Community Assembly and Stress Response of Grassland Phyllosphere Bacteria

Emily Bechtold

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Community Assembly and Stress Response of Grassland Phyllosphere Bacteria

A Dissertation Presented

By

Emily K. Bechtold

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements of the degree of

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Department of Microbiology
Community Assembly and Stress Response of Grassland Phyllosphere Bacteria

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Emily K. Bechtold

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Grasslands are an important ecosystem with potential to help stabilize food security and reduce greenhouse gas levels. As global temperatures rise, weather patterns are predicted to drastically change. The resulting increase in intensity, duration, and frequency of drought in important grassland areas will not only affect agricultural production, but also increase grassland susceptibility to fire, disease, and soil erosion. Thus, developing ways to sustainably promote grassland health and production is essential to increase food security and reduce environmental strain. Microbes in the phyllosphere, or aerial surface of plants, promote host fitness through phytohormone and nutrient production, increased stress tolerance, and protection against pathogens. However, what drives phyllosphere community assembly is not well understood. Because plant-health is reliant on microbial communities, understanding the ecological impacts of climate change on plant-microbe relationships is important to develop strategies to counteract the associated negative effects. The effects of drought on microbial community assembly on tropical and temperate grass hosts is described in this dissertation through four different research studies. We found that phyllosphere communities on the leaf
surface are dynamic and are strongly selected for by their host species, that selection increased throughout the experiments driving communities to become more distinct from each other over time, and we found that microbial communities were significantly impacted by drought stress. Microbial communities responded to drought stress before measured plant traits showed a response to stress. Additionally, we found that changes in microbial community structure correlated with traits and stress response strategies of the host. Furthermore, we found that phyllosphere communities fix nitrogen and that this process is stable over time and under the stress condition of water reduction. Understanding the roles of phyllosphere bacteria in plant health and global biogeochemical cycles will allow us to leverage plant-microbe relationships to promote sustainable farming practices and reduce greenhouse gas emissions in the future.
PREFACE

Agricultural production, through crops and grazing, is key to global food security. Grasslands are an important ecosystem with potential to help stabilize food security and reduce greenhouse gas levels by sequestering and storing carbon. Current agricultural intensification and expansion practices have taken a serious toll on the environment, causing degradation of land, water, and biodiversity while increasing greenhouse gas emission. As global temperature continues to rise, weather patterns are predicted to drastically change increasing intensity, duration, and frequency of drought in important grassland areas over the next century. This will not only affect agricultural production, but also increase susceptibility to fire, disease, and soil erosion which can all impact carbon cycling. Thus, developing ways to sustainably promote grassland health and production is essential to increase food security and reduce environmental strain.

The phyllosphere, or aerial surface of plants, is estimated to cover over $10^9$ km$^2$ and contain $10^{26}$ bacterial cells, making it one of the largest microbial habitats on earth. Microbes in the phyllosphere promote host fitness through phytohormone and nutrient production, increased stress tolerance, and protection against pathogens. Due to temperature fluctuations, changing precipitation patterns, and UV exposure, the phyllosphere is an extreme habitat for microbes. Despite these harsh conditions, phyllosphere communities exhibit strong seasonal and temporal patterns. Previous studies of tree phyllospheres identified host species as an important driver of leaf microbial community structure, but recent work in herbaceous plants found that microbial community structures were similar across host species. These differing
trends suggest phyllosphere community assembly is not a completely stochastic process, and what drives assembly is not well understood. Because plant-health is reliant on microbial communities, understanding the ecological impacts of climate change on plant-microbe relationships is important to develop strategies to counteract the associated negative effects. The effects of drought on microbial community assembly on tropical and temperate grass hosts is described in this dissertation through four different research studies.

In the first study, phyllosphere community assembly and response to drought stress was assessed on three tropical and two temperate forage grass species grown under optimal conditions in a greenhouse. Plants were subjected to well-watered control, mild drought, and severe drought conditions over a course of 22 days (tropical grasses) or 35 days (temperate grasses). Using 16S rRNA sequencing, studies showed Gammaproteobacteria was the dominant class of bacteria on both tropical and temperate hosts and dominance of these bacteria increased under severe drought conditions while overall community diversity declined. Bacterial community diversity, structure, and response to drought were significantly different between grass species and these species differences became more defined under stress. Furthermore, community structure was significantly correlated with plant host traits such as chlorophyll, relative water content, and plant growth. These findings raise questions about symbiotic evolutionary relationships between plant hosts and their associated microbial communities that could be further explored by testing a larger number of host species and
looking at how a larger range of host traits such as metabolites, leaf structures, and leaf physiology correspond to microbial community composition and function.

In the second study, phyllosphere community assembly and response to drought stress was assessed on three tropical and three temperate forage grass species grown under modified field conditions. Plants, grown in pots, were subjected to well-watered control or severe drought conditions over a 38-day period. 16S rRNA sequencing revealed host species imposed strong selection on community assembly processes. Additionally, we found evidence of phylosymbiosis which increased significantly under drought stress, indicating phyllosphere communities and their response to stress relate to grass species phylogeny. Furthermore, native temperate grasses displayed stronger cophylogenetic relationships between grass hosts and their microbial communities and had increased selection by host species over time compared to non-native tropical hosts. In addition to 16S rRNA community analysis, abundance of the functional marker gene *nifH* was assessed using quantitative PCR (qPCR). Results showed differential abundance across host species, but showed no changes as a result of drought. We thus concluded that host identity and provenance impacts phyllosphere community assembly and response to drought.

In the third study, results from the greenhouse study were compared to results from the modified field study for two temperate host species to understand if the environmental setting was influencing community assembly processes. Plants in the two studies were grown in the same soil and drought stress was imposed for a similar amount of time.
Microbial communities were assessed through 16S rRNA sequencing and qPCR of the \textit{nifH} gene. Microbial community profiles were markedly different at the class taxonomy level, with field samples dominated by \textit{Alphaproteobacteria} and greenhouse communities dominated by \textit{Gammaproteobacteria}. Moreover, host species had a greater effect on community structure in the field than in the greenhouse, while the impact of drought on community structure was stronger in the greenhouse environment than in the field environment. Additionally, network analysis revealed that microbial communities from field grown plants exhibited stronger selection and were more stress-tolerant than greenhouse communities. We concluded that environmental differences had a strong effect on community assembly and response to drought in this study, evident by the observed differences in community structure and stability.

The fourth study focused to assess community stability by determining composition and functional resistance and resilience. Resistance is the degree to which communities remain unchanged as a result of disturbance and resilience is the ability of a community to recover to pre-disturbance conditions. The three species of temperate grass were planted in the field where they were exposed to either 10 weeks of no watering followed by three weeks of watering or kept under well-watered conditions for the 13-week period. Compositional stability was assessed using 16S rRNA gene sequencing and functional stability was assessed for two traits, nitrogen fixation and bacterial biomass production. Additionally, processes of community assembly were assessed using ecological null modelling. This method measures the effect of stochasticity and determinism (selection) in shaping microbial communities. In this study, phyllosphere
compositional stability was highly related to plant host species phylogeny and, to a lesser extent, known stress tolerances. Similarly, we found that specific community assembly processes were different between the host species, but that each host species was governed by undominated and stochastic processes with only minor levels of deterministic assembly. However, functional stability was observed across each host species. Phyllosphere community assembly and stability is a result of complex interactions of ecological processes that are differentially imposed by host species.

Overall, these findings demonstrate that plant hosts play an important role in shaping microbial community composition and that this effect amplifies during disturbance events. Furthermore, certain community functions appear to be more stable than community composition. Next steps should include further analysis of community functionality both under normal and stressed conditions, and investigation of direct interactions between microbes and their plant hosts. Climate change will continue causing widespread disturbance events across many ecosystems. Understanding the effects these disturbance events will have on microbial communities and understanding how microbial communities impact plant and ecosystem health could be used to improve plant and ecosystem health and productivity in the face of changing climates.
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species based on NCBI taxonomy [1] shows that *B. decumbens* and *B. brizantha*
are more closely related to each other than they are to Orchardgrass (*Dactylis glomerata*) and Tall Fescue (*Festuca arundinacea*) and orchardgrass and tall fescue are to each other. The cladogram was generated using phyloT and visualized with iTOL [2].

Figure 3.1. Average relative abundance of bacteria from 57 plants (27 control and 30 drought) sampled at 5 separate time points over 38 days. (A) The most dominant bacterial classes changed over time, between host species, and as a result of drought. To understand the composition of these classes, the average relative abundance of the genera from the three most abundant classes were plotted. Genera included were present in greater than 0.25% average relative abundance. At the end of 38 days when drought effect was strongest, we observed significant differences as a result of drought in *Actinobacteria* (*P*<0.001), *Bacilli* (*P*=0.006), and *Cytophagia* (*P*=0.001) (calculated using TukeyHSD). Additionally, strong differences were observed between host species with significant differences observed in *Actinobacteria* (*P*<0.001), *Alphaproteobacteria* (*P*<0.001), *Bacilli* (*P*<0.001), *Betaproteobacteria* (*P*<0.001), *Cytophagia* (*P*<0.001), *Deltaproteobacteria* (*P*=0.001), and *Gammaproteobacteria* (*P*=0.008) (B) The class Alphaproteobacteria was dominated by the genera *Sphingomonas* and *Methylobacterium*, (C) Gammaproteobacteria was not consistently dominated by any individual genera, and (D) the class Cytophagia was dominated by the genus *Hymenobacter*.

Figure 3.2. Bacterial communities from each host species became more distinct over time and were significantly impacted by drought stress. NMDS ordination was plotted for each sampling day using weighted UniFrac distances. PERMANOVA was conducted for each corresponding day to determine how communities were changing over time and when drought stress altered community structure.

Figure 3.3. Phyllosphere communities became more similar over time regardless of treatment on the temperate host species but stayed the same on the tropical host species. Average distance between samples from the same host species were calculated for each sampling day using weighted UniFrac distance. Average distance within each host species significantly decreased in the communities from temperate hosts but did not change in the communities from the tropical hosts.

Figure 3.4. The top eight ASVs important for predicting if samples were from control or drought stressed plant hosts. Average relative abundance of each of the eight ASVs is given for control (n=27) and drought stressed plants (n=30) on day 38 of the experiment. ASV identities provided in Table 3.

Figure 3.5. Cophylogenetic relationship analysis was conducted for (A) control plants (n=27) and (B) drought stressed plants (n=30). Blue lines in this tanglegram represent significant associations between phyllosphere bacteria on the left and their plant hosts on the right measured using ParaFitGlobal, which
were determined if either of the ParaFit F statistics were below 0.05. Numbers under host species identity indicate the number of significant associations that a host species has with the bacterial phylogenetic tree. The bacterial phylogenetic tree was constructed in QIIME2 using FastTree which infers approximately-maximum-likelihood phylogenetic trees. The maximum-likelihood tree for the grass host phylogeny was constructed in MEGA. Only five grass species were included because host sequence information was not available for the *Brachiaria* hybrid.

Figure 3.6. Abundance of the *nifH* gene was significantly different between host species and changed over time. However, it was not significantly impacted by drought stress. *nifH* abundance was measured using qPCR and standardized to number of copies per gram of leaf material.

Figure 3.7. Pots were organized in a randomized block design. Half the plants were kept under rain shelters to induce drought and the other half were in the open and their soil moisture was maintained above 80% field capacity.

Figure 3.8. Alpha diversity of phyllosphere communities did not significantly change as a result of drought stress. Alpha diversity was different between host species and varied over time. Alpha diversity was measured using Shannon Diversity Index, which takes into account both abundance and evenness of species present. We fit an interactive GLMM with treatment, time, and host species as fixed effects and sample ID as a random effect.

Figure 3.9. Average distance between samples from each host species were calculated for each sampling day using weighted UniFrac distance. Average distance within each host species significantly decreased in the communities from temperate hosts but had varying trends from each tropical host.

Figure 3.9. Bacterial community changes over time and as a result of drought on each species were determined using a PERMANOVA of weighted UniFrac distances (G-L) and visualized using NMDS (A-F). Images of control plants next to plants exposed to drought treatment at the end of the experimental period are given in figures M-R.

Figure 3.10. Machine learning using a random forest model resulted in high predictive power for determining if communities were from control or drought stressed plants. Any points occurring at levels higher than 0.5 in the area under the receiver operating characteristic curve (AUROC) indicates model prediction was better than by chance. Cross-validation and Test data (AUROC of 0.87) resulted in similar AUROC values indicating the model had high predictive power.

Figure 3.11. Two bacterial species were highly important for accurately predicting if a community came from a tropical or temperate host species on the
last day of drought. *Methylobacterium organophilum* occurred at a higher relative abundance on tropical grasses compared to temperate grasses. *Sphingomonas mali* was present in low relative abundances on tropical grasses, but it was not detected on any temperate grass host.

Figure 3.12. Average relative abundance of genera belonging to the class Betaproteobacteria over the 38-day experimental period for each control and drought stressed plant host species. The host plant *Brachiaria decumbens* had a significant increase in the genus *Massilia* under severe drought conditions, which is known to have nitrogen fixation potential [3].

Figure 4.1. Plant health measurements displayed similar trends as a result of drought stress in the field and the greenhouse. (A) Leaf relative water content (RWC) decreased as a result of drought and (B) electrolyte leakage increased under drought stress.

Figure 4.2. Alpha diversity of the phyllosphere community was significantly impacted by drought in the greenhouse but not in the field. The diversity measure shown here was calculated using Faith’s phylogenetic distance (PD). Asterisks indicate significant differences between the control and drought treatment (significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Figure 4.3. Average relative abundance of the dominant phyllosphere bacteria collected over the experimental period. Asterisks on the right indicate significant differences between the treatments based on relative abundance of all sample days and treatments. Green asterisks indicate significant differences between the greenhouse and field environment for orchardgrass, and yellow asterisks indicate significant differences of phyllosphere communities for tall fescue. Changes in class by environment were tested using an ANOVA followed by a post hoc Tukey multiple comparison test. Additional comparisons for treatments and days are given in Table S1. Significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 4.4. (A) *Sphingomonas* and (B) *Methylobacterium* had higher relative abundance in the field compared to the greenhouse. Average relative abundance of each genus was plotted over time and by watering treatment for each species in the greenhouse and in the field.

Figure 4.5. Host species was a stronger driver of community structure in the field environment (A) compared to the greenhouse environment (B). Nonmetric multidimensional scaling (NMDS) ordination was based on weighted UniFrac distances of all 94 field samples and 70 greenhouse samples. Points are colored based on host species (green for orchardgrass; yellow for tall fescue). Ellipses represent 95% confidence intervals around samples.
Figure 4.6. Network and occupancy analysis revealed more stable phyllosphere communities in the field compared to the greenhouse. (A-D) Network analysis showed that field communities were more complex than greenhouse communities, had a higher ratio of negative to positive interactions, and were more able to withstand drought stress. A connection indicates a significant interaction (p<0.05) between two ASVs determined by calculating the Spearman correlation. Node size corresponds to the number of connections, or degrees. (E-H) Occupancy analysis using presence/absence data showed higher overlap between control and drought treatment plants in the field compared to the greenhouse. Value is the percent overlap between the treatment groups on each species.

Figure 4.7. Abundance of the *nifH* gene was significantly different between environments. *nifH* abundance was measured using qPCR and standardized to number of copies per gram of leaf material. Significance levels are assigned as p > 0.05 (not significant); *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

Figure 4.8. Total aboveground plant biomass showed similar decreasing trends as a result of drought stress in both greenhouse and field experiments. Biomass was destructively measured at the end of the experimental period by removing all above ground plant material, drying it at 70 °C and then determining mass.

Figure 4.9. Differences in microbial community dynamics between the field and greenhouse environments were determined by the effect of environment, drought, and time on alpha diversity. Alpha diversity was measured using (A) richness or total number of ASVs and (B) Shannon diversity index.

Figure 4.10. Comparison of genera from three of the dominant classes found in the greenhouse and field environments to understand differences in community structure. Graphs depict the average relative abundance of bacteria from (A – C) 20 plants (10 control and 10 drought) sampled at 5 separate time points over 38 days grown in the field and (D – F) 19 plants (9 control and 10 drought) sampled at 4 separate time points over 36 days grown in the greenhouse. * F indicates family level resolution in graph C and F.

Figure 4.11. Host species had a stronger influence on community structure in the field compared to the greenhouse, and greenhouse communities were more influenced by drought stress. NMDS ordination was plotted using weighted UniFrac distances on samples collected from the last day of drought treatment when drought effect was strongest.

Figure 5.1. Phyllosphere community resistance and resilience in response to water-reductions stress on temperate forage grass hosts was measured during a 13 week field experiment.

Figure 5.2. Plant hosts showed few measured changes during the 10-week water reduction period despite significant decrease in soil moisture in each of the 3 host
species (A). Plant measurements taken throughout the experimental period include (B) relative water content, (C) relative chlorophyll content, and (D) electrolyte leakage. (E) Proline content was measured at the end of the water reduction period (WRP) and at the end of the recovery period (RP). (F) Root mass was measured at three different depths at the end of the experimental period before re-watering occurred.

Figure 5.3. Microbial community diversity was measured for each species and treatment over the experimental period using species (A) richness and (B) evenness. Community evenness on tall fescue hosts was the only alpha diversity metric to show a significant response to water reduction (p=0.001). Differences in diversity levels between host species is represented by the lower-case letters at the top of each plot. Significant differences are represented by different letters and no difference is represented by the same letter. Trends over time were determined using linear models. R^2 values are represented at the bottom of the plots for significant trends. N.S., not significant.

