. In addition, the spatial scale of sampling could be expanded to include a broader range of environments, particularly those with even more extreme drought conditions or the geographic margins of *A. gerardii'*s distribution [741,818–820]. It would be particularly informative to assess whether the observed *"home-field advantage"* and selection by host plants remain consistent or strengthen with more extreme conditions, or whether these relationships break down at a certain point. Exploring these questions at both temporal and spatial scales could provide a more comprehensive

understanding of the ecological and evolutionary factors that shape plant-associated microbial communities [821].

While these broader ecological questions are compelling, my interest was in the mechanisms underlying functional selection and the role of the observed *"home-field advantage"* in microbiome recruitment. Specifically, I was interested in the following question: What roles do locally recruited microbes play in enhancing host performance in their native environments? Through what mechanisms do plants select beneficial microbes? What genes and metabolic products are responsible for this recruitment? Is *"home-field advantage"* recruitment triggered primarily by environmental stress, or does it represent a deeper co-adaptive relationship between hosts and microbes? Do local microbial communities evolve traits that improve their compatibility with local hosts? These questions lie at the heart of understanding how plant-microbe interactions contribute to the host's adaptation and resilience in challenging environments.

To investigate some of these questions, I conducted metagenomic sequencing with subsequent gene- and genome-centric analyses in the second data chapter to characterize the functional potential of rhizosphere microbial communities associated with different *A. gerardii* ecotypes. Notably, the 16S metabarcode analyses described in the first data chapter provided valuable insights into the rhizosphere community composition, which guided our sample selection for the metagenome sequencing described in the second data chapter, thereby allowing for improved metagenomic coverage. The broad gene-centric approach provided a comprehensive, community-level overview of functional potential by identifying and quantifying genes across metagenomic short reads [669,670,822]. Using the gene-centric approach, I also profiled the taxon content [668,670].

In contrast, using the genome-centric approach, I assembled metagenome-assembled genomes (MAGs), which enabled the recovery of genomes of individual microbial populations [678,692]. This method provided insights into which specific microbial population possessed particular functional traits [692]. In parallel, I conducted metabolomic analyses of the rhizosphere to identify plant metabolic outputs that may be involved in regulating plant-microbe interactions [372,576]. These data suggest that in drier environments, plants appeared to preferentially recruit microbes with functional traits related to stress tolerance, nutrient acquisition, and environmental survival, potentially contributing to the reduced number of stress-related metabolites. One key finding was a positive correlation between lower plant metabolite trimethyllysine (TML) levels and the abundance of five MAGs, particularly in dry ecotypes growing under arid *"home"* conditions. Although the role of TML in plants remains poorly understood, I hypothesized that it is associated with stress responses [651,656]. Reduced TML levels may indicate lesser stress in locally adapted plants, potentially due to associations with *"unique"* beneficial microbes and their active contribution to host stress mitigation [651,656]. For example, MAGs detected in the dry environment had a variety of genes potentially involved in converting various nitrogen substrates into ammonia, supporting their potential role in facilitating host nutrient uptake and overall resilience to harsh conditions. Alternatively, these microbes may directly metabolize TML, converting it into downstream compounds such as carnitine, trimethylamine (TMA), or trimethylamine N-oxide (TMAO) [823]. Although similar to TML, the function of these compounds in the plant system is not well studied. Emerging evidence suggests they may improve stress tolerance [651,652,656,824]. Further investigation into these microbial pathways and plant responses could shed light on novel mechanisms of microbially mediated plant adaptation to environmental stress.

Like any study, this work has its limitations, and it is essential to acknowledge them. I wish to highlight three. First, I conducted metagenomic sequencing on 24 rhizosphere samples. Yet, I was only able to assemble 34 non-redundant MAGs. This low MAG recovery reflects the high overall diversity, within-taxon heterogeneity, and lack of comprehensive reference genomes in current databases for soil microbiomes, all of which complicate accurate MAG reconstruction [825–831]. Although MAGs offer valuable insights into the functional potential of specific microbial populations, it is essential to consider whether a relatively small number of MAGs supports robust ecological conclusions in an inherently complex soil or holobiont system [832,833]. Gene-centric analysis, on the other hand, provides a broader community-level view, but can lack specificity [822]. These limitations highlight the importance of carefully selecting methodological approaches to address research objectives.