Figure 5.4. Relative abundances of bacterial classes were significantly different between the plant host treatments and had different responses to water reduction. Communities from tall fescue and ryegrass hosts were more similar to each other than they were to orchardgrass. The red triangle separates the water reduction period from the recovery period. Legend column A represents classes from orchardgrass hosts that were significantly different from both ryegrass and tall fescue. B represents classes that were significantly different between ryegrass and fescue in both control and water reduction treatment. C represents classes that were different between ryegrass and tall fescue water reduction treatment but not the control treatment. Significance levels are assigned as p > 0.05 (not significant); *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

Figure 5.5. Bacterial communities from each host species became more distinct over time and were significantly impacted by water reduction stress. NMDS ordination was plotted using Bray-Curtis distances for (A) the beginning of the experimental period (week 1), (B) early water reduction period (week 3), (C) late water reduction period (week 9), and (D) end of the recovery period (week 13). (E) PERMANOVAs were conducted for each corresponding period to understand community stability by determining when communities under disturbance showed signs of change and if they were able to recover from the disturbance event.

Figure 5.6. Community assembly processes were different on each of the different host species, but all three had high levels of undominated processes. Boxplots show the βNTI values (A, C, E) and RCBC values (B, D, F) comparing communities from the control treatment to communities from the water reduction treatment for each sampling week. Dashed red lined represent the significance cutoffs for βNTI (|βNTI|>2) and RCBC (|RCBC| > 0.95). RCBC values that fall between the dashed red lines represent undominated processes.
Figure 5.7. Treatment effects on community assembly processes for each of the host species. While undominated processes were prevalent on each host species, the defined processes varied. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.

Figure 5.8. The effects of host species and treatment on bacterial biomass over time. The total number of bacterial cells per of leaf material from each host species was calculated by washing bacterial cells off the surface of leaves, counted using epifluorescence microscopy, and compared to leaf areas calculated using ImageJ. Each watering treatment is represented by 5 biological replicates for each host species on each sampling day. After the first sampling day, orchardgrass and tall fescue showed stable bacterial biomass, but ryegrass had more day-to-day variation.

Figure 5.9. Rate of nitrogen fixation (A) and abundance of the nifH gene (B) were significantly different between host species, but were not affected by the water reduction disturbance. Nitrogen fixation rate was measured using stable isotope probing. The abundance of nifH genes was measured using qPCR and standardized to number of copies per gram of leaf material. Significant differences between host species, determined by linear models with TukeyHSD post-hoc analysis, is represented by lowercase letters in the upper left corner of each plot. Different letters indicate statistical differences.

Figure 5.10. Alpha diversity was calculated using several different metrics including (A) Chao1, (B) Faith’s PD, and (C) Shannon Diversity Index. Trends from the observed and estimated richness measures were similar. Observed ASV richness and evenness had different trends over time resulting in different trends when measuring Shannon diversity index.

Figure 5.11. Host species had the strongest impact on community structure. NMDS ordination was plotted using Bray-Curtis distances for every sampling day.

Figure 5.12. Heatmaps representing pairwise all comparisons for BNTI (A) and RC_{BC} (B). (C) Contribution of each process to community assembly was calculated for all host species, treatments, and sampling days by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.

Figure 5.13. Boxplots show the BNTI values (A-B) and RC_{BC} values (C-D) comparing communities from each sampling week to communities sampled in week 1 for both the control and water reductions treatment for each host species. Dashed red lines represent the significance cutoffs for BNTI (|BNTI|>2) and RC_{BC} (|RC_{BC}| > 0.95). (D) Bar plots show the overall contribution of each process to community assembly process differences between treatments on each of the
host species. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.

Figure 5.14. Boxplots show the BNTI values (A-B) and RC_{BC} values (C-D) comparing communities from each sampling week to communities sampled in the previous week for both the control and water reductions treatment for each host species. Dashed red lines represent the significance cutoffs for BNTI (|BNTI|>2) and RC_{BC} (|RC_{BC}| > 0.95). (D) Bar plots show the overall contribution of each process to community assembly process differences between treatments on each of the host species. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.

Figure 6.1. Microbe-microbe and plant-microbe interactions that occur on the surface of leaves have the potential to influence plant and ecosystem health.

Figure A1. Drought experiments were conducted either under greenhouse, modified field, or field conditions. Community diversity and composition were assessed using several different univariate and multivariate statistics, microbial biomass was assessed using fluorescence microscopy, and rate of nitrogen fixation was measured using stable isotope probing.

Figure B1. Relative abundance species distributions of isolates sequenced using the same primers used in the MiSeq community sequencing project. Identity was assigned by comparing isolate sequence to the representative sequence database created in the MiSeq analysis.

Figure C.1. Cultivars used in the (A) 2020 and (B) 2021 phyllosphere study and their location in the randomized block design. Xs indicate cultivars that were not sampled.

Figure C.2. Alpha diversity showed decreasing trends as a result of drought for several cultivars but only two cultivars showed significant declines in alpha diversity as a result of drought. Significant relationships are represented by capital letters above boxplots, where different letters indicate significant differences. In cultivars where treatment did not result in significant differences, no letters are displayed.

Figure C.3. NMDS and PERMANOVA based on weighted UniFrac distance metrics shows that community structure changes as a result of drought, but is most influenced by host species.

Figure C.4. Taxa barplots at the (A) class level show that (B) Alphaproteobacteria and (C) Gammaproteobacteria are the most dominant genera. The two Kentucky bluegrass cultivars are given in blue labels and the 6 tall fescue have pink labels.
Differences in community structure and response to drought are seen between the two host species and the different cultivars within each host species. Of note from Alphaproteobacteria (B), the genus Agrobacterium showed an increase in abundance during recovery while the genus Sphingomonas was susceptible to drought and showed little signs of recovery. In Gammaproteobacteria (C), Enterobacter increased in abundance during drought while Xanthomonas and Pseudomonas showed sharp increases during recovery.

Figure C.5. Alpha diversity was measured for each cultivar using the Shannon Diversity Index. Kentucky bluegrass cultivars had a stronger response to drought with several of the cultivars decreasing under drought stress. Many tall fescue cultivars showed no response or only shifted during the first drought period. One explanation for the stronger shift during the first drought period could be due to a priority effect in which microbes that survived the first drought period alter community profiles resulting in communities more tolerant to the second drought. For each host species, five cultivars (DRT) were exposed to repeating drought and recovery periods and one cultivar was a control irrigated over the entire experimental period (IRR). Sample Day 1 corresponds to pre-drought, Sample Day 2 to the end of the first drought period, Sample Day 3 to the end of recovery period 1, Sample Day 4 to the end of drought 2, and Sample Day 5 to the end of the final recovery period.

Figure C.6. NMDS and PERMANOVA based on weighted UniFrac distance metrics shows that community structure is different between host species but become more similar after several drought and recovery periods. Additionally, differences between irrigated controls and drought stressed plants measured on the same day had significantly different community structures, but their structures were not different on pre-drought or recovery days indicating that communities are resilient to drought stress.

Figure C.7 Taxa barplots at the (A) class and for dominant genera including (B) Actinobacteria, (C) Alphaproteobacteria, and (D) Gammaproteobacteria for each tall fescue cultivar. Gammaproteobacteria shows an increase in relative abundance during the drought days (2 and 4) which correspond with increases in Enterobacteriaceae family or Erwinia genus, while Agrobacterium from the Alphaproteobacteria class appear to have increased relative abundance on non-drought days (1, 3, 5). Other notable changes include a decrease in Rhodococcus (B) and an increase in Sphingomonas (C) over time.

Figure C.8 Taxa barplots at the (A) class and for dominant genera including (B) Actinobacteria, (C) Alphaproteobacteria, and (D) Gammaproteobacteria for each Kentucky bluegrass cultivar. Similarities in the abundance profiles compared to tall fescue include increased Gammaproteobacteria, specifically Enterobacteriaceae and Erwinia, during drought stress. Notable differences include overall higher abundance of Methylobacterium (C) and a large increase on the last sampling day.
CHAPTER 1
INTRODUCTION

1.1 Grassland Ecosystem Importance

The human population is predicted to increase by 2 billion people over the next 30 years reaching a global population of 9.7 billion people by 2050 [4]. With an increasing population, humanity faces a growing problem of how to provide enough food to feed the population. Already, one in seven people either lack access to food or are malnourished [5], a number that will continue to increase unless crop production increases [6]. Over the past several decades, agricultural yields have increased through expansion and intensification practices. These practices can have negative effects on the environment, evident by the 30-35% of global greenhouse gas emissions that are produced by the agricultural industry [6–10]. One important intensification practice is the application of fertilizers. Within the past 50 years, total fertilizer application has increased by over 500% while nitrogen fertilizer application has increased by 800% [11, 12]. Some of the problems resulting from such intensification practices include degradation of water quality and increased pollution [12, 13]. Agricultural expansion, the conversion of nonagricultural land is converted into agricultural land. Expansion practices can have severe negative ecological consequences including degradation of important habitats, loss of biodiversity, and decrease in carbon storage and soil health [6]. Currently, the tropics are experiencing a high degree of agricultural expansion, where an estimated 80% of croplands have replaced important tropical forests [14].
One important ecosystem that has been affected by both intensification and expansion practices are grasslands. Grasslands account for 37% of earth’s terrestrial area, with meadows and pastures comprising 69% of global agricultural land. To date, an estimated 70% of natural grasslands have been converted into agricultural land [6, 15, 16]. This conversion can have several negative consequences as grasslands provide important ecological services including promotion of carbon sequestration and storage, promotion of biodiversity, erosion prevention, recharging of water tables, and enhanced soil fertility [17, 18]. Beyond ecological impacts, the important socioeconomic impacts grasslands have involve the control of global food and income security by acting as the foundation for ruminant milk and meat production, and providing livelihoods for over 800 million people [16].

Despite grasslands’ significant role in food production and global biomass, they are at risk of degradation due to human activity and are susceptible to climate change [6, 16]. Grasslands around the world are facing changing temperature and moisture patterns, which will require broader management approaches to maintain production levels that meet food needs. Elevated atmospheric CO₂ can have a range of effects on grasslands including increased photosynthesis resulting in increased productivity, but can reduce forage quality by altering the crude protein concentration and C:N ratio [19, 20]. Additionally, temperature and drought stress can significantly decrease ecosystem functionality both by decreasing productivity and decreasing carbon sequestration [16, 21]. A decrease in carbon sequestration in European grasslands was observed in separate instances during summer heat waves, and were attributed to the decrease in
photosynthetic uptake that resulted from the drought conditions [22]. As grassland conditions decrease or are converted to urban environments, new land must be cleared to maintain current agricultural outputs. Creating new agricultural grasslands has two major consequences: (1) Important ecosystems are degraded and (2) new grasslands are established on poorer quality soil in areas often more prone to extreme weather conditions, which results in decreased output [6]. Increasing pasture productivity and lifespan is crucial to stopping agricultural expansion and creating sustainable farming practices that can keep up with the growing global population, thus slowing the rate of global warming and the associated negative effects [6, 16].

Grasslands around the world are made up of species that have a wide range of physiological and morphological features such as different carbon fixation mechanisms, heat and cold tolerances, drought tolerances, and nutritional content [23]. In tropical regions, the dominant grasses which utilize C₄ photosynthesis include Napier grass (*Pennisetum purpureum*), and species from the *Brachiaria* and *Panicum* genera [23]. In temperate regions, however, dominant grasses utilize C₃ photosynthesis with prominent species commonly from the genera *Agrostis* (bentgrass), *Festuca* (Fescue), *Lolium* (ryegrass), and *Dactylis* (orchardgrass) [23].

1.1.1 Forage Systems in Brazil

One region experiencing an influx in pasture grasslands is Brazil, where Amazon Rainforest areas are shifting from highly diverse rainforests to homogenized cattle pastures. This shift not only represents a loss of biological diversity, but also an increase
in GHG emissions, responsible for 12% of annual anthropogenic CO$_2$ emissions [6, 24]. Cattle pastures are established by logging of forests, followed by the cutting and burning of remaining vegetation. Pastures are maintained until productivity declines, at which point they are abandoned for new pastures [25]. The grasses being planted in these pastures belong to the genus *Brachiaria*. These grass species are native to sub-Saharan Africa and were brought to Brazil in the mid-1900s due to their high productivity, tolerance to extreme weather events such as drought, and ability to grow in nutrient poor, acidic soil [26]. Species commonly used include *Brachiaria brizantha*, *Brachiaria decumbens*, and various hybrids of these species with other *Brachiaria* species [27].

1.1.2 Forage Systems in the United States

In the United States, a recent emphasis has been placed on developing more resilient forage systems, as biotic and abiotic variables will have an increased impact on agricultural production [28]. Studies on grasslands are shifting to reflect not only the need to increase productivity, but understand management impacts on ecosystem services. For example, while grasslands can be carbon sinks, several studies have shown that land use history and management practices can result in shifts from carbon sinks to sources or shifts resulting in neither sink nor source [29]. Common grass species used in temperate forage systems include tall fescue, orchardgrass, and perennial ryegrass. Tall fescue is a robust cool season grass with a long grazing season capable of surviving in diverse conditions including in acidic soil, soil with poor drainage, and are considered drought tolerant [30]. Tall fescue’s drought tolerance can be enhanced by the fungal endophyte *Neotyphodium coenophialum*. However, toxic alkaloids produced by the endophyte can
reduce livestock fitness, so work has focused on finding alternative endophytes that provide the same fitness advantages to the grasses without the negative impacts on the animals [31]. Orchardgrass is a high yield grass that has similar drought tolerance compared to endophyte free tall fescue varieties [30]. Additionally, orchardgrass is a high-quality forage grass that can grow in a wide range of soil types and qualities making it a commonly used forage grass in the United States. Perennial ryegrass is native to Europe, Asia, and north Africa, but has a high abundance in pastures in the United States. Perennial ryegrass has a lower drought tolerance than orchardgrass or tall fescue, but has a higher quality forage and has high yielding potential [32, 33].

1.1.3 Selected Forage Grasses

The following research was carried out on globally dominant tropical (Brachiaria brizantha, Brachiaria ducumbens, Brachiaria hybrid) and temperate forage grasses (endophyte-free tall fescue, orchardgrass, ryegrass). Both temperate and tropical systems are facing needs to increase productivity while decreasing negative environmental impacts often associated with increased agricultural production. Therefore, grasses were chosen due to their importance in temperate or tropical forage systems, their range of growth conditions, and susceptibility to drought (Table 1). By studying a range of host species, we hoped to gain a better understanding of ecological principles underpinning plant-microbe interactions with the eventual goal of leveraging these relationships to promote sustainable agricultural practices.
Table 1.1 Grass species traits under optimal growing conditions.

<table>
<thead>
<tr>
<th>Grass Species</th>
<th>Carbon Fixation</th>
<th>Height (cm)</th>
<th>Leaf Hairs</th>
<th>Leaf Width (mm)</th>
<th>Leaf Length (cm)</th>
<th>Optimal pH</th>
<th>Optimal Temp (°C)</th>
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<td>40-100</td>
<td>5-6</td>
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<td>yes</td>
<td>7-20</td>
<td>5-25</td>
<td>5-6</td>
<td>25-35</td>
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<td>5.5-6</td>
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</table>

1.2 The Phyllosphere as a Microbial Habitat

The phyllosphere is the aerial surface of plants, which hosts diverse microbial communities composed of bacteria, fungi, archaea, and viruses [39, 40]. Though the phyllosphere is dominated by leaves, it can be subdivided based on plant anatomy to include the surface of leaves (phylloplane), leaf waterscape (phyllotelma), stems (cauloplane), flowers (anthoplane), and fruits (carpoplane) [39, 40]. The leaf surface is a vast global habitat for microbes. Models have estimated that the global leaf area is $5 \times 10^8$ km$^2$ [41]. As microbes inhabit the top and bottom of leaves, this corresponds to $1 \times 10^9$ km$^2$ of habitat for microbes [40]. With an estimated average density of $10^6$-$10^7$ bacteria per cm of leaf surface, the global phyllosphere population is estimated to $10^{26}$ bacteria with unknown levels of other microorganisms [40, 42].

1.2.1 Leaf Biogeography and Physiology

Across the leaf surface as a whole, bacterial growth is highly variable [43]. Leaf surfaces are covered in a hydrophobic waxy cuticle which protects the plant from metabolite leaching and evaporation of water, but results in a nutrient poor, or oligotrophic,
environment for the microbes [40]. At a microscopic level, the leaf surface is a heterogenous terrain composed of stomata, hydathodes, trichomes, veins, and epidermal cell grooves [39, 44]. Studies have found microbes disproportionately present around these surfaces as they can offer protection, nutrients, and moisture [39, 42–45]. Stomata and hydathodes are openings into the plant that are present across leaf surfaces. Stomata are composed of guard cells that can open or close the opening which allows them to control gas exchange and water loss in the plant [39]. Hydathodes are pores that are permanently open and are involved in guttation, which is the exudation of liquid from the plant which contains organic and inorganic compounds [46]. Trichomes are epidermal outgrowths on the surface of leaves, which are involved in controlling leaf temperature, protection from UV light, and protection from herbivores and pathogen development through excretion of secondary metabolites [39]. These three structures promote microbial growth because they are the site where nutrients and moisture utilized by microbial communities are released from the plant. Veins and cell grooves similarly promote bacterial growth because resources can pool in these areas thus allowing microbial utilization.

In addition to leaf anatomy, microbial communities are also greatly influenced by plant physiology. Nutrient availability on the leaf surface is heavily linked with photosynthesis [39]. Photosynthesis produces carbohydrates which are moved through the phloem of the
plant in the form of sucrose. However, some sugars never reach the phloem and instead diffuse through the cuticle in a process called leaching whereby they become available for phyllosphere bacteria [47]. The process can also occur with other organic and inorganic nutrients [39]. Bacteria can further promote nutrient availability through the production of biosurfactants, which increase leaf wettability resulting in increased leaching [48]. Photosynthesis, respiration, and evapotranspiration shape bacterial growth through fluxes in CO₂, O₂, and water vapor, and emission of volatile organic compounds (VOCs) [39]. Water vapor that diffuses through the stomata can create microscopic leaf wetness that protect microbes from desiccation [49]. Emission of VOCs is a normal part of plant cell growth and also can be a result of biotic and abiotic stress [39]. For example, methanol is a byproduct of cell wall metabolism that is regularly released through the stomata and is a well-documented nutrient for groups of phyllosphere bacteria known as methylotrophs [50, 51]. Another VOC that is a common phyllosphere nutrient is isoprene [52]. Isoprenes are important for the plant because they are thought to help protect them from high temperatures [53]. Photosynthesis, respiration, and evapotranspiration paired with leaf anatomy, help account for the observed spatial distribution of microbes in the phyllosphere.

1.2.2 Biofilm Formation

Within the heterogenous distribution of microbes across the leaf-scape, approximately 70% of bacteria are found in large aggregates [44, 45]. These aggregates are found within extracellular polymeric substances (EPS), and are considered to be biofilms [40, 42, 54, 55]. Bacterial growth within a biofilm provides a selective advantage by protecting from
desiccation, promoting genetic and metabolic exchange, protecting against predators, and allowing for coordinated responses by intra and interspecies communication through quorum sensing [40, 42, 55]. Incorporation into the biofilm is important for bacterial survival because it provides protection from the harsh phyllosphere environment prone to rapid temperature fluctuations, changing precipitation patterns, UV exposure, low nutrient availability, and inconsistent water availability [40, 56, 57].

1.2.3 Drivers of Structure and Patterns of Assembly

What has become clear from the last two decades of phyllosphere research, which have utilized both culture dependent and independent methods, is that phyllosphere communities are not a random assembly of microorganisms. Phyllosphere communities are diverse assemblages of microbes characterized by a few dominant and many rare groups, but with lower species richness compared to nearby rhizosphere and soil communities [58, 59]. This likely relates to the short lifespan of leaves, as soil microbes can survive in dormant conditions for decades [40]. Despite the transient nature of plant leaves, microbial communities follow semi-predictable successional patterns within and across seasons suggesting strong selection for community composition and consistent sources of microbial colonizers [60–63]. Additionally, leaf age plays an important role in phyllosphere community structure. Young leaves have a lower number of total bacteria, lower diversity, and different community assemblages compared to mature leaves [62, 64–66]. This likely relates to changing nutrient availability on the leaf surface as leaf chemistry changes. For example, leaching of
nutrients increases as leaves age because older leaves have higher wettability, which makes more nutrients available on older leaves [47].

Due to the nature of the phyllosphere, there are stochastic airborne colonizers that either cannot survive and therefore exist for only short periods of time or are able to locally establish but not exist within temporal or geographic trends [62, 67]. However, the strong temporal trends suggest other stable reservoirs of bacterial colonizers. Several studies have found soil is a major reservoir, which would provide a stable source of colonizers each year as leaves emerge [61, 68–70]. Additionally, recent studies have shown that vertical transmission through the seeds are an important source of microbial colonizers [71].

In addition to having successional patterns, phyllosphere communities are highly specific to plant host species. Several studies found this pattern across tree species [72–75], herbaceous plants [76], and grass species [61, 77]. The differences across species can relate to differences in leaf morphology and leaf surface properties such as differences in cuticle formation [78]. In this study by Bodenhausen et al., *Arabidopsis thaliana* genotypes characterized by more permeable cuticles showed significantly different microbial communities compared to the wild type. Cuticles are waxy substances found on the outside of epidermal cells, which act as a diffusion barrier to help prevent water loss and protect against abiotic stresses. The differences in cuticle permeability resulted in changes in bacterial community composition and abundance because there was an increased availability of nutrients and decreased risk of
desiccation [78]. Furthermore, leaf wax levels can influence adhesion of microbes influencing which microbes are able to attach to the leaves [42]. Differences in leaf morphology can also alter air flow, availability of water soluble nutrients, and topology [79]. In addition to structural differences, differences in hormone signaling can result in differences in phyllosphere community composition. Studies in A. thaliana found differences in ethylene signaling, and salicylic acid and jasmonic acid signaling defense pathways altered community composition [78, 80].

Geographical location is another important component of phyllosphere community structure, though there is debate if geography or host species has a greater influence. One study found lower community variability within pine tree species across large distances compared to higher variability between different host species within a single site [73]. Similar trends were seen in temperate trees that were sampled at different urban intensities. While urban intensity did influence community structure, the effect was not as strong as the effect of host species [74]. However, separate studies in Tamarix trees found that geographic location had a greater impact on microbial communities than host species [81, 82]. This finding was confirmed in a study on Arabidopsis plants and their surrounding plants which found location had a larger impact on Methylobacterium community composition than host species [63]. What causes these discrepancies is unclear, but explanations could include differences in selection strength by host species or stochastic community assembly.
Despite species and site level differences, strong trends exist across phyllosphere communities. Most communities are dominated by non-pathogenic bacteria belonging to the phyla Proteobacteria, Bacteriodetes, Actinobacteria, and Firmicutes [40, 83]. Within the phylum Proetobacteria, Alphaproteobacteria and Gammaproteobacteria are frequently the most dominant phyllosphere classes [61, 74, 84, 85]. Dominant Alphaproteobacteria genera include *Sphingomonas* and *Methylobacterium*, and members of the *Pseudomonas* genus are frequently the dominant Gammaproteobacteria [39]. *Sphingomonas* survive on a wide range of substrates due to high abundance of TonB receptors, while *Methylobacterium* can grow on one-carbon compounds such as methanol, a byproduct of host cell-wall metabolism [58, 86]. Some prominent genera found in many phyllosphere studies contain pathogenic species, including *Agrobacterium, Burkholderia, Pantoea, Pseudomonas*, and *Xanthomonas* [40]. *Pseudomonas* species include well-studied foliar pathogens such as *Pseudomonas syringae* which is capable of infecting many different plant species [87], but can also promote plant health through phytohormone production and protection from pathogens [88–90].

1.2.4 Bacterial Survival Strategies

Given the biotic and abiotic stresses microbes experience in the phyllosphere, this harsh environment imposes strong degrees of environmental selection on microbes and/or microorganisms must be adapted to establish and survive. Production of pigments and DNA repair strategies such as photolyases help protect bacteria from UV damage [91, 92]. Aggregate formation and production of bioactive compounds such as
biosurfactants help protect bacteria from desiccation by maintaining moisture on the leaf surface and increasing wettability [40]. Bacterial aggregate formation is also supported by bacterial adhesion, which can help prevent removal by abiotic factors such as rainfall and wind. Many commensal bacteria utilize aggregate formation compared to some phytopathogens, which utilize flagella to move towards more favorable sites such as towards nutrients [58, 93]. Additionally, successful establishment in the phyllosphere often requires evasion of antimicrobial compounds, a common biotic stress produced by both the plant host and other microbes [40]. Because nutrients on the leaf surface are limited, microbial survival in the phyllosphere is promoted in bacteria that are able to utilize a wide variety of substrates [58]. Additional microbial survival strategies are to increase availability of nutrients through production of compounds and hormones such as biosurfactants and indole-3-acetic acid which can increase substrate leaching by increasing leaf wettability or cell wall loosening, respectively [42, 48].

1.2.5 Benefits to Plant Hosts

Plant-microbe interactions can result from parasitic, commensal, and mutualistic relationships. While the benefits provided by the plant hosts to the microbial community are obvious, we are just beginning to understand the benefits provided by the microbes to the plant host. Phyllosphere microbes acting as biocontrol agents protect plant hosts from phytopathogens directly through antibiotic production or indirectly through niche exclusion, activation of the plant immune system, and siderophore production [83]. One study found that axenic plants were more susceptible
to infection and that *Sphingomonas* spp. isolates helped protect the plant hosts from bacterial pathogens [93]. Potential of phyllosphere biocontrol agents against leaf blight (*Exserohilum turcicum*) on maize showed that *Bacillus* and *Pantoea* species inhibited growth of the pathogen through competitive exclusion [94]. In other studies, *Bacillus* spp. are linked with production of secondary metabolites by the plant host resulting in disease protection and increased growth [95, 96]. *Pseudomonas protegens* CS1, a non-pathogenic bacterium from the lemon phyllosphere, helps protect hosts from citrus canker through generation of reactive oxygen species (ROS) linked to the siderophore pyochelin [97].

Phyllosphere microbes have shown additional plant growth-promoting traits such as nitrogen fixation and hormone production. One study found that phyllosphere epiphytes could account for 10-25% of the nitrogen content found in their host species, indicating phyllosphere microbes are likely an important source of nitrogen [98]. These levels were first determined through laboratory experiments in which leaves and their epiphytes were incubated together and separately in the presence of $^{15}$N. Following the incubation period, incorporation of $^{15}$N was determined using isotope ratio mass spectrometry. Epiphytes were removed from the leaf sample before measuring incorporation to ensure that only $^{15}$N incorporated into the leaf sample was being determined. Additionally, epiphyte incorporation of nitrogen was confirmed in the field by attaching a gas chamber to individual leaves and incubating the leaf with $^{15}$N. Nitrogen incorporation was then determined by measuring $^{15}$N levels in both the leaf attached to the gas chamber and an adjacent leaf. Phyllosphere nitrogen fixation has been measured in several systems
including temperate and tropical forests, agricultural systems, and on urban plants [99–104]. Studies in agricultural systems that inoculated plants with nitrogen fixing isolates found improved plant yields indicating nitrogen fixing bacteria could act as useful alternatives to chemical fertilizers [102, 103, 105]. Similarly, inoculation of phytohormone producing methylotrophic bacteria resulted in increased plant biomass [106, 107]. In these studies, methylotrophic bacteria were screened and chosen as inoculants for their ability to synthesize cytokinin or indole-3-acetic acid (IAA). IAA, which is linked to plant growth in several studies, is one of the most common auxins produced by phyllosphere microbes [108–110].

1.2.6 Ecosystem Benefits

Plant-microbe relationships do not just influence individual plant health, these shared processes have the capability to influence ecosystem level productivity and global biogeochemical cycles. A 2017 study by Laforest-Lapointe found that leaf bacterial diversity was positively linked to ecosystem productivity [111]. Furthermore, it is expected that phyllosphere bacteria are important for biogeochemical cycling given their abundance, vast habitat, and presence of important genes [58]. Phyllosphere communities have a high abundance of methylotrophic bacteria, which means they are able to use methanol as a carbon source. Methanol is a volatile organic compound and the methanol released as a byproduct of plant growth is an important contributor to atmospheric methanol [86]. Given the prevalence of methylotrophs in the phyllosphere, microbes in this habitat are thought to have an important role in the carbon cycle by regulating methanol emissions from plants [58, 61]. Phyllosphere
bacteria are also involved in the nitrogen cycle. Numerous studies have shown nitrogen fixation in the phyllosphere and have shown that inoculation with nitrogen fixers can support plant growth [99–104].

Plants depend on nitrogen for growth as a major component in both chlorophyll and amino acids; therefore, sufficient nitrogen levels promote plant growth by increasing rates of photosynthesis and development of proteins. But, many crops are nitrogen deficient [112]. Even though atmospheric nitrogen is the most abundant element in earth’s atmosphere, it cannot be directly used by plants, making nitrogen one of plants most limiting nutrients. Plants acquire nitrogen through application of fertilizer or naturally through biological nitrogen fixation (BNF) [113]. BNF currently does not provide enough nitrogen to support agricultural production. As a result, agricultural management frequently involves the application of nitrogen fertilizer, but applied nitrogen fertilizer has a low use efficiency; studies estimate only 33% of applied nitrogen fertilizer is used by the plants [112, 114]. The excess nitrogen leaches out of the soil resulting in significant environmental damage including eutrophication, loss of diversity, surface and groundwater pollution, soil acidification, and emission of nitrous oxide, a powerful greenhouse and ozone depleting gas [115]. Since phyllosphere microbes are capable of BNF and the phyllosphere is one of the vastest habitats on earth, it has the potential to influence the global nitrogen cycle and agricultural productivity by acting as a biofertilizer [40, 84].
1.3 Drivers of Phyllosphere Community Assembly

Community assembly is the process in which local communities are established through colonization events and maintained through continuous immigration events and the resulting interactions [116]. One framework used to describe community assembly proposes it is composed of four ecological processes: selection, diversification, dispersal, and ecological drift [117, 118]. This framework combines niche-based theory and neutral theory. Niche-based theory contends that community structure results from deterministic processes such as environmental filtering and biological interactions such as competition, predation, and mutualism [116]. In contrast, neutral theory proposes that no competitive difference exists between species and thus community dynamics result from stochastic processes [119]. The Vellend framework acknowledges stochastic and deterministic processes often occur in parallel to shape community assembly [116, 117, 120].

Environmental selection, also known as habitat filtering, is a deterministic process in which species not adapted to the environment are filtered out of a community.

Diversification is a mostly stochastic process in which new species are created. Dispersal, which describes the movement of organisms through space, can be either stochastic or deterministic. Drift is a purely stochastic process resulting from a collection of random changes in species abundance. Each of these four processes has an important role in shaping phyllosphere communities.

1.3.1 Selection in the Phyllosphere

Selection results from fitness differences between organisms and is considered one of driving forces of phyllosphere community assembly. Selection in the phyllosphere can be
categorized as external selection in which environmental factors such as leaf physiology and local climate favor certain microbes or as internal selection which results from microbe-microbe interactions [39]. Host species influence on phyllosphere communities is well documented [61, 72–74, 76, 77, 121, 122]. Some studies have linked the observed differences to specific plant traits. In tropical trees, leaf phosphorous content influenced bacterial community structure [72], and in Mediterranean trees and shrubs leaf water content was the primary influencer of community structure [65]. As already discussed, the phyllosphere is a harsh environment, so microbes with certain functions, such as pigment production to protect from UV damage, ability to aggregate and form biofilms, and ability to survive on a wide range of nutrients or those commonly emitted by plants such as methanol, provide a distinct selective advantage [40]. In a study using sterile Arabidopsis plants, researchers demonstrated host selection as initial colonizers on the plants closely resembled the air microbiome, but by the end of the experiment plants had their own distinct communities [62]. Internal selection or microbe-microbe imposed selection is also important in structuring phyllosphere communities as microbes either need to outcompete organisms occupying a similar niche or coexist through niche partitioning [56].

1.3.2 Diversification in the Phyllosphere

Diversification was originally described in the Vellend framework as speciation but was later modified to diversification by Nemergut [117, 118]. Diversification is important for shaping the microbial functional diversity in plant leaf communities though it is difficult to study [39, 116, 123]. The leaf surface has been considered a hot spot for horizontal
gene transfer with large potential for substantial gene mixing [42]. Additionally, phyllosphere communities are expected to have high mutation rates because of the degree and types of stress they experience (UV radiation, ROS) [39]. Due to large potential of horizontal gene transfer and mutation, diversification in the phyllosphere is predicted to be high [39, 42].

1.3.3 Dispersal in the Phyllosphere

Microbes can arrive at the leaf surface via several different types of dispersal events that can vary based on plant age and development. The earliest colonizers of the phyllosphere can come from the seed or germination tissue [71, 124]. After leaf emergence, leaf colonizers can come from a variety of sources, including bioaerosols, neighboring plants, rain/irrigation events, or animals such as herbivores and pollinators [39, 125, 126]. Bioaerosols can be composed of single bacterial cells, an aggregate of bacterial cells, fungi, and spores, which can be by themselves or attached to dust particles or pollen. The source of the microbes in the bioaerosols is expansive and can include soil, neighboring plants, water sources, and animals [126]. Aerosolization frequently occurs through passive processes such as wind erosion and splashing from rain [127]. After microbes have been aerosolized, they are transported through the air either to nearby plants or can travel up to hundreds of miles [128]. Dispersal as a whole in microbial communities is not a well understood process [116]. A concept that has persisted within microbial ecology theory for decades is the Baas Becking hypothesis which states, “Everything is everywhere, but the environment selects”. This is often used as a null hypothesis, which tests the relative strength of the forces of dispersal and environmental selection in
shaping community structure. The strong geographic patterns that exist within phyllosphere communities provide evidence of the import role of dispersal in patterns of phyllosphere community assembly [81, 82, 116].

1.3.4 Drift in the Phyllosphere

Ecological drift occurs when the relative abundance of species within a community change over time as a result of random birth, death, and reproduction rates [116, 118, 130]. Microbial communities are susceptible to drift because their large numbers of rare taxa are more effected by small changes in abundance [118]. Since phyllosphere communities generally have a few abundant taxa and many rare taxa, drift plays an important role shaping phyllosphere communities [39].

1.4 Understanding Microbial Community Stability

Though microbial communities widely promote ecosystem productivity, many ecosystem simulation models do not include microbial communities and their associated processes [131]. Because of microbial community importance in ecosystem functioning, understanding how microbial community functional and compositional profiles respond to disturbances will help us predict community and ecosystem responses to future climate conditions [132, 133]. Disturbances are events that either alter the environment thus affecting the inhabiting communities or directly change a community through processes such as modified relative abundances of certain members [133]. Depending on their duration, disturbances can be classified as pulses or presses [134]. Pulses are distinct, short-term events, while presses are continuous, long-term events [133]. How
communities respond to disturbance is known as stability, which can be broken down into two measurable states: resistance and resilience (Fig. 2) [133, 135]. Resistance is the degree to which communities remain unchanged as a result of disturbance and resilience is the ability of a community to recover to pre-disturbance conditions [133]. Stability can be determined by looking at community composition, function, or the interrelatedness of the two metrics. Disturbance does not always result in the same degree of change in terms of community composition and function. For example, communities that display a high degree of functional redundancy could develop a stable functional profile but a changing compositional profile resulting in unchanged effects on ecosystem functioning [135].