Second, conclusions about causal feedback between the host and its microbiome remain largely speculative. For example, although metabolomic analysis revealed correlations between low levels of the plant-derived metabolite TML and the abundance of five MAGs, the current data do not definitively determine the source, fate, or functional role of TML in the plant-microbe system. Future research should investigate the mechanisms underlying TML production and transformation. How does TML biosynthesis respond to varying levels of environmental stress? Do microbial communities associated with the *"home-field advantage"* utilize TML directly, or do they influence its exudation by the host? Addressing these questions is critical for understanding whether TML actively mediates plant-microbe interactions or simply reflects broader shifts in plant physiological status.

Finally, the first two data chapters are based entirely on sequencing and computational analyses. While these approaches are powerful for generating hypotheses and identifying patterns,

they are inherently limited by the absence of direct experimental validation [386]. Of course, the transition from computational to empirical experimentation has its challenges, but they are necessary to validate hypotheses generated based on the computational findings. For example, when using my data, I was able to predict that some genes are involved in stress-related pathways or that specific metabolites may influence recruitment. However, without direct biochemical or physiological evidence, these interpretations remain speculative [834,835]. I believe that metagenome sequencing technologies and analytical tools will continue to advance, and the integration of gene- and genome-centric approaches will become more accessible and practical. For now, thoughtful sample selection, careful experimental design, a clear understanding of each tool's limitations, and realistic, well-defined objectives are essential for the proper utilization of metagenomic data in holobiont studies.

Despite these limitations, it is also essential to mention the endless potential of metagenomics, metabolomics, and multi-omics. For example, while in my second data chapter I used metagenome analysis to focus on the bacterial communities, the main strength of shotgun metagenome sequencing is that it captures the complete genomic content of a sample, extending beyond bacteria to include archaea, fungi, protists, viruses, plasmids, and even recently described elements like borgs [558,816,826,836–840]. This approach opens the door for exploring the multi-kingdom interactions within the holobiont system.

In the final data chapter, I explored a validation of my metagenomic findings through a culturomics approach. The overarching aim of this work is to harness the potential of the plant holobiont to enhance crop productivity through the development of plant-oriented synthetic communities (SynComs). My final data chapter focuses on the first foundational steps of this process: culturing, identifying, and functionally characterizing rhizosphere microbes and their

associated metabolic products. By physically isolating these microbes using a self-assembly method, evaluating their functional potential, and linking them to specific metabolic products, particularly those potentially involved in drought stress management, I investigated the relationship between the genetic potential of microbial isolates and their realized metabolic outputs [324,841]. One key finding of this study was the high abundance of the microbial metabolite LPE 17:1 in the consortium from the dry environment. LPE 17:1 is a type of lipid present in plant cell membranes. Although further research is needed to address the specific function of LPE 17:1, I hypothesized that it may play a role in priming the plant immune system and potentially contribute to enhanced stress resistance [727,728,799,801]. This finding raises several important questions for future research: What is the specific role of LPE 17:1 in plant stress response pathways? What triggers the production of LPE 17:1? How does microbial LPE 17:1 influence the host? What microbial genes are responsible for the biosynthesis of LPE 17:1, and which plant genes mediate its recognition and response? Moreover, given that LPE 17:1 is a relatively large lipid molecule with a 17-carbon chain, typically embedded in plant membranes, how does a microbially produced LPE interact with the host? What mechanisms allow it to move through the plant cell barrier or signal across the membrane to influence intracellular responses?

Additionally, future research should aim to identify both plant and microbial receptors involved in these interactions, as well as the signaling pathways that regulate them. From the microbial perspective, I think it would be exciting to investigate *"sender"* and *"receiver"* channels of chemical signals involving acyl-homoserine lactones (acyl-HSLs) [842–846]. Acyl-HSLs are central to quorum sensing in many bacteria and recognized as key mediators of inter-kingdom communication [843,846,847]. They are critical in establishing and maintaining symbiotic relationships, particularly with gram-negative bacteria, by modulating plant gene expression,

immune responses, and root colonization behavior [843,846,847]. Biofilms on root surfaces are typically rich in acyl-HSLs, highlighting the dense and active communication networks in the rhizosphere. For example, among the cultured bacteria, I observed multiple isolates with acyl-HSLs and biofilm-producing genetic potential, suggesting their potential involvement in quorum sensing and the establishment of long-term host colonization [844]. These findings raise important questions for future research: Is there a predominant type or profile of acyl-HSL signals produced by microbial communities from wet versus dry environments? Is developing the understanding of the special acyl-HSL signals a part of the host's *"home-field advantage?"* Which isolates are most effective at producing biofilms under drought stress conditions, and what genetic or physiological traits contribute to this ability? What environmental or host-derived cues trigger and regulate acyl-HSL production? How do specific acyl-HSL signals interact with plant receptors to modulate host responses? Is there a structured signaling pattern that emerges within complex, multispecies rhizosphere communities? And finally, is biofilm formation by key microbes essential for the successful assembly, stability, and cooperative function of a SynCom community designed to support plant health?