1.4.1 Individual Survival Strategies

Community stability is influenced by individual, population, and community level dynamics, in which community-level stability cannot occur without survival of individuals cells followed by persistence of populations (groups of the same species in an environment) [133]. Individual survival is influenced by plasticity, stress response, and dormancy. Bacteria with high degrees of plasticity are able to respond to changes
in their environment through adaptive gene expression. For example, *Rhodobacter sphaeroides* can shift from an anaerobic phototrophic lifestyle to an aerobic chemoheterotrophic lifestyle [133]. Mixotrophy, defined as the ability of an organism to utilize many different carbon sources, is common among many microbial communities [136] including those in the phyllosphere [137, 138]. This ability to switch based on environmental conditions likely increases the compositional stability of the community [133]. In addition to adapting to changing environments, cellular response to stress can promote individual survival. Several proteins associated with stress response have been found in phyllosphere communities including superoxide dismutase, catalase, DNA protection proteins, and proteins involved in the formation of trehalose (an osmoprotectant) [58]. In the absence or failure of plasticity or stress response, community structure can be preserved through dormancy. Dormancy occurs when under stressful or unfavorable conditions, organisms enter a state of reduced metabolic activity that they are able to reverse under favorable environmental conditions [139]. It is proposed that dormancy aids community stability through its role in recovery after a disturbance event [139]. Therefore, dormancy likely has a greater stabilizing effect during shorter pulse disturbances compared to continuously occurring press events where individuals are less likely to reverse the dormant state [133].

1.4.2 Population Survival Strategies

In addition to survival of individual cells, persistence of microbial populations is needed for community stability. Properties that allow population persistence include
evolutionary adaptation, microbial growth, and dispersal [133]. Microorganisms ability to grow quickly, reach high population densities, high mutation rates, and their ability to undergo horizontal gene transfer can help community response to disturbance through rapid adaptation and evolution [140, 141]. Growth rates are tied to resource use efficiency, which can have important implications for community stability. Communities with fast growers (r-strategists) are more resilient but have less resistance, while slower growing microbes (K-strategists) and oligotrophs which have higher resource use efficiency have increased resistance [133]. This dynamic was observed in a study looking at resistance and resilience of grassland soil communities in response to drought. This study found the slow growing fungi were more resistant while the faster growing bacteria were more resilient [142]. Furthermore, a communities ability to recover after a disturbance event is greatly influenced by rates of dispersal. A community with continuous dispersal from a local reservoir will have a high degree of resilience as microbial taxa are able to recolonize the disturbed habitat [133]. Alternatively, dispersal following a wide-spread disturbance event could reduce resilience through colonization of taxa tolerant to the disturbance, which could ultimately influence future community resistance [143]. Furthermore, disturbance events can change available niches in an environment, which could allow non-native community members to establish early and affect the success of subsequent colonizers in a process known as priority effect [133].

1.4.3 Community Survival Strategies

At the community level, stability is influenced by diversity, microbial turnover, and community interactions [133]. The role microbial diversity has in resistance and
resilience is not well understood and studies have revealed different trends between environments [133, 144, 145]. One prominent theory is that diverse communities are more likely to be functionally redundant, which increases community resistance [135]. Functional redundancy, which is when multiple microbial taxa are able to carry out the same process at the same rate, can promote stability because it increases the likelihood that niches are filled as stress alters environmental conditions [146]. Many microbial communities, including phyllosphere communities, have clear temporal and seasonal patterns [60, 61, 73]. The rate of turnover (replacement of community members over time) within environments can be useful for understanding stability both as a measure of the effect of disturbance and predicting resilience [133]. Finally, interactions between community members are an important aspect of community stability. One microcosm study in which three bacterial species were chosen for their individual stress tolerances found that disturbance alone did not account for extinction but was a result of interspecies competition in combination with disturbance intensity [147]. An increasing popular method to understand and predict community stability patterns is through the use of co-occurrence networks.

Network analysis can provide insight into communities following disturbance events by providing understanding of community interactions and the resulting loss or gain of taxa [133]. Relative community stability can then be inferred by comparing network properties. For example, communities with highly complex networks resulting from higher diversity and more interconnectivity, are predicted to be more resistant and resilient [148–150]. Co-occurrence networks can additionally shed light onto the types of
interactions between community members. Positive interactions may result from cooperative interactions or from shared niches while negative interactions can represent competition or indicate taxa occupy different niches [148, 150]. Thus, higher ratios of negative to positive interactions increases network complexity which is an indicator for more stable communities [150].

1.5 Rationale

Despite the ecological importance of grasslands, little knowledge exists on grassland phyllosphere community dynamics. Less than a handful of studies have investigated their phyllosphere. One study suggests soil acts as a reservoir for phyllosphere microbes and that strong seasonal patterns influence phyllosphere community structure [61]. Additionally, increased temperature experiments with grasses showed changes in phyllosphere community diversity where plant beneficial bacteria were replaced by potential plant and human pathogens [84]. Little else is understood about grassland phyllosphere community dynamics, which made this dissertation necessary.

As global temperatures are rising, drastically changing weather patterns are predicted to increase intensity, duration, and frequency of drought in important grassland areas [151, 152]. Since the phyllosphere is one of the vastest habitats on earth, it has the potential to influence agricultural productivity by acting as biofertilizer [40, 74]. Though microbial communities widely promote ecosystem productivity, many ecosystem simulation models do not include microbial communities and their associated processes [131]. Because of the importance of microbial communities in ecosystem functioning,
understanding how microbial community functional and compositional profiles of microbial communities respond to disturbances will help us predict community and ecosystem responses to future climate conditions [132, 133].

This dissertation focuses on unanswered questions regarding phyllosphere community assembly and response to stress in grassland ecosystems. Phyllosphere community assembly and response to drought was assessed using six common grass species found in tropical and temperate environments in two separate greenhouse experiments and two separate field experiments. **In the first aim (Ch. 2),** we addressed phyllosphere community dynamics and response to drought stress by studying three species of tropical grasses in a greenhouse environment and subjecting them to three watering conditions followed by a second greenhouse study in which two species of temperate grass were studied. **In our second aim (Ch. 3),** we looked at phyllosphere community assembly and response to drought in a separate field experiment in which three species of temperate grasses and three species of tropical grasses were grown under the exact same conditions and subjected to two watering conditions: control and drought. **In our third aim (Ch. 4),** we assessed if microbial community assembly dynamics were similar between the two environments by comparing the results from the temperate greenhouse study to the results from the temperate grasses grown in the field experiment to understand how environment influences community dynamics and stress tolerance. **Finally in our fourth aim (Ch. 5),** we addressed resistance and resilience of phyllosphere community composition and function in response to water-reduction stress. Community function was assessed by
looking at changes in biomass and rate of nitrogen fixation. A visual overview of the experimental design is available in Appendix A.
CHAPTER 2

PHYLLOSHERE COMMUNITY ASSEMBLY AND RESPONSE TO DROUGHT STRESS ON COMMON TROPICAL AND TEMPERATE FORAGE GRASS


2.1 Abstract

Grasslands represent a critical ecosystem important for global food production, soil carbon storage, and water regulation. Current intensification and expansion practices add to the degradation of grasslands and dramatically increase greenhouse gas emissions and pollution. Thus, new ways to sustain and improve their productivity are needed. Research efforts focus on the plant-leaf microbiome, or phyllosphere, because its microbial members impact ecosystem function by influencing pathogen resistance, plant hormone production, and nutrient availability through processes including nitrogen fixation. However, little is known about grassland phyllospheres and their response to environmental stress. In this study, globally dominant temperate and tropical forage grass species were grown in a greenhouse under current climate conditions and drought conditions that mimic future climate predictions to understand if (i) plant host taxa influence microbial community assembly, (ii) microbial communities respond to drought stress, and (iii) phyllosphere community changes correlate to changes in plant host traits and stress-response strategies. Community analysis using high resolution sequencing revealed *Gammaproteobacteria* as the dominant bacterial class, which increased under severe drought stress on both temperate and tropical grasses while overall bacterial community diversity declined. Bacterial community diversity, structure, and response to drought were significantly different between grass species. This
community dependence on plant host species correlated with differences in grass species traits, which became more defined under drought stress conditions, suggesting symbiotic evolutionary relationships between plant hosts and their associated microbial community. Further understanding these strategies and the functions microbes provide to plants will help us utilize microbes to promote agricultural and ecosystem productivity in the future.

2.2 Importance
Globally important grassland ecosystems are at risk of degradation due to poor management practices compounded by predicted increases in severity and duration of drought over the next century. Finding new ways to support grassland productivity is critical to maintaining their ecological and agricultural benefits. Discerning how grassland microbial communities change in response to climate stress will help us understand how plant-microbe relationships may be useful to sustainably support grasslands in the future. In this study, phyllosphere community diversity and composition was significantly altered under drought conditions. The significance of our research is demonstrating how severe climate stress reduces bacterial community diversity, which previously was directly associated with decreased plant productivity. These findings guide future questions about functional plant-microbe interactions under stress conditions, greatly enhancing our understanding of how bacteria can increase food security by promoting grassland growth and resilience.
2.3 Introduction

Agricultural production, through crops and grazing, is key to global food security. Despite current advances, the number of undernourished people is increasing [153]. Grasslands are an important ecosystem with potential to help promote food security through their role in ruminant milk and meat production and reduce greenhouse gas levels by sequestering and storing carbon. Current intensification and expansion practices have taken a serious toll on the environment, causing degradation of land, water, and biodiversity while increasing greenhouse gas emissions [6, 16, 154]. As global temperatures continue to rise, weather patterns are predicted to drastically change increasing intensity, duration, and frequency of drought in important grassland areas over the next century [151, 152, 155, 156]. This will not only affect agricultural production, but also increase levels of atmospheric CO$_2$ further destabilizing current weather patterns [157]. Thus, finding ways to promote grassland health and production in sustainable ways is essential for increasing food security and reducing environmental strain.

The phyllosphere, or aerial surface of plants, is estimated to cover over $10^9$ km$^2$ and contain $10^{26}$ bacterial cells, making it one of the largest microbial habitats on earth [42]. Microbes in the phyllosphere promote host fitness through phytohormone and nutrient production, increased stress tolerance, and protection against pathogens [40, 56, 158]. As a result, phyllosphere communities can support ecosystem functioning [40, 42, 93, 99, 111]. In order to leverage plant-microbe relationships to promote ecosystem health and agricultural production [61, 159, 160], we need a better understanding of which microbes are associated with plant hosts, what drives their
community structure, how they support their host, and how microbial communities are changing as a result of climate stress.

Due to temperature fluctuations, changing precipitation patterns, and UV exposure, the phyllosphere is an extreme habitat for microbes. Despite these harsh conditions, phyllosphere communities exhibit strong seasonal and temporal patterns and according to the literature are consistently dominated by *Gammaproteobacteria* and *Alphaproteobacteria* [61, 85, 161]. Within *Alphaproteobacteria*, members from the genera *Methylobacterium* and *Sphingomonas* are ubiquitous across many phyllosphere studies. They are hypothesized to be generalists able to survive off of a low abundance of many different substrates making them ideal members of the continuously changing phyllosphere ecosystem [58, 61, 84]. Although studies of grassland and tree phyllospheres identified host species as an important driver of community structure [72–74, 77], a recent study found that the bacteria species detected in the phyllosphere were shared across the different host species studied suggesting that phyllosphere communities are composed of generalist bacteria capable of surviving variable environmental conditions [76]. These differing trends suggest community assembly is not a completely stochastic process, and what drives assembly is not well understood [160, 161]. Because plant-health is reliant on microbial communities, understanding the ecological impacts of climate change on plant-microbe relationships is important to develop strategies that counteract the associated negative effects [160, 162].
In spite of the ecological importance of grasses, few studies have used culture-independent methods to investigate bacterial phyllosphere communities on grasslands. Studies suggest soil acts as a reservoir for phyllosphere microbes and that strong seasonal, environmental, and temporal patterns influence phyllosphere community structure [61, 163]. Despite grassland phyllosphere communities showing significant shifts over time, community assembly is also impacted by host identity [77, 163]. Experiments with grasses at elevated temperatures showed significant shifts in phyllosphere communities with plant beneficial bacteria decreasing and potential plant and human pathogens increasing [84, 164]. Additionally, grass phyllosphere communities are greatly impacted by urbanization [165]. More targeted studies are needed to understand how continued climate stress will impact grassland phyllosphere community dynamics.

Grasslands around the world have a wide range of physiological and morphological features including different carbon fixation mechanisms [23]. Many tropical grass species utilize the C₄-pathway, with a 50% improved photosynthetic efficiency over the C₃-pathway utilized by temperate grass species [166]. These physiological differences result in different levels of stress tolerance and response strategies. For example, C₄ plants have lower stomatal conductance and perform photosynthesis while stomata are closed, resulting in lower transpiration rates and continued biomass production under drought stress [167]. Understanding if differences in plant physiology relate to differences in phyllosphere community structure under changing climate conditions will shed light onto drivers of microbial community assembly and
determine if universal strategies using microbes to promote grassland health can be developed. Three closely related species of C₄ tropical grasses and two species of C₃ temperate grasses were grown under optimal temperature conditions in greenhouses and subjected to different watering regimes to understand (i) how plant host identity influences microbial community assembly, (ii) how microbial communities respond to drought stress, and (iii) if the changes correlate to changes in plant host traits and plant stress response strategies. We hypothesized that (1) each species of plant has distinct phyllosphere communities and more closely related plant species have more similar communities. (2) Drought results in decreased microbial community diversity and changes in microbial community structure on all plant species. Additionally, (3) bacterial communities from more drought tolerant species show less change due to their ability to better grow and survive under drought conditions.

2.4 Materials and Methods

Three closely related C₄ tropical forage grass species (*Brachiaria brizantha*, *Brachiaria decumbens*, and *Brachiaria* hybrid cv Cobra (CIAT 1794)) and two C₃ temperate forage grass species (*Festuca arundinacea* and *Dactylis glomerata*), were grown under three different watering conditions: well-watered control, mild drought (MD), and severe drought (SD). Grass species were chosen because they are widely used in forage systems in either tropical or temperate climates. Experiments were set up in the College of Natural Sciences Research and Education Greenhouse at the University of Massachusetts-Amherst. Grass host species and their relatedness were compared to understand if they influenced bacterial community diversity and structure, and to
determine if different plant responses to drought stress influence changes in microbial community structure. We sampled mature, but not senescing, leaves for each experiment because preliminary work showed that leaf age (young vs mature vs senescing) influenced microbial community structure (unpublished data from 2017). Drought severity was standardized based on the leaf relative water content (Figure 2.7B and 2.8A).

2.4.1 Plant Growth Conditions

2.4.1.1 Temperate Grasses: Endophyte free *Dactylis glomerata* (orchardgrass) and *Festuca arundinacea* (tall fescue) seeds were acquired from Albert Lea Seed Company (Albert Lea, MN, USA). Seeds were germinated in Pro-mix commercial potting media (Quakertown, PA, USA) in October 2018. After 6 weeks, each individual plant was transplanted into its own plastic pot (13 cm diameter by 23 cm height) so that each pot contained a plant from one seed. Pots were filled with soil collected from natural grass fields in Amherst, MA. The top 15 cm of topsoil was collected, all rocks and roots were removed, and soil was immediately transported to the greenhouse for planting. Soil nutrients were tested at the University of Massachusetts Soil and Plant Nutrient Testing Laboratory (Table 2.4). Plants were maintained under greenhouse conditions at 21°C for 16 hours light provided by 1000W metal Halide and 50% 600W High pressure sodium lights and at 18°C for 8 hours during the nighttime. Humidity was set at 50%. Drought experiments began once plants reached 5 months of growth.
2.4.1.2 Tropical Grasses: *Brachiaria brizantha* (CIAT 26564), *Brachiaria decumbens* (CIAT 6370), and *Brachiaria* hybrid (CIAT 1794) seeds were acquired from CIAT (Cali, Columbia). Seeds underwent acid scarification before germination in Pro-mix commercial potting medium in January 2018. After 4 months, individual plants were transplanted into plastic pots (13 cm diameter by 23 cm height) so that each pot contained one plant. Pots were filled with soil designed to mimic tropical soil. Soil from a forest in Amherst, MA underwent nutrient testing (Table 2.4) to determine nutrient similarity to tropical soil. Soil was collected, rocks and roots were removed, soil was then amended with kaolinite in order to replicate the high clay content found in Amazonian soil, and then soil was transported to the greenhouse for immediate planting. Plants were maintained under greenhouse conditions at 30°C for 16 hours light provided by 1000W metal Halide and 50% 600W High pressure sodium lights and at 26°C for 8 hours during the nighttime. Humidity was set at 60%. Drought experiments began 5 months after seeds were planted.

2.4.2 Drought Experiment

Fully grown grasses were divided into a control group and two independent drought treatment groups. Control plants were watered to maintain approximately 25 to 30% volumetric soil moisture content, MD at 10-15%, and SD at 3-5% by the end of the experimental period. Plants were organized in a randomized block design. Field capacity was determined before the start of the experiment by flooding the soil with water, allowing it to drain for 24 hours and then measuring soil moisture with a soil moisture
In temperate grass experiments, each of the three watering treatments consisted of 5 biological replicates for each of the two plant host species, except for the Tall Fescue control treatment which had 4 replicates, for a total of 29 individual plants. For 36 days (28 Jan 2019 – 04 March 2019), the soil moisture content was measured every three days using a MiniTrase TDR with Buriable probe (Soilmoisture Equipment Corp., Goleta, CA, USA). In tropical grass experiments, each of the three watering treatments consisted of 3 biological replicates for each of the three plant host species totaling 27 individual plants. Over 22 days (03 July 2018 – 24 July 2018), soil moisture content was measured weekly using an Extech Soil Moisture Meter (Extech, Waltham, MA, USA). The amount of supplemental water was determined based on soil moisture readings for each pot. Because individual plants differed slightly in total biomass, the rate of transpiration was different between each plant and therefore each plant required varying amounts of water to maintain equal soil moisture.

2.4.3 Plant Health Measurements

Throughout each drought experiment, several plant health measurements were used to assess plant response to drought. These measurements were taken from the same plants from which phyllosphere communities were collected in order to directly correlate plant traits with microbial communities. Leaf relative water content of each grass species was measured weekly [168]. Leaf chlorophyll concentration was determined weekly by
extracting chlorophyll from the leaves using dimethyl sulfoxide and measuring spectrophotometrically [169]. Additionally, leaf cellular membrane stability was determined by measuring electrolyte leakage in temperate grasses [170] three times throughout the experiments on days coinciding with leaf sampling. Leaf relative water content, chlorophyll concentration, and membrane stability were measured in the laboratory after aseptically removing three mature but not senescent whole leaves from each plant. Photosynthetic capabilities and efficiency were further assessed weekly in tropical grasses using a chlorophyll fluorometer (Opti-Sciences Inc., Hudson, NH, USA) following established protocols [171].

Plant growth was non-destructively determined by measuring leaf width on a weekly basis. At the end of the experiment, biomass was measured by harvesting the plants and dividing them into five categories: stems, dead material, roots, mature leaves, and young leaves. Fresh mass was taken for each sample, then dried in an incubator at 70°C for 5 days after which dry mass was determined.

2.4.4 Bacterial Community Analysis

Phyllosphere communities collected from well-watered control and drought-exposed plants were characterized using 16S rRNA marker gene sequences. In the tropical drought experiment, leaves from each plant were collected for total cell counts and DNA extraction on days 1, 12, and 22, totaling 81 samples. In the temperate drought experiment, leaves were collected on days 1, 14, 23, and 36, totaling 112 samples (one Tall Fescue and one Orchardgrass control sample were removed due to pest infestation).
2.4.4.1 Bacterial Cell Counts. Three leaves (~1-2 grams) were placed in 50 ml conical tubes with 10 ml of phosphate-buffered saline (PBS), incubated at room temperature for 1 hour, then vortexed horizontally (vortex-adaptor, Qiagen, Germantown, MD, USA) at full speed for 10 min. The PBS leaf wash was collected and the process repeated. Samples were then fixed with 3.7% paraformaldehyde and stored at 4°C. Samples were filtered onto black polycarbonate membrane filters (pore size 0.2 µm, 25 mm diameter) (Steriltech Corporation, Kent, WA, USA), stained using 0.1% acridine orange for 3 min, and analyzed with epifluorescence microscopy by counting 20 fields using SimplePCI (Hamamatsu, Japan) [172, 173].

2.4.4.2 DNA Extraction and Sequencing. Whole leaves were aseptically removed from plants in the greenhouse and stored at 4°C until bacterial community extraction was carried out the same day. For temperate drought, bacterial DNA was extracted using the Nucleospin Plant II Extraction Kit (Machery-Nagel, Düren, Germany) with 3 modifications. Three whole leaves (~1-2 g) were placed into 15 ml conicals containing 1ml of NucleoSpin Type-B beads and 1.6 ml of Buffer PL1. Tubes were vortexed horizontally for 5 min at room temperature. Aydogan et al. (2018) found that vortexing whole leaf samples in tubes with lysis buffer and beads extracted important community members from biofilms with minimal plant DNA co-extraction [84]. The lysate was incubated for 60 min at 65°C, placed in a NucleoSpin Filter tube, and centrifuged for 2 min at 11000xg following manufacturer instructions. The filtrate was added to 1.6 ml of Buffer PC and extraction continued following recommended protocol steps. For tropical drought, DNA extractions were performed using the QIAGEN RNeasy Power Water Kit,
which extracts total RNA and DNA. This method utilizes the same principle used in temperate grasses in that vortexing whole leaves with beads and lysis buffer increases bacterial DNA extractions while minimizing plant DNA co-extraction. Instead of a membrane filter, a single leaf (~0.5-2 g) was used for extraction following the manufacturer’s protocol to isolate DNA. All final products were stored at -80°C. The extraction methods were compared side by side using leaves collected on the same day from the same plant hosts and leaves which had similar total biomass. In this test, we found similar total yields of DNA which we tested using a qubit and followed up with PCR of the 16S rRNA gene (data not shown).

Samples underwent a two-step PCR amplification to attach Illumina adaptor sequences and barcodes as detailed in Supplemental Materials. The first PCR step used chloroplast excluding primers with linker sequences 799F (5’TACACTGACGACATGGTTCTACA AACMGGATTAGATACCCKG-3’) and 1115R (5’TACGGTAGCAGAGACTTGGTCTCAGGGTTGCGCTCGTTG-3’) targeting the V5-V6 region of the 16S rRNA gene where the underlined portion is the linker sequence followed by the primer sequence [73]. The product from the first PCR was used as template for the second PCR to attach unique Access Array Barcodes (Fluidigm, San Francisco, CA, USA) using previously published methods [174]. The amplicons were pooled and sequenced on Illumina MiSeq Platform, with 251 bp paired-end sequencing chemistry. Illumina PhiX was spiked-in (~25%) to account for the low base diversity. Sequencing was performed at the Genomics Resource Laboratory (University of Massachusetts-Amherst).
2.4.5 Sequence Analysis

Using the QIIME2 [175] pipeline, paired-end reads were demultiplexed, merged, trimmed to 315 bp, and binned using DADA2 [175] inferring amplicon sequence variants (ASVs). Taxonomic identities were assigned using the naïve Bayes sklearn classifier trained with the 799F/1115R region of the Greengenes 13_8 database.

In temperate grass species, 2,142,917 high quality paired-end reads were kept while 560,063 (20.7%) reads failed quality control and were removed. After filtering out chloroplast and mitochondria sequences, 1,853,078 reads were left (4,340-75,714 reads per sample). Seven samples were removed from analysis due to inadequate coverage, leaving 36,277 ASVs representing 39 different phyla.

In tropical grass species, 3,604,936 high quality paired-end reads were kept while 1,555,207 (30.1%) reads failed quality control and were removed. After filtering out chloroplast and mitochondria sequences, 3,522,914 reads remained (10,478-116,876 reads per sample). Two samples were removed from analysis due to inadequate coverage, resulting in 6,400 ASVs representing 28 different phyla. All tropical and temperate samples were rarefied to 4000 reads which sufficiently captured the diversity in both tropical and temperate systems.

2.4.6 Statistical Analyses

Because of the differences in experimental procedures, all analyses were performed separately on samples from tropical and temperate grasses, and models were never made
combining the data. Alpha diversity was calculated using the Shannon Diversity Index, Chao1, Pielou’s Evenness, and ASV Richness. Differences in each alpha diversity metric as a result of species, treatment, and time were calculated using Kruskal-Wallis pairwise comparisons in QIIME2. We additionally assessed changes in Shannon Diversity Index as a result of drought and host species at the end of the experimental period using generalized linear models (GLMs) in R [176]. To then understand these changes over time, we used generalized linear mixed models (GLMMs) with plant ID included as a random effect to account for the repeated measures over the course of the experiment (lme4 package [177]). For both GLMs and GLMMs a gamma distribution with a log link was used because alpha diversity is a continuous variable bounded at zero. Correlation of variables was tested using the Car package [178] and the best model was selected using AICcmodavg [179]. Differences in alpha diversity metrics Chao1, Pielou’s Evenness, and ASV Richness as a result of species, treatment, and time were calculated using Kruskal-Wallis pairwise comparisons in QIIME2. Alpha diversity was assessed separately for the temperate and tropical grasses.

Beta diversity was assessed using weighted UniFrac and Bray-Curtis distances as implemented in QIIME2. Permutational multivariate analysis of variance (PERMANOVA) using the adonis function in the vegan package [180] was used to determine the influence of host species, watering condition, and time on phyllosphere community structure. Post-hoc analysis for pairwise multilevel comparisons was performed using pairwise_Adonis [181]. Separate analyses were run for tropical and temperate grass hosts. Results were visualized by creating nonmetric multidimensional
scaling (NMDS) plots using vegan and ggplot2 [182]. Plant health traits were correlated to community structure and visually overlaid in the NMDS plots using envifit function in the vegan package. Differences in beta diversity were further assessed by identifying bacterial indicator genera for each treatment. Indicators were determined individually for the tropical and temperate grasses using the indicspecies package [183] and visualized by creating ternary plots using the ggtern package [184].

2.4.7 16S rRNA Gene Copy Normalization

Because there is a large range of 16S rRNA gene copy numbers between microbial species, we additionally normalized our data based on 16S rRNA gene copy number to determine if this influenced our results [185]. 16S rRNA gene copy number was normalized using the q2-gcn-norm plugin in QIIME2. After normalization samples were rarefied to 2000 reads to account for the changes in read number, allowing incorporation of maximum samples and sufficient depth to capture community diversity. All alpha and beta diversity analyses were repeated using gene copy number normalized community data.

2.5 Results

2.5.1 Alpha and Beta Diversity

To characterize the response of grassland phyllospheres to drought we grew two species of temperate grass (tall fescue and orchardgrass) and three species of tropical grass (Brachiaria brizantha, Brachiaria decumbens, and Brachiaria hybrid) under three different watering conditions: well-watered control, mild drought, and severe drought.
Because there is a large range of 16S rRNA gene copy number between microbial species, all analyses were conducted using both gene copy number normalized and non-normalized data. No major differences were found between the two methods, so all data reported directly in the paper are from non-normalized data, but corresponding analyses can be found in the supplemental information (Table 2.5, Figures 2.9-2.10).

2.5.1.1 Temperate Grass

Phyllosphere communities on selected temperate grass species were altered as a result of severe drought but the degree to which they changed was dependent on host species. Assessment of alpha diversity using Shannon Diversity Index, Chao 1, Pielou’s Evenness, and ASV richness revealed similar trends regardless of the alpha diversity metric used (Figure 2.11). Because Shannon Diversity Index incorporates both richness and evenness, we decided to use it to model how alpha diversity was impacted over time by drought severity and host species. We fit 20 different GLMMs, which included possible combinations of host species, leaf relative water content, sampling day, and watering treatment as predictor variables and with host ID as a random effect. The best model, selected using the Akaike information criterion [186], was an interactive GLMM comparing host species and leaf relative water content indicating that alpha diversity was impacted by host species identity and decreasing relative water content, but did not change over time (Table 2.6). To further understand the differences, we built a GLM to look at the combined impact of drought and host species on the last sampling day in order to understand the impact of drought when the drought effect was strongest. This model revealed a significant decrease in alpha diversity as a result of severe drought (p =0.01).
but not from mild drought (p=0.6) and that alpha diversity was more impacted by severe
drought on tall fescue than on orchardgrass (Figure 2.1A).

The influence of host species and watering condition on phyllosphere community
structure over time was determined using permutational multivariate analysis of variance
(PERMANOVA) on weighted UniFrac and Bray-Curtis distances. UniFrac metrics
incorporate information on relative relatedness of community members based on
phylogenetic distances. We did not observe strong differences between analyses that used
weighted UniFrac or Bray-Curtis distances, which likely relates to how these
phyllosphere communities are dominated by a few abundant taxa and many rare taxa.
Results from the PERMANOVA revealed sample day as the strongest driver of
community structure and changes in rare taxa (Table 2.1). Host species and watering
treatment were also significant drivers though each explained a smaller amount of
variability (Table 2.1). We performed NMDS ordination of weighted UniFrac and Bray-
Curtis distances to visualize differences in community structure on the last day of the
drought (Figure 2.12). Bacterial community structures were correlated to different
measured plant traits for each host species. On orchardgrass, changes in community
structure were correlated with higher relative water content, higher overall biomass, and a
higher proportion of root mass in the control and mild drought groups while severe
drought had higher proportions of dead leaves (Figure 2.1B). On tall fescue, community
structures of control communities were correlated with higher RWC, mild drought
communities with higher proportion of mature leaf mass, and severe drought showed
increased electrolyte leakage indicating increased level of stress induced injury to plant tissue (Figure 2.1C).

Figure 2.1. Changes in alpha and beta diversity in temperate grass hosts. (A) Phyllosphere community diversity (alpha-diversity) was significantly impacted by severe drought and host species on the temperate grass hosts. The diversity measure is based on the Shannon Diversity Index accounting for both abundance and evenness of the species present. Significant differences between treatments within a host species are indicated by different letters above each boxplot. Changes in bacterial community structure correlate with changes in plant traits on (B) orchardgrass and (C) tall fescue. Ordination was calculated using weighted UniFac Distance on samples collected on the last day of drought treatment and visualized by NMDS. Arrows represent significant correlation (P<0.05). Mature leaves, dead leaves, and roots represent the ratio of total biomass. RWC stands for leaf relative water content, EL for electrolyte leakage.

To determine when in the drought period bacterial communities from each host species were significantly affected, we conducted PERMANOVA analyses on each host species to understand how microbial communities were impacted by drought on each sampling day. By looking at each individual host species, we can better understand how susceptible
to drought each community is based on its host species. Changes in community structure were first observed on orchardgrass on the second day of sampling, day 14, but were not seen until the last day of sampling, day 36, on Tall Fescue (Table 2.2) (Figures 2.13-2.14).

Table 2.1. PERMANOVA revealed sampling day was the strongest driver of community structure.

<table>
<thead>
<tr>
<th>Potential Drivers</th>
<th>Weighted UniFrac</th>
<th>Bray-Curtis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>R²</td>
</tr>
<tr>
<td>Species</td>
<td>2.512</td>
<td>0.025</td>
</tr>
<tr>
<td>Day</td>
<td><strong>10.260</strong></td>
<td><strong>0.154</strong></td>
</tr>
<tr>
<td>Treatment</td>
<td>2.268</td>
<td>0.022</td>
</tr>
<tr>
<td>Species:Day</td>
<td>0.719</td>
<td>0.021</td>
</tr>
<tr>
<td>Species:Treatment</td>
<td>1.111</td>
<td>0.022</td>
</tr>
<tr>
<td>Day:Treatment</td>
<td>1.195</td>
<td>0.035</td>
</tr>
<tr>
<td>Species:Day:Treatment</td>
<td>1.371</td>
<td>0.082</td>
</tr>
</tbody>
</table>

* Compositional dissimilarities between bacterial community structures on the temperate grasses were explained by host species, watering treatment, sampling day, and their interactions using a PERMANOVA on weighted UniFrac and on Bray-Curtis distance measures. Because of the significant three-way interactions, independent PERMANOVAs were conducted for each watering treatment and sampling day to better understand the impact of host species, drought treatment, and time on community structure. Data for the strongest driver are in boldface. *P ≤0.05; **P≤0.01; *** P≤0.001.

Table 2.2. Tall fescue and orchardgrass were significantly affected by severe drought.

Orchardgrass showed earlier changes as a result of drought (Sample Day 14), but by the end of the drought period tall fescue had greater changes as a result of drought.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Sampling Day</th>
<th>Weighted UniFrac</th>
<th>Bray-Curtis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F value</td>
<td>R²</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>1</td>
<td>0.540</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td><strong>2.036</strong></td>
<td><strong>0.270</strong></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>2.548</td>
<td>0.316</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>2.688</td>
<td>0.328</td>
</tr>
<tr>
<td>Tall Fescue</td>
<td>1</td>
<td>0.943</td>
<td>0.158</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.097</td>
<td>0.166</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1.282</td>
<td>0.221</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td><strong>4.273</strong></td>
<td><strong>0.487</strong></td>
</tr>
</tbody>
</table>
2.5.1.2 Tropical Grass

Phyllosphere communities from all tropical grasses were altered as a result of severe drought but no statistical differences were detected between host species. To determine which variable affects phyllosphere communities we conducted the same modelling process used on the temperate grass hosts. Each of the alpha diversity metrics resulted in similar trends, so Shannon Diversity index was chosen for the modelling process (Figure 2.14). The best model was a GLMM comparing a single categorical fixed effect, watering treatment, with host ID as a random effect (Table 2.7). This model indicates that alpha diversity significantly decreased as a result of severe drought (p=0.002), but that mild drought and time had no significant impact. To further understand the effect of drought, we built a GLM to look at the impact of drought and host species on the last sampling day. This model revealed a significant decrease in alpha diversity as a result of severe drought compared to the control treatment (p<0.001), but showed no significant difference between host species (Figure 2.2A).
Figure 2.2. Changes in alpha and beta diversity in tropical grass hosts. (A) Phyllosphere community diversity (alpha-diversity) was significantly impacted by severe drought, but showed no difference between host species on the tropical grass hosts. The diversity measure is based on the Shannon Diversity Index accounting for both abundance and evenness of the species present. Significant differences between treatments within a host species are indicated by different letters above each boxplot. (B) Bacterial communities from control and mild drought treatments correlated with plant traits on tropical grass species. The NMDS ordination was calculated using weighted UniFrac Distance of community samples collected on the last day of drought treatment. Arrows represent significant correlation (P<0.05). RWC stands for leaf relative water content, CC for chlorophyll content measured by chlorophyll extraction, and yield for photochemical efficiency.

The influence that host species and watering condition had over time on beta diversity was determined using PERMANOVA. Results from the PERMANOVA using weighted UniFrac and Bray-Curtis distances revealed watering treatment as the only significant predictor of community structure (Table 2.2). Post hoc analysis on weighted UniFrac analyses revealed, only severe drought was significantly different from the control.
(R^2=0.297, p=0.022) and from the mild drought communities (R^2=0.351, p=0.001), but that control and mild drought treatments were not different from each other (R^2=0.094, p=0.196). Performing an NMDS ordination of the weighted UniFrac and Bray-Curtis distances allowed us to visualize differences in community structure on the last day of the drought. Because no significant difference between host species communities were observed, NMDS was plotted together. When community structure was correlated to measured plant traits, communities under control and mild drought conditions were associated with higher chlorophyll content and photochemical efficiency (yield), higher relative water content, and a higher proportion of young leaves (Fig. 2.2B) (Figure 2.16).