The biofilm formation could be extended to exploring rhizosheaths. Rhizosheaths are aggregates of soil particles bound to roots by mucilage and root hairs [848]. Like biofilms, rhizosheaths create microenvironments enriched with plant and microbial exudates that facilitate microbial adhesion, communication, and survival [849]. These conditions suggest that rhizosheaths may also confer a selective advantage to native microbial communities [59,850]. Interestingly, rhizosheaths are prevalent in arid and semi-arid ecosystems, where prolonged droughts can suppress the abundance and activity of rhizosphere microbial communities [849–851]. Given their structural and functional parallels to biofilms, rhizosheaths represent a

compelling avenue for future research. Some future research directions include: Do rhizosheaths preferentially recruit native or function-specific microbial taxa? And how do their structure and the overall functional potential shift across precipitation gradients?

Finally, future studies should explore plant–microbe interactions and the more complex networks of plant–microbe–microbe and microbe–microbe relationships [345]. These multi-species interactions likely play an essential role in shaping plant immunity, stress resilience, and overall plant health. For example, one intriguing outcome of my thesis research was the isolation of a weak plant pathogen from dry consortium soil. This raised some interesting questions I would be interested in addressing: could the controlled introduction of such weak pathogens, as part of a microbial consortium, benefit the holobiont system by activating the host immune system? If so, can this activation enhance the host's ability to recruit beneficial microbes more effectively or more selectively? Does the presence of a weak pathogen alter the metabolic activity, gene expression, or colonization behavior of neighboring microbes or the host? Finally, how does spatial organization, such as the physical proximity of weak pathogens to beneficial microbes on the root surface, affect the function of the community?

Over the course of this thesis research, I have gained many valuable insights through the use of multi-omics approaches. One of the key strengths of these methods I wish to point out is the remarkable flexibility and reusability of -omics datasets. While my research focused on the functional potential of microbial communities, specifically their overall survival strategies, metabolic functions, and communication, my dataset offers countless opportunities for further exploration in a wide range of directions. For instance, beyond my core research focus, I also detected an abundance of various genes related to antibiotic production and resistance [852–855]. These findings raise compelling questions for future investigations using this dataset with a focus

on antibiotic production-related genes. The presence of these genes may not be incidental; rather, they may reflect ecologically important processes. Do these genes differ systematically between ecotypes or locations? Could plant microbial recruitment reveal a defense strategy involving microbes with antimicrobial capabilities? Understanding such patterns could illuminate how microbial communities are shaped by competitive interactions and environmental pressures, as well as their contribution to host defense. Is there evidence of microbial selection for specific antimicrobial compounds that supports the *"home-field advantage"* hypothesis? This line of inquiry may reveal how certain microbial traits confer fitness benefits in specific contexts, potentially influencing community assembly and stability. Ultimately, these insights could have applications not only for ecological theories but also for agriculture, biotechnology, and conservation, especially in the development of microbiome-informed approaches to plant health and soil management.

Building on the findings of this dissertation, several exciting research avenues could further advance our understanding of plant-microbe interactions and their potential to enhance plant resilience. These future directions could involve addressing new questions outlined in this thesis and expanding upon the topics discussed throughout this dissertation. My findings advance our understanding of the complex plant holobiont system and pave the way for future studies exploring how these relationships can be harnessed to enhance plant performance in the face of environmental challenges, particularly those driven by climate change.