**Figure 2.3.** Average relative abundance of the dominant class of phyllosphere bacteria collected at the end of the experimental period. Each treatment was calculated based on 5 biological replicates in the temperate grass species and 3 biological replicates in the tropical grass species. Changes in bacterial classes by watering treatment were tested using an ANOVA followed by a post hoc Tukey multiple comparison test (Table 2.10). Gammaproteobacteria significantly increased as a result of severe drought on tall fescue (p.adj=0.0002), Orchardgrass (p.adj=0.02), and on the tropical grass species (p.adj=0.002).
2.5.2 Taxonomic Analysis

To gain a deeper understanding of community changes seen between watering conditions at the end of the drought, we compared changes in taxonomy to observed changes in community structure. Using NMDS ordination of Bray-Curtis and weighted UniFrac distances (Figure 2.17) and taxa barplots comparing relative abundance (ANOVA with Tukey’s test post hoc analysis), *Gammaproteobacteria* significantly increased under severe drought in all six host species (Figure 2.3) (Table 2.8). Next, we compared changes in dominant genera between treatments. In temperate grasses, a significant decrease was observed in *Bacillus* and *Pseudomonas* between control and severe drought conditions (Figure 2.4A). The abundancies of *Acinetobacter, Bacillus, Sphingomonas,* and *Staphylococcus* all showed an increasing trend in mild drought and a significant decrease in severe drought communities. *Buchnera* significantly increased and four genera (*Cornyebacterium, Delftia, Erwinia, Streptococcus*) remained unchanged between watering conditions. In the phyllosphere of tropical grasses, six taxonomic groups showed no difference, while *Methylobacterium, Sphingomonas,* and *Hymenobacter* significantly decreased under mild and severe drought, and *Deinococcus* under severe drought (Figure 2.4B). In order to understand how changes in relative abundance related to bacterial load on plant leaves, cell counts per leaf area were conducted. In temperate grasses, a decreasing but not significant trend was observed under severe drought conditions (Figure 2.5A). For tropical grasses, all three species showed a significant decrease in cell counts on hosts under severe drought compared to both control and mild drought conditions (Figure 2.5B).
Figure 2.4. **Average relative abundance of the top 10 most dominant genera in bacterial communities from temperate and tropical grass species.** (A) In temperate grass species each treatment is represented by 10 biological replicates, and in (B) tropical species by 9 biological replicates. Differences were calculated using analysis of variance (ANOVA) followed by a *post hoc* Tukey multiple comparison test. Data are displayed using boxplots, which visualizes the distribution of the data. The box represents the interquartile range with the line in the middle representing the mean value of the data. The whiskers coming from the sides of the boxes represent the minimum and maximum quartiles, and the circles represent outliers. Additionally, significance levels are assigned as $P > 0.05$ (not significant); $* P \leq 0.05$; $** P \leq 0.01$; $*** P \leq 0.001$. 

**Temperate**

- Acinetobacter
- Bacillus
- Buchnera
- Corynebacterium
- Delftia
- Erwinia
- Pseudomonas
- Sphingomonas
- Staphylococcus
- Streptococcus

**Watering Treatment**

- Control
- Mild Drought
- Severe Drought

**Tropical**

- Ammoniphilus
- Bacillus
- Deinococcus
- Erwinia
- Hymenobacter
- Kaistobacter
- Methylobacterium
- Pseudomonas
- Sphingomonas
- Staphylococcus

**Relative Abundance**

0 25 50 75 100
Figure 2.5. The effects of host species and treatment on bacterial biomass. The total number of bacterial cells per leaf area in (A) temperate and (B) tropical grass species. Each watering treatment is represented by 5 biological replicates from the temperate grass hosts and 3 biological replicates for the tropical grass hosts. Number of bacterial cells on tropical grasses decreased significantly as a result of severe drought for each individual species. Significant differences between treatments within a host species are indicated by different letters above each boxplot. Bacterial cells per leaf area (mm$^2$) were calculated by washing bacterial cells off the surface of leaves, counted using epifluorescence microscopy, and compared to leaf areas calculated using ImageJ.

2.5.3 Indicator Analysis

To determine if any phyllosphere bacteria are indicators of watering condition, we performed an indicator analysis (detailed in Table 2.9) [74, 187]. In the temperate grass species, 57 genera were indicators for control treatments, 9 were indicators in mild drought, 23 were indicators shared between control and mild drought, and 2 were indicators of severe drought. The relative abundance distribution of genera between treatments was visualized in ternary plots for the 9 dominant phyla (Figure 2.6A). Several of the most dominant genera were indicator taxa. *Pseudomonas* was an indicator of the
control community; *Sphingomonas* was an indicator of the mild drought community;

*Bacillus, Hymenobacter, and Methylobacterium* were indicators of both control and mild drought communities; and *Buchnera* was an indicator only of severe drought (Table 2.3).

In the tropical grasses, 25 indicators were associated with control treatments, 3 with mild drought, 3 for control and mild drought combined, and none for severe drought. The distribution of genera between treatments was visualized in ternary plots for the 9 most dominant phyla (Figure 2.6B). Indicator taxa that were also dominant phyllosphere genera were *Hymenobacter, Kaistobacter, Methylobacterium, and Sphingomonas* which were indicators of the control condition and *Deinococcus* which were an indicator of control and mild drought conditions.
2.6 Discussion

This study investigated how phyllosphere microbial communities from five different forage grass species responded to drought. Three species of C₄ tropical grasses and two species of C₃ temperate grasses were grown as well-watered controls and under different drought conditions in a greenhouse to investigate how plant taxonomy and traits
influence bacterial community assembly and response to stress. Few studies have compared phyllosphere studies in the greenhouse to studies in the field, but they consistently show that bacterial communities in the greenhouse had lower numbers of bacteria, reduced bacterial diversity, and a shift in dominant bacterial class [188, 189]. Despite these differences, these studies concluded that greenhouse studies provide an important controlled framework to study phyllosphere community assembly and succession.

Common characteristics in the microbial communities across each grass species were observed, such as high abundance of *Gammaproteobacteria* and *Alphaproteobacteria* consistent with previous grassland phyllosphere studies [61, 84]. Other groups, including *Actinobacteria*, *Deinococci*, *Betaproteobacteria*, and *Bacilli*, were among the most dominant classes of phyllosphere bacteria on every grass species, but occurred at different abundances between host species. Previous work comparing phyllosphere communities from temperate and tropical tree species also found that some phyla were consistently present on all tree species and are likely common phyllosphere residents, indicative of potentially beneficial effects on plant hosts [122]. Phyllosphere microbes directly promote plant growth and stress tolerance through phytohormone and nutrient production and protection from UV damage [99, 109, 190]. Identifying microbes, both taxonomically and functionally, that can continue to survive and promote plant health under changing climatic conditions will be important for creating resilient agricultural systems.
2.6.1 Host Species Impact on Community Structure

Consistent with previous studies, we found that plant species affected microbial community structure, but aspects of community structure varied by host species. The microbial communities from the temperate grass species were distinguishable from each other under severe drought conditions while the three tropical grasses were not distinguishable when using weighted UniFrac and Bray-Curtis distances. The tropical grass species are more closely related to each other than the temperate grass species (Figure 2.18). Phylogenetic relationships of the host could therefore account for the varying effects each plant species has on microbial community structure. Kembel et al. [72] found that different bacterial community structures could be explained by different host attributes including resource uptake strategies, leaf morphology, and physiology. This confirmed our first hypothesis that host identity influences microbial community assembly and that communities coming from closely related plants are more similar to each other.

2.6.2 Bacterial Stress Response Correlates to Plant Stress Response

Different grass species employed dissimilar strategies for dealing with drought stress. While some universal trends were observed in phyllosphere response to drought, the differences seen in resource uptake strategies between host species under control conditions were exacerbated under drought conditions. Three methods of drought tolerance used by plants are commonly identified: drought escape, drought avoidance, and tolerance of dehydration [191]. Drought escape is often seen as dormancy of the plant through dehydration of tissue. Instead of complete dormancy, orchardgrass and tall
fescue enter a state of inactivity where no growth occurs and leaves senesce [191]. Conversely, tropical grass species remain productive throughout the dry season and previous greenhouse studies that were consistent with ours showed only minor decreases in biomass under drought conditions [34, 192]. None of the grasses compared in this project utilize the dormancy strategy, but instead employ some combination of the other two strategies (Table 2.10). Every grass species showed a decrease in phyllosphere alpha-diversity, but the extent varied greatly, with the tropical grass species experiencing a smaller reduction than temperate species. The tropical grasses used in this study each utilize drought avoidance by forming deep-rooting networks to acquire water (Table 2.10). Their roots, reaching two meter depths, have high extraction efficiency allowing continuous water uptake during dry conditions [34, 192]. Additionally, because of their continued growth and color retention during low water stress, we propose they exhibit dehydration tolerance. One mechanism the tropical grasses use to tolerate dehydration is stomatal closure at relatively high leaf water potential [192–194]. This is likely the reason that tropical grasses showed a significant decrease in the number of cells present on their leaf while temperate grasses remained unchanged. Since stomata provide an interface for nutrient and water acquisition by the microbes, less plant-provided nutrients and water are available on the leaf surface for the microbes as drought stress increases and plants close their stomata to reduce transpiration [42, 50, 195]. Because tropical species have high drought tolerance and employ similar methods to counteract drought stress, we only observed minor changes in their phyllosphere community diversity and structure between treatments.
Conversely, previous work on orchardgrass and tall fescue found the two species have different drought survival strategies which parallels the increased differences observed in their microbial communities [191]. Tall fescue utilized drought avoidance by growing extensive and deep-rooted networks while orchardgrass relied on water uptake in low soil moisture conditions. Additionally, orchardgrass shows tolerance of dehydration by protecting the meristem against dehydration and promoting membrane stabilization. In our study, phyllosphere communities found on orchardgrass showed early changes in community structure as a result of drought, before any plant response was observed. However, by the end of the drought period, we observed a greater decrease in alpha-diversity and community structure was more impacted by severe drought stress even though Tall Fescue plants showed similar signs of stress compared to orchardgrass (Figure 2.14). Early response by the orchardgrass communities could indicate a host-microbe response resulting in increased stress resilience. Overall, changes in diversity as a result of stress were host species dependent, thereby supporting hypothesis 3 and simultaneously raising the question: are phyllosphere communities and the functions they provide a plant trait?

### 2.6.3 Drought Stress Changes Phyllosphere Community Diversity and Structure

Under severe drought stress, bacterial communities on leaf surfaces showed some common trends. Confirming hypothesis 2, alpha-diversity decreased, and community structure shifted, resulting in increased relative abundance of *Gammaproteobacteria*. Previous work found that increased phyllosphere bacterial diversity resulted in higher ecosystem productivity, likely relating to the complementarity effect, a situation in which
more diverse communities use more available resources as a result of niche partitioning and are therefore more productive [111, 196]. Additionally, high diversity frequently results in functional redundancy, which helps promote resiliency and resistance of communities by increasing the likelihood that niches are filled under various environmental conditions [146, 197]. Therefore, a loss of bacterial community diversity and change in community structure could indicate decreased plant and bacterial community health.

Potentially beneficial genera found on several grass species were identified as indicators of control or mild drought conditions and were suppressed under severe drought (Table 2.3). Of particular interest were the indicators of mild drought, either singly or in combination with control, because these are bacteria able to withstand some level of climate stress and could therefore be good biofertilizer targets. The dominant mild drought indicators include *Bacillus, Deinococcus, Hymenobacter, Methylobacterium, Pseudomonas*, and *Sphingomonas* (Table 2.3). While their interaction with plants in this study is unknown, previous work has shown several different beneficial relationships with plant hosts. Soybean plants inoculated with *Sphingomonas* demonstrated increased growth and drought tolerance [198], while *Methylobacterium* residing in the phyllosphere have been linked to nitrogen-fixation and biomass production [50, 105]. Additionally, some *Methylobacterium* and *Deinococcus* are resistant to UV radiation which gives them a selective advantage on the leaf surface and also helps to protect the plant from UV damage [199, 200].
In drought stressed temperate and tropical grasses, the dominant *Gammaproteobacteria* included bacteria from genera known to be potential plant pathogens, such as *Erwinia* [84, 201]. These results are consistent with previous research looking at grasslands under climate stress. Aydogan et al. (2018) observed that with the rise in *Gammaproteobacteria*, caused by elevated ambient temperatures in grasslands, the appearance and growing preeminence of potential plant and human pathogens also increased [84].

**Table 2.3.** Functions that have been observed in the dominant genera found on tropical and temperate grass species. These genera were also identified as indicator genera in this study.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Indicator</th>
<th>Observed Functions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus</em></td>
<td>Temperate control-mild</td>
<td>Biological control agent; in rhizosphere increase biomass and growth, phytohormone production (IAA, GAs, cytokinins); under drought conditions promote plant growth through increased availability of water, nutrients and hormones and regulate drought response genes.</td>
<td>[202–204]</td>
</tr>
<tr>
<td><em>Deinococcus</em></td>
<td>Tropical control-mild</td>
<td>Resistant to UV damage and radiation; degradation of aromatic compounds</td>
<td>[200, 206, 207]</td>
</tr>
<tr>
<td><em>Hymenobacter</em></td>
<td>Temperate control-mild Tropical control</td>
<td>Potential phyllosphere “hub” taxa; can produce carotenoids which can be involved in photosynthesis, stabilize cellular membranes, and act as antioxidants; and have genes involved in UV-damage DNA repair</td>
<td>[84, 208, 209]</td>
</tr>
<tr>
<td><em>Kaistobacter</em></td>
<td>Tropical control</td>
<td>Potential disease suppression; associated with healthy plants in rhizosphere and phyllosphere studies but function unknown</td>
<td>[210–212]</td>
</tr>
<tr>
<td><em>Methylobacterium</em></td>
<td>Temperate control-mild Tropical control</td>
<td>Promote plant growth; nitrogen fixation; phytohormone production; protect against desiccation by producing osmo-protectants</td>
<td>[105, 213–215]</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Temperate control</td>
<td>Produce phytohormones (IAA, cytokinins, and gibberellins), protect against diseases (through antibiosis, niche exclusion, quorum sensing, and induction of systemic resistance) Some pseudomonads are also plant pathogens</td>
<td>[88, 90, 216]</td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>Temperate mild Tropical control</td>
<td>Increase water uptake under drought through enhanced root growth, pathogen protection</td>
<td>[84, 93, 217]</td>
</tr>
</tbody>
</table>
Temperate grass species also saw changes in the genus *Buchnera*, for which high abundance was an indicator genus of the severe drought treatment (Table 2.9A). *Buchnera*, an aphid endosymbiont [218], has previously been found in grassland phyllospheres under climate stress [84]. Throughout our study, aphids were found on the temperate study plants but the higher abundance of *Buchnera* on severe drought plants is consistent with previous studies looking at the effects of drought or temperature stress on plants, which found they are more susceptible to aphid infestation as a result of changes in secondary metabolite production [219, 220]. However, we cannot discount the idea that increased pest presence under stress conditions results from changing phyllosphere communities [84]. By increasing abundance of potential pathogens and decreasing abundance of commensals and bacterial diversity, drought stress weakened the phyllosphere’s ability to promote plant health and growth.

### 2.6.4 Conclusion

Plant productivity is directly influenced by microbes in the phyllosphere, but what factors govern these symbiotic relationships is still unknown. To date, this is the first study to show the significant effects drought has on phyllosphere bacterial communities of grasses. Five commonly used grass species, specifically selected for their variation in native climate zone, taxonomy, and pathways of carbon fixation, were used in order to understand how grassland phyllosphere communities are composed and whether community response to stress is similar. Some similarities in bacterial communities and their response to stress were observed, and the overall trends may be representative for other temperate and tropical grasslands. Bacterial community abundance and diversity
decreased while relative abundance of potentially pathogenic bacteria increased, indicating reduced bacterial community health. Additionally, differences in microbial communities in relation to plant traits became more defined under stress conditions. Still, the question remains if bacterial communities can act as a stress response trait for the host plants. Finally, we found that some bacteria indicators of mild drought conditions are potential plant symbionts and are therefore useful targets for biofertilizers designed to promote agricultural systems under climate stress. Future studies should focus on functional interactions between communities and plant host in response to stress conditions in order to understand how bacteria promote plant growth and stress tolerance. By understanding microbe-microbe and plant-microbe interactions, we can better support agricultural practices and discover ways to promote ecosystem productivity through the use of bacteria.

Studying microbial community assembly and response to drought in greenhouse environments enables us to exhibit high levels of control on the system, thus allowing us to distill patterns in assembly processes. However, because of the environmental control in the greenhouse, plant growth and microbial community assembly patterns do not always replicate what is happening in the field. We, therefore, used the information we had learned in the greenhouse studies to develop hypotheses to explore and test in a field study. In the next chapter, we employ many of the same principles used in this chapter to study microbial community dynamics and response to drought in a modified field experiment. In the following chapter, all plants were planted in the greenhouse at the same time as plants from this chapter, but were moved to the field environment for a
separate experiment. Both experiments were conducted in pots using soil from the same natural grassland in Amherst, MA.

2.7 Data Availability

The 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA727243 for tropical grasses and PRJNA727101 for temperate hosts.

2.8 Supplemental Methods

2.8.1 High Throughput Sequencing with Barcodes

All phyllosphere community DNA samples underwent a two-step PCR amplification to attach barcodes and Illumina adaptor sequences. In the first PCR step, chloroplast excluding primers targeting the V5-V6 region of the 16S rRNA gene with linker sequences were used. The primer pair consists of the forward primer (799F):

\[ 5'\text{ACACTGACGACATGGTTCT ACA ACMGGATTAGATACCCKG-3', and the } \]

Reverse primer (1115R): \[ \text{TACGGTAG CAGAGACTTGTTCT AGGGTTGCCTCGTG-3', where the underlined portion is the linker sequence followed by the primer sequence. The first PCR step was a 30 µl reaction containing 3 µl of 10X OmniKlentaq buffer (DNA Polymerase Technology, St. Louis, MO), 2.4 µl dNTPs (10 µM), 0.75 µl forward primer (5 µM), 0.75 µl reverse primer (5 µM), 0.5 µl bovine serum albumin (BSA), 0.2 µl OmniKlentaq LA, 3 µl DNA, and 19.4 µl molecular-grade water. The PCR reaction had an initial denaturation step at 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 60°C for 30 s, 68°C for 30 s, and a final elongation step for 3 min at 68°C. The} \]
product from this PCR was used as the DNA template for the second PCR step, which attached individual barcodes to each sample to allow separation of samples in downstream analyses. The second PCR step was a 20 μl reaction containing 10 μl of 2X Dreamtaq Mastermix (Thermo Fisher Scientific, Waltham, MA), 4 μl water, 2 μl DNA, and 4 μl of Access Array Barcode primer pools (Fluidigm, San Francisco, CA). The second PCR reaction consisted of an initial 5 min denaturation step at 95°C; 8 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final 7 min elongation step at 72°C. The amplicons were pooled and sequenced on Illumina MiSeq Platform, with 251 bp paired-end sequencing chemistry. Illumina PhiX was spiked-in (~ 25%) to account for the low base diversity. Sequencing was performed at the Genomics Resource Laboratory (University of Massachusetts-Amherst).
2.9 Supplemental Tables

Table 2.4. Soil physical-chemical analysis for experiments with tropical and temperate grasses. Macronutrient and micronutrient concentrations were determined using Modified Morgan extractables.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Temperate</th>
<th>Tropical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil pH</td>
<td>5.4</td>
<td>4.3</td>
</tr>
<tr>
<td>Macronutrients (ppm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>8.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>170</td>
<td>79</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>411</td>
<td>138</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>78</td>
<td>34</td>
</tr>
<tr>
<td>Sulfur (S)</td>
<td>6.1</td>
<td>16.7</td>
</tr>
<tr>
<td>Micronutrients (ppm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boron (B)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>3.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
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<td>2.6</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>12.3</td>
<td>44.3</td>
</tr>
<tr>
<td>Aluminum (Al)</td>
<td>50</td>
<td>167</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>6.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Cation Exch. (meg/100 g)</td>
<td>10.5</td>
<td>17.5</td>
</tr>
<tr>
<td>Exch. Acidity (meg/100 g)</td>
<td>7.4</td>
<td>16.4</td>
</tr>
<tr>
<td>Soil Organic Matter (%)</td>
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<td>7.7</td>
</tr>
<tr>
<td>C:N Ratio</td>
<td>12.3</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Table 2.5. PERMANOVA was conducted on weighted UniFrac distances of Gene copy number normalized data. Similar drivers were significant in the GCN normalized data as in the unnormalized data.

<table>
<thead>
<tr>
<th>Potential Drivers</th>
<th>Temperate Hosts</th>
<th>Tropical Hosts</th>
<th>Weighted UniFrac</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>R²</td>
<td>P value</td>
</tr>
<tr>
<td>Species</td>
<td>2.409</td>
<td>0.017</td>
<td>0.040*</td>
</tr>
<tr>
<td>Day</td>
<td>11.050</td>
<td>0.234</td>
<td>0.001***</td>
</tr>
<tr>
<td>Treatment</td>
<td>3.710</td>
<td>0.052</td>
<td>0.001***</td>
</tr>
<tr>
<td>Species:Day</td>
<td>1.052</td>
<td>0.022</td>
<td>0.398</td>
</tr>
<tr>
<td>Species:Treatment</td>
<td>1.020</td>
<td>0.014</td>
<td>0.420</td>
</tr>
<tr>
<td>Day:Treatment</td>
<td>1.709</td>
<td>0.072</td>
<td>0.014*</td>
</tr>
<tr>
<td>Species:Day:Treatment</td>
<td>1.353</td>
<td>0.057</td>
<td>0.092</td>
</tr>
</tbody>
</table>
Table 2.6. GLMM model selection on temperate grass species. Models with combinations of fixed effects were tested and compared using AIC to determine which model best explained trends in alpha diversity over the experimental period.

<table>
<thead>
<tr>
<th>GLM</th>
<th>AIC score</th>
</tr>
</thead>
<tbody>
<tr>
<td>glmer(shannon ~ RWC* Species+ (1</td>
<td>ID), data = alpha_field, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC+ Species+ (1</td>
<td>ID), data = alpha_field, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC* Day* Species + (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ RWC+ (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ RWC+ Day+ Species+ (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ RWC+ Day+ (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ Day*Treatment + (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ Species<em>Treatment</em>Day + (1</td>
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<tr>
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<td>glm(shannon ~ 1, data = alpha_field, family=Gamma(link=log))</td>
<td>440.7882</td>
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<td>glmer(shannon ~ Species + (1</td>
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<tr>
<td>glmer(shannon ~ Treatment + (1</td>
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</tr>
</tbody>
</table>
Table 2.7. GLMM model selection on tropical grass species. Models with combinations of fixed effects were tested and compared using AIC to determine which model best explained trends in alpha diversity over the experimental period.

<table>
<thead>
<tr>
<th>GLM</th>
<th>AIC score</th>
</tr>
</thead>
<tbody>
<tr>
<td>glmer(shannon ~ Day+Treatment + (1</td>
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<td>glmer(shannon ~ Treatment + (1</td>
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</tr>
<tr>
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<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC+ Day+ (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ Day*Treatment + (1</td>
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<tr>
<td>glmer(shannon ~ Species+Treatment + (1</td>
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</tr>
<tr>
<td>glmer(shannon ~ Species+Treatment+Day + (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ Species+Day + (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC* Day+ Species+ (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ Species* (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC+ Species+ (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC* Species+ (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glm(shannon ~1, data = alpha_trop, family=Gamma(link=log))</td>
<td>242.7032</td>
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</tr>
<tr>
<td>glmer(shannon ~ 1 + (1</td>
<td>Day), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ RWC* Day* Species + (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
<tr>
<td>glmer(shannon ~ Species<em>Treatment</em>Day + (1</td>
<td>ID), data = alpha_trop, family=Gamma(link=log))</td>
</tr>
</tbody>
</table>
Table 2.8. Differences in relative abundance of each bacterial class were determined for (A) Tall Fescue, (B) Orchardgrass, and (C) Tropical grasses. Analyses on temperate grasses were conducted separately because PERMANOVA analyses revealed microbial communities were significantly different between the host species. No significant difference was detected on communities from tropical grass hosts, so class analyses were conducted together. Differences between treatment for each class was determined using an ANOVA with TukeyHSD analyses. The column labelled diff is the difference between average relative abundance of the two treatments and lwr and upr represent the lower and upper 95% confidence intervals. P values were calculated for multiple comparisons. Significant p-values are in bold font.

<table>
<thead>
<tr>
<th>Bacterial Class</th>
<th>Comparison</th>
<th>diff</th>
<th>lwr</th>
<th>upr</th>
<th>P adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammaproteobacteria</td>
<td>Mild-Control</td>
<td>-0.0013</td>
<td>-0.3009</td>
<td>0.2983</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Severe-Control</td>
<td>0.5790</td>
<td>0.2925</td>
<td>0.8655</td>
<td>&gt;0.001</td>
</tr>
<tr>
<td></td>
<td>Severe Mild</td>
<td>0.5803</td>
<td>0.3172</td>
<td>0.8435</td>
<td>&gt;0.001</td>
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Table 2.9. Indicator analysis identified indicator genera for each treatment from the (A) temperate climate group, and (B) tropical climate group. Genera were categorized as an indicator group if the indicator value was higher than 0.3 and P-value <0.05.

(A) Indicator genera of the temperate climate group

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### (B) Indicator genera or the tropical climate group

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Table 2.10. Average plant traits expected under optimal growth conditions.

<table>
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<tr>
<th>Grass Species</th>
<th>Carbon Fixation</th>
<th>Height (cm)</th>
<th>Root Depth (m)</th>
<th>Leaf Hairs</th>
<th>Leaf Width (mm)</th>
<th>Leaf Length (cm)</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>Annual Water (mm)</th>
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<td>2</td>
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<td>40-100</td>
<td>5-6</td>
<td>25-35</td>
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<tr>
<td>B. decumbens</td>
<td>C₄</td>
<td>30-150</td>
<td>2</td>
<td>yes</td>
<td>7-20</td>
<td>5-25</td>
<td>5-6</td>
<td>25-35</td>
<td>&gt;1500</td>
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<td>C₄</td>
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<td>yes</td>
<td>25</td>
<td>40-65</td>
<td>5.5</td>
<td>25-35</td>
<td>&gt;700</td>
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<tr>
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<td>45-120</td>
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<td>10-60</td>
<td>5.5</td>
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<td>10-45</td>
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Table 2.11. Plant dry mass at the end of the drought for each species and treatment (each replicate is represented). Plants were separated into each mass group on harvest day, dried for 5 days at 70°C, and then weighed to understand differences in plant traits and stress response strategies between the plant species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Mature Leaf Mass (g)</th>
<th>Young Leaf Mass (g)</th>
<th>Dead Leaf Mass (g)</th>
<th>Stem Mass (g)</th>
<th>Root Mass (g)</th>
<th>Total Biomass (g)</th>
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<td>3.53</td>
<td>2.24</td>
<td>13.75</td>
<td>9.29</td>
<td>33.88</td>
</tr>
<tr>
<td>B. brizantha</td>
<td>Control</td>
<td>6.45</td>
<td>5.69</td>
<td>3.29</td>
<td>21.13</td>
<td>12.11</td>
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<td>6.28</td>
<td>6.88</td>
<td>19.85</td>
<td>13.02</td>
<td>51.53</td>
</tr>
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<td>4.84</td>
<td>4.28</td>
<td>7.48</td>
<td>18.48</td>
<td>8.93</td>
<td>44.01</td>
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<tr>
<td>B. decumbens</td>
<td>Control</td>
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<td>2.60</td>
<td>3.08</td>
<td>7.21</td>
<td>3.65</td>
<td>18.74</td>
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<tr>
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<td>Control</td>
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<td>5.06</td>
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<td>19.25</td>
<td>8.85</td>
<td>41.02</td>
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<td>Root Mass (g)</td>
<td>Total Biomass (g)</td>
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2.10 Supplemental Figures

**Figure 2.7.** Temperate grass measurements taken during the drought period revealed different plant response strategies over time. (A) soil moisture content, (B) relative water content, (C) Electrolyte leakage, (D) Leaf width, and (E) Chlorophyll content.

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<td><strong>Electrolyte Leakage (%)</strong></td>
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**Figure 2.8.** Tropical grass measurements taken during the drought period were (A) relative water content, (B) Chlorophyll content, (C) Light adapted photosynthetic efficiency (Yield), and (D) Dark adapted photosynthetic efficiency (Fv/Fm). Measurements revealed changes over time as a result of drought.

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<td><strong>Dark Adapted Photosynthetic Efficiency (Fv/Fm)</strong></td>
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Figure 2.9. Alpha diversity calculated using 16S rRNA gene copy number (GCN) normalized data had similar results as non-normalized data.
Figure 2.10. Community profiles based on relative abundance and their response to drought calculated using 16S rRNA gene copy number normalized data were similar to the profiles calculated using non-16S rRNA gene copy number normalized data.

Figure 2.11. Alpha diversity in orchardgrass and tall fescue was determined on the last sampling day when drought effect was strongest using (A) Chao 1, (B) Pielou’s Evenness, and (C) Richness measured by total number of observed ASVs. In each method, mild drought was not significantly different from the control treatment while alpha diversity significantly decreased as a result of drought. Different letters indicate significant differences measured using Kruskal-Wallis pairwise comparisons.
Figure 2.12. NMDS based on Bray-Curtis distance metrics shows that community structure changes as a result of severe drought increasing differences between communities found on orchardgrass and tall fescue.

Figure 2.13. NMDS of (A-D) Bray-Curtis and (E-H) weighted UniFrac distances on phyllosphere communities removed from orchardgrass comparing watering condition for each of the sampling days. Significant effects from drought were first observed on day 14.
Figure 2.14. NMDS of (A-D) Bray-Curtis and (E-H) weighted UniFrac distances on phyllosphere communities removed from Tall fescue comparing watering condition for each of the sampling days. Significant effects from drought were not observed until day 36.
Figure 2.15. Alpha diversity in *Brachiaria brizantha*, *Brachiaria decumbens*, and *Brachiaria* hybrid was determined on the last sampling day when drought effect was strongest using (A) Chao 1, (B) Pielou’s Evenness, and (C) Richness measured by total number of observed ASVs. Phyllosphere communities sampled from *Brachiaria brizantha* were the only communities to show a significant reduction as a result of drought.

Figure 2.16. NMDS based on Bray-Curtis distance metrics shows that community structure changes as a result of severe drought, but no effect was observed as a result of host species.
Figure 2.17. Changes in relative abundance of bacterial genera as a result of drought stress correlate with changes in bacterial community structure in (A-B) temperate grass species and (C-D) tropical grass species. The relative abundance data were calculated from rarefied community data. Ordination was calculated using Bray-Curtis (A & C) and weighted UniFrac Distance (B & D) on samples collected on the last day of drought treatment. Arrows represent significant correlation ($P<0.01$).
Figure 2.18. Cladogram depicting the taxonomic relationship of several grass species based on NCBI taxonomy [1] shows that *B. decumbens* and *B. brizantha* are more closely related to each other than they are to Orchardgrass (*Dactylis glomerata*) and Tall Fescue (*Festuca arundinacea*) and orchardgrass and tall fescue are to each other. The cladogram was generated using phyloT and visualized with iTOL [2].
CHAPTER 3
EVOLUTIONARY HISTORY IMPACTS PHYLLOSHERE COMMUNITY ASSEMBLY ON FORAGE GRASSES

3.1 Abstract

Benefits leaf bacterial communities provide to plant hosts are reduced by external stress. Understanding how plant hosts impact phyllosphere community assembly, how microbes influence plant traits, and how this interaction changes under stress will advance our insight into the evolutionary relationship between plants and their microbial communities. We investigated phyllosphere community assembly change over time, between host species, and under drought stress on three native temperate grasses and three non-native tropical grasses. By growing them together, effects of host geography and differences in environmental variables were eliminated allowing us to test evolutionary history on community assembly. We found evidence of phylosymbiosis which increased significantly under drought stress, indicating phyllosphere communities and their response to stress relate to grass species phylogeny. We also show native temperate grasses displayed stronger cophylogenetic relationships between grass hosts and their microbial communities and had increased selection by host species over time compared to non-native tropical hosts. Interestingly, the functional marker gene nifH, though differentially present on all host species was not susceptible to drought. The evidence of shared evolutionary history, presence of functionally important bacteria, and responses to drought suggest that microbial communities are important plant traits that coevolve alongside their plant hosts.
### 3.2 Introduction

As one of the largest terrestrial habitats, grasslands make up nearly 70% of global agricultural land and contribute important ecosystem services including impacting water quality, erosion prevention, and climate regulation through carbon sequestration and greenhouse gas mitigation [221]. The important agricultural and ecological functions grasslands provide are threatened due to projected decreases in water availability as drought frequency and severity continue to increase [152, 156, 222]. This will have drastic effects on grassland productivity, ultimately reducing global food security and increasing climate change [223].

Grass leave surfaces harbor diverse microbial communities, termed the phyllosphere, which provide important functions to their host including disease prevention, stress tolerance, ecosystem productivity, and nutrient cycling through processes such as nitrogen fixation [99, 111, 224, 225]. In return, plants provide nutrients to the bacteria creating a symbiotic relationship, but what drives these relationships is not completely understood. Previous studies show that while plant host identity plays an important role in microbial community assembly, phyllosphere communities are broadly dominated by similar taxa including *Proteobacteria, Bacteroidetes*, and *Actinobacteria* [58, 72, 73, 84].

Common theories to explain phyllosphere community assembly include the existence of a functional core community, in which phyllosphere community members provide consistent functional traits across host species [58, 226], and the hologenome theory of evolution, which postulates evolution occurs between hosts and microbes together [227].
Many core functions support epiphytic bacterial growth under harsh conditions indicating microbial adaptation to the phyllosphere. These include pigmentation and DNA repair systems to protect from UV radiation, production of extracellular polysaccharides to promote biofilm formation which protects against osmotic stress, and motility-related proteins for movement towards nutrients [42, 58, 225, 228]. Additional functional traits are important for plant health and ecosystem functioning, by promoting global carbon and nitrogen cycles, photosynthetic strategies, resource acquisition, and plant defense [58, 226, 229]. Nitrogen fixation by bacteria called diazotrophs, frequently associated with the rhizosphere, occurs in the phyllosphere contributing to total nitrogen input in an ecosystem [58, 99, 104]. The observed differences in relative abundance of functional genes and taxonomic identity despite low variability between host species [72, 226], suggest the functional core exists within the hologenome theory. For example, phyllosphere bacteria can have rhodopsins which provide energy and protection from UV damage. These pigments absorb different wavelengths of light than their plant host allowing for optimal utilization of resources thus indicating shared evolutionary history [159, 230, 231].

How functional profiles and plant-microbe relationships change under stress conditions is still unknown. Therefore, we do not understand if response to stress is a stochastic process dependent largely on atmospheric conditions, a response to changes in plant physiology, or a response characterizing joint plant-microbe interactions. One method to explore host-microbe relationships is phylosymbiosis, which determines if significant associations between microbial communities and the phylogeny of their host species exist.
Phylosymbiosis can be determined using a Mantel test to compare a host phylogenetic distance matrix to a microbial community distance matrix. When phylosymbiosis occurs, phylogenetically related host species have more similar microbial communities than less phylogenetically related hosts. Phylosymbiosis can result from coevolution, which occurs when plant-microbe systems act as reciprocal selective forces on each other [234, 235]. However, it can also result from differences in host geography, host traits, or codiversification, which occurs when hosts and microbes exhibit parallel divergence during continued associations [236]. A second method used to understand how host phylogeny relates to microbial communities is cophylogeny, which tests the concordance of the host phylogeny with the phylogeny of the associated microbial community [237, 238]. Cophylogenetic occurrences indicate shared evolutionary history between hosts and microbial groups [238, 239]. While cophylogeny can result from processes such as biogeographical distance, presence of cophylogeny is consistent with host-microbe coevolution [238, 240, 241]. Previous work suggests that cophylogenetic associations are more likely to exhibit microbe-to-host interactions [93, 226, 242]. Therefore, identifying these associations can help identify evolutionarily important and ecologically active plant-microbe relationships.