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Appendix A - Supplementary Tables

Table 4.1 Abbreviation key columns: *Ai - Acinetobacter indicus; Av - Acinetobacter venetianus; As - Agromyces subbeticus; Ag - Arthrobacter globiformis; Ar - Arthrobacter sp; Bc - Bacillus cereus; Bf - Bacillus flexus; Cel - Cellulomonas sp.; Chr - Chryseobacterium sp.; Cur - Curtobacterium sp.; Ean - Elizabethkingia anopheli; Eac - Exiguobacterium acetylicum; La - Leifsonia aquatica; Lf - Lysinibacillus fusiformis; Ld - Lysobacter defluvii; Mb - Microbacterium barkeri; Mh - Microbacterium hydrocarbonoxydans; Mp - Microbacterium paraoxydans; Pf - Pseudomonas fuscovaginae; Unk - Unknown (no taxonomic assignment)*

Abbreviation key rows: Overall - all draft genomes isolated; L-Ectoine - L-Ectoine production; EnvZ - Osmolarity sensory histidine kinase EnvZ system; OmpR - Two-component system response regulator OmpR; ACC deaminase - 1-aminocyclopropane-1-carboxylate deaminase enzyme; AHLs - N-acyl-l-homoserine lactone hydrolase enzyme; pgaABCD - complete pgaABCD operon; QQ - Quorum-Quenching genes; IPDC - Indole-3-pyruvate decarboxylase enzyme; NIT - Nitrilase enzyme; Plant-induced NIT - Plant-induced Nitrilase enzyme; NHase - Nitrile hydratase enzyme; IGPS - Indole-3-glycerol phosphate synthase enzyme.

Supplementary Table 1. Locations of seeds and populations collected from *Andropogon gerardii*.

Supplementary Table 2. Samples names and details for the soil cores collected for this study.

Supplementary Table 3. Raw sequence analysis by QIIME 2 Version 2019.7. The number of counts of bacteria and fungi initially obtained, and the counts that were considered after primer trimming and DADA2 quality control per sample. Bacterial identifications along with the number of counts per sample.

Supplementary Table 4. Soil chemistry parameters and analysis (pH, %C, %N, water content, and soil texture) across all sites (Hays, Manhattan and Carbondale).

Supplementary Table 5. Indicator taxon analysis on different locations (irrespective of ecotypes) - Hays, Manhattan and Carbondale.

Supplementary Table 6. Unique taxa for locations and ecotypes.

Supplementary Table 7. Indicator taxon analysis on ecotypes (irrespective of locations) - Dry, Mesic and Wet.

Supplementary Table 8. Indicator taxon analysis on locations by individual ecotypes.

Supplementary Table 9. Indicator taxon analysis on ecotypes by individual locations.

Supplementary Table 10. Genus-level oligotyping analysis

Supplementary Table 11. Description of the *Andropogon gerardii* rhizosphere collection samples used for the metagenome and metabolomics analysis

Supplementary Table 12. Metabolomic analysis results include the overall analysis, analysis by location (Carbondale and Hays), ecotype (Dry and Wet), population (DES, FUL, WEB, and CDB), and location and ecotype interactions.

Supplementary Table 13. Gene-centric community profiling (MetaPhlAn), including the overall output and the subset resolved to the genus level analysis by location (Carbondale and Hays), ecotype (Dry and Wet), and location and ecotype interactions.

Supplementary Table 14. Gene-centric metabolic profiling (HUMAnN) including the overall output and analysis by location (Carbondale and Hays), ecotype (Dry and Wet), population (DES, FUL, WEB, and CDB), and location and ecotype interactions.

Supplementary Table 15. Shotgun metagenome sequencing data obtained from the 24 rhizosphere samples used for the gene- and genome-centric analysis

Supplementary Table 16. Description of the 34 MAGs with the ANVI'O taxonomic assignment.

Supplementary Table 17. The subset of genes/subsystems with the function to promote plant host growth or communicate with plant host growth or other microbes observed in the 34 MAGs.

Supplementary Table 18. Locations of soil cores collected for the preparation of the microbial consortium.

Supplementary Table 19. A) The total number of sequences after primer trimming and DADA2 quality control per sample, the bacterial annotation, and counts of amplicon-sequence variances (ASVs) per sample. B) Genus-level oligotyping analysis for *Acinotobacter* and *Pseudomonas.*

Supplementary Table 20. A) Single cell sorting results from the consortium. B) Single cell sequencing and assembly statistics of each draft genome. C) Similar genomes downloaded from BV-BRC that were used for phylogenomics analysis.

Supplementary Table 21. A) All list of microbial metabolites found in the Dry, Wet consortium and R2A broth control. B) List of the microbial metabolites predominantly associated with Dry isolates, sorted by the role of interest following crucial functions: survival, function, and communication.

Appendix B - Permissions

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