To understand plant-microbe interactions we need to understand rules of assembly and functional processes. Our objective was to investigate if phyllosphere communities are an adapted plant trait. To address this objective, we explored the questions: (i) How does host phylogeny influence microbial community assembly? (ii) How does host identity or phylogeny influence microbial community response to drought stress? (iii) How is
diazotroph abundance related to microbial community structure and response to stress? To answer these questions, we investigated how microbial community assembly changed over time, between host species, and under drought stress. We chose three species of grasses commonly used in temperate forage systems and three species commonly used in tropical forage systems. By growing all species in the same common garden experiment, we eliminated effects of host geography and differences in environmental variables on community assembly. Additionally, by growing native temperate and foreign tropical species, we tested the influence of evolutionary history on community assembly. Comparing the evolutionary history of phyllosphere communities to that of their hosts and determining how communities change under drought stress, allowed us to understand if phyllosphere microbes are a plant trait and begin to understand how to leverage microbes to promote plant growth and stress tolerance.

3.3 Materials and Methods

3.3.1 Study system

Seeds for three non-native tropical grasses, *Brachiaria brizantha* (CIAT 26564), *Brachiaria decumbens* (CIAT 6370), and *Brachiaria* hybrid (CIAT 1794), were acquired from CIAT (Cali, Columbia). Native temperate grass species seeds, *Festuca arundinacea* (endophyte free Tall Fescue), *Dactylis glomerata* (Orchardgrass), and *Lolium perenne* (Ryegrass), were acquired from Albert Lea Seed Company (Albert Lea, MN, USA). Seeds were germinated in Pro-mix commercial potting medium (Quakertown, PA, USA) in 2018 and grown in the College of Natural Sciences Research and Education Greenhouse at the University of Massachusetts-Amherst. In June 2019, individual plants
were transplanted into 15x30cm pots filled with soil collected from natural grass fields in Amherst, MA (Supplementary Methods, Table 3.2). Pots were moved outside, organized in a randomized block design, and allowed to re-establish. Ten plant replicates of each temperate species were divided between ‘control’ and ‘drought’ treatments. Drought treatment plants were placed under a 10 ft high rain shelter made of greenhouse plastic allowing maximal airflow and high UV light penetration (Figure 3.7). Drought conditions were imposed over 38 days (21 AUG - 27 SEPT 2019). Plants in the control group were given supplemental water to maintain soil moisture above 80% field capacity. Plants in the drought group were given supplemental water when necessary to maintain an even dry-down rate, determined from soil-moisture readings measured twice weekly using a MiniTrase TDR with Buriable probe (Soilmoisture Equipment Corp., Goleta, CA, USA).

3.3.2 Plant Health Measurements

Plant health measurements were taken on days 1, 19, 26, 33, and 38 to understand the effect of drought on the plant host. Plant measurements taken were leaf relative water content (RWC), chlorophyll concentration, and leaf cellular membrane stability determined by measuring electrolyte leakage [168, 170, 171]. At the end of the drought period, above ground biomass was measured by dividing plant material into five categories: stems, flowers, dead, mature, and young leaves. After determining fresh mass, samples were dried in an incubator at 70°C for 5 days and dry mass was measured.
3.3.3 Bacteria Community Sampling

At each timestep, bacterial community DNA was extracted using the Nucleospin Plant II Extraction Kit (Machery-Nagel, Düren, Germany) following a modified protocol. Five whole ryegrass leaves or three whole leaves of each other species were aseptically removed from the plant host and placed into a 15 ml conical tube with 1.5 ml of NucleoSpin Type-B beads and 4X volume of Buffer PL1. Tubes were vortexed horizontally for 5 min at room temperature. The lysate was incubated for 60 min at 65°C, placed in a NucleoSpin Filter tube, and centrifuged for 2 min at 11,000xg. The filtrate was added to 4X Buffer PC and extraction continued following the manual. Aydogan et al. found that vortexing whole leaf samples in tubes with lysis buffer and beads extracted important community members from biofilms with minimal plant DNA co-extraction [84]. Extracted DNA samples underwent a two-step PCR amplification to attach Illumina adaptor sequences and barcodes (Supplemental Methods). The first PCR step used chloroplast excluding primers 799F and 1115R targeting the V5-V6 region of the 16S rRNA gene [73] with linker sequences to attach Access Array Barcodes (Fluidigm, San Francisco, CA, USA) [174]. Amplicons were pooled and sequenced on Illumina MiSeq Platform, with 251 bp paired-end sequencing chemistry at the Genomics Resource Laboratory (University of Massachusetts-Amherst). The abundance of nitrogen-fixing bacteria was determined using qPCR quantifying the nifH gene using the PoL and PoR primers [243].
3.3.4 Sequence Analysis

Using the QIIME2 [175] pipeline, paired-end reads were demultiplexed, merged, trimmed to 315 base pairs, and binned inferring amplicon sequence variants (ASVs). Taxonomic identities were assigned using the naïve Bayes sklearn classifier trained with the 799F/1115R region of the Greengenes 13_8 database.

The data contained 9,207 ASVs from 280 samples containing a total of 15,218,029 reads. Samples were rarefied to 4,000 reads, resulting in a loss of 16 samples. Alpha diversity was calculated using Shannon Diversity Index and beta diversity using Weighted UniFrac and Bray-Curtis distance metrics.

3.3.5 Machine Learning

We used the mikropml R package to conduct machine learning (ML) analyses [176, 244, 245]. For each model, we used random forest classification with 75% of the microbial communities for each plant host (42 replicates) used to train the model and the remaining 25% (14 plant replicates) to test the model. 75% of the communities were used to train the data because it allowed for enough data to be used so that the model was able to learn the intricacies of the data provided, and the remaining 25% allows for enough data to be left to get a good average of testing performance. ML was used on data collected the last day of drought to predict if: (1) communities are from control or drought treated plant hosts regardless of host species, and (2) bacterial communities came from tropical or temperate grass hosts regardless of treatment. Model performance was evaluated using the area under the operating characteristic curve (AUC) value. Models yielding AUC
values above 0.6 were determined to have good predictive power. Additionally, the mikropml pipeline enables determination of bacterial features important for prediction and how much they contribute to AUC values.

3.3.6 Phylosymbiosis and Cophylogeny

Phylosymbiosis was determined using a Mantel test with matrices of grass species’ phylogenetic distances and microbial community beta diversities calculated using Bray-Curtis and weighted UniFrac distances. Grass host phylogenetic distances were calculated using MEGAX [246]. Sequences of the chloroplast reference genome for each host species were retrieved from NCBI (NC_009950.1, NC_027473.1, NC_011713.2, NC_03007.1, NC_030066.1) [1] and aligned using MUSCLE [247]. The Brachiaria hybrid species was excluded from the analysis because its chloroplast genome was not present in the NCBI database. A phylogenetic tree was constructed using the maximum likelihood method. A Mantel test was performed with the Spearman’s Rank correlation with 9999 permutations using the Vegan package in R [180].

We tested for cophylogeny to understand if coevolution between microbial communities and their plant host exists. Two separate global fit methods were employed: ParaFit as carried out in the ape package [248] and PACo using the R package paco [239]. Microbial data used to test for cophylogeny were filtered to only include data collected on the last sampling day with at least 100 reads across all samples resulting in 359 ASVs. Both methods were performed with host phylogeny, microbial 16S rRNA phylogeny, and a presence/absence matrix for each host and ASV. Additionally, both methods used the
Cailliez correction method to account for negative eigenvalues [249, 250]. This method corrects negative eigenvalues by adding a constant to all dissimilarities. PACo analysis was performed with 1000 permutations using the most conservative quasiswap method, which is used when it is uncertain if the host is tracking symbiont evolution or symbionts are tracking host evolution. ParaFit was performed using 999 permutations. Significant associations were plotted using the cophyloplot function in the ape package.

3.3.7 Statistical Methods

Separate generalized linear mixed models (GLMMs) were created to assess changes in alpha diversity and nifH abundance using gamma distributions with a log link using the lme4 R package [177]. Drought treatment, host species, and time were fixed effects and sample ID a random effect to account for sampling over time. Effects of each variable was determined using Tukey tests for comparison using lsmeans [251]. Effects of host species, drought treatment, time, and their interactions on microbial community structure were determined using permutational analysis of variance (PERMANOVA) and analysis of multivariate homogeneity of group dispersions (PERMDISP2) with weighted UniFrac distances. Results were visualized using non-metric multidimensional scaling (NMDS). PERMANOVA, PERMDISP2, and NMDS were conducted using the vegan package and visualized using ggplot2 [182].

3.4 Results

Phyllosphere communities varied between host species, over time, and as a result of drought. Across all sample days and host species, Alphaproteobacteria was the dominant
class in both control (34.2%) and drought samples (34.6%), but community dynamics over time and as a result of drought were different between host species (Figure 3.1A). At the start of the experiment, *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant groups, but by the end of the experiment *Cytophagia* was the dominant class under control conditions. While *Cytophagia* increased in relative abundance under drought conditions, *Alphaproteobacteria* remained the dominant group on drought stressed hosts.

*Alphaproteobacteria* was dominated by *Sphingomonas* and *Methylobacterium* for each species, but trends in relative abundance over time and as a result of drought were different between the host species (Figure 3.1B). Genera from the class *Gammaproteobacteria* were more diverse and variable between treatments, host species, and over time, but *Pseudomonas* and unidentified *Enterobacteriaceae* were present across many samples (Figure 3.1C). The increase in *Cytophagia* was accounted for almost exclusively by the genus *Hymenobacter* (Figure 3.1D).
Figure 3.1. Average relative abundance of bacteria from 57 plants (27 control and 30 drought) sampled at 5 separate time points over 38 days. (A) The most dominant bacterial classes changed over time, between host species, and as a result of drought. To understand the composition of these classes, the average relative abundance of the genera from the three most abundant classes were plotted. Genera included were present in greater than 0.25% average relative abundance. At the end of 38 days when drought effect was strongest, we observed significant differences as a result of drought in Actinobacteria (P<0.001), Bacilli (P=0.006), and Cytophagia (P=0.001) (calculated using TukeyHSD). Additionally, strong differences were observed between host species with significant differences observed in Actinobacteria (P<0.001), Alphaproteobacteria (P<0.001), Betaproteobacteria (P<0.001), Gammaproteobacteria (P=0.008) (B) The class Alphaproteobacteria was dominated by the genera Sphingomonas and Methylobacterium, (C) Gammaproteobacteria was not consistently dominated by any individual genera, and (D) the class Cytophagia was dominated by the genus Hymenobacter.

Table 3.1. Phyllosphere community structure on native temperate and non-native tropical grasses change over time (day) and are impacted by host species and drought treatment. Impact of each variable on community structure was determined using a PERMANOVA on weighted UniFrac distance measures.

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<td>Treatment<em>Day</em>Species</td>
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3.4.1 Host Species, Time, and Drought Drive Changes in Community Structure

To evaluate the role plant species and drought had on microbial community diversity, we modeled how alpha diversity changed over time, as a result of drought, and based on host species (Figure 3.8). Alpha diversity was not affected by drought treatment but was significantly different based on host species identity.

Phyllosphere community structures were impacted by time, host species, and drought. Additionally, the degree microbial communities changed as a result of drought related to known drought tolerances of their host species. The strongest driver of phyllosphere community structure was plant host species (R²= 0.19, p=0.00; PERMANOVA on weighted UniFrac distances) (Table 3.1). Sample day (R²=0.14, p=0.001) and watering condition (R²=0.02, p=0.001) were also significant drivers. All three two-way interactions were significant, with the strongest interaction between sampling day and host species (R²=0.10, p=0.001). However, the three-way interaction was not significant (R²=0.04, p=0.0572). PERMDISP2 was conducted to ensure significant PERMANOVA results were caused by shifts in community structure instead of differences in dispersion within treatments. PERMDISP2 analyses were not significant (p=0.07), indicating that significant results from the PERMANOVA analyses are important factors for community structure. Because of significant two-way interactions, we conducted individual analyses on host species and sampling day to understand how microbial communities from each host changed over time and were impacted by drought. Overall community response was first detected 33 days into the experimental period. Additionally, host species effect on community structure increased over time (Figure 3.2). Separate PERMANOVAs run on
control samples from Day 1 (R²=0.38, p=0.01) and Day 38 (R²=0.57, p=0.001) show increased effect of host species on community assembly under non-stressed conditions. Additionally, influence of host species within the temperate (R²=0.34, p=0.039) and tropical (R²=0.31, p=0.094) groups was similar at the start of the experiment, but temperate species (R²=0.72, p=0.001) explained greater variability by the end of the experiment than the tropical species (R²=0.39, p=0.024) (Table 3.2).

Table 3.2. The effect of host species on phyllosphere community composition on non-stressed hosts increased over time. The impact of host species on community structure was measured for communities from the well-watered control host plants at the beginning (day 1) and end (day 38) of the drought period. Influence of host species was determined for all hosts together and separately for the native temperate grasses and non-native tropical grasses using a PERMANOVA of weighted UniFrac distances.

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<td>0.34</td>
<td>0.039</td>
<td>0.31</td>
<td>0.094</td>
</tr>
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<td></td>
<td>0.65</td>
<td></td>
<td>0.69</td>
<td></td>
</tr>
<tr>
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<td>0.72</td>
<td>0.001</td>
<td>0.39</td>
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<td></td>
<td>0.28</td>
<td></td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

To further understand changes in community structure, average community distance over time was modelled. On temperate grasses, average distance of communities from the same host species significantly decreased over time in both control and drought treatments (p.adj<0.001, TukeyHSD posthoc analyses). However, community distance on individual tropical grass hosts remained stable over time. Additionally, significant differences were not observed between temperate and tropical control groups but were observed between drought samples (p.adj=0.04) (Figure 3.3) (Figure 3.9).
We analyzed individual host species to understand how susceptible to drought each bacterial community was based on host species. Ryegrass microbial communities were the first to show changes between control and drought treatments; significant differences were first observed on day 26 ($R^2 = 0.37$, $P=0.012$) (Figure 3.10). *B. brizantha* ($R^2 = 0.47$, $P=0.031$), tall fescue ($R^2 = 0.27$, $P=0.035$), and orchardgrass ($R^2 = 0.25$, $P=0.033$) first showed significant differences on day 33; *Brachiaria* hybrid ($R^2 = 0.30$, $P=0.006$) on day 38; and *B. decumbens* never displayed significant differences.
Figure 3.2. Bacterial communities from each host species became more distinct over time and were significantly impacted by drought stress. NMDS ordination was plotted for each sampling day using weighted UniFrac distances. PERMANOVA was conducted for each corresponding day to determine how communities were changing over time and when drought stress altered community structure.
3.4.2 Machine Learning Allows Accurate Prediction of Microbial Communities in Drought

Machine learning (ML) allows detection of trends missed by traditional methods such as PERMANOVA [245], and allows identification of features that enable its predicative power. We used ML to test for a common response to drought among host species despite plant host selection on microbial communities. ML revealed high predictive power in determining if microbial communities were from the control or drought treatment (AUC=0.87) (Figure 3.11) and that the top eight ASVs contributed 0.07 to our AUC value (Figure 3.4, Table 3.3).
Additionally, ML had high predictive power in determining if communities were from temperate or tropical hosts (AUC=0.89) at the end of the experiment regardless of drought treatment. The model identified that 2 features, *Sphingomonas mali* and *Methylobacterium organophilum*, contributed 0.107 to our AUC values, indicating their presence was important in model performance (Figure 3.12). Since tropical grasses are more related to each other than to the temperate grasses, this analysis helped determine if community assembly is stochastic or deterministic and identifies features associated with the two grass types.

![Figure 3.4. The top eight ASVs important for predicting if samples were from control or drought stressed plant hosts.](chart)

Average relative abundance of each of the eight ASVs is given for control (n=27) and drought stressed plants (n=30) on day 38 of the experiment. ASV identities provided in Table 3.3.
3.4.3 Grass Host Phylogeny Influences Phyllosphere Communities

Host species impact on community assembly and response to drought was tested using phylosymbiosis, which occurs when significant association between host species phylogeny and associated microbial communities occur [232]. Mantel tests on Bray-Curtis dissimilarities showed more closely related host species had more similar microbial communities (Mantel r=0.117, p=0.0001). Additionally, microbial communities were more related to host phylogeny during drought stress (Mantel r=0.202, p=0.007) than under control conditions (Mantel r=0.158, p=0.02) at the end of the drought period. Tests of phylosymbiosis using Weighted UniFrac measures showed similar trends but with weaker associations (All: Mantel r=0.064, p=0.0002; Drought: Mantel r=0.114, p=0.05; Control: Mantel r=0.057, p=0.19). Because weighted UniFrac incorporates phylogenetic information, it reduces nuanced variations at the tips of the bacterial phylogenetic trees [234].

To further explore evolutionary relationships between host phylogeny and bacterial communities, cophylogeny was tested with two separate global-fit methods. Global-fit methods test congruence between host phylogenetic trees and the corresponding microbial phylogeny and allow for identification of significant associations. PACo (Procrustes Approach to Cophylogeny) uses Procrustes analyses to test the dependency of one phylogeny on the other [238, 239]. ParaFit compares two distance matrices constructed from host and microbial phylogenetic distances and tests for random associations between the groups [252]. Positive correlations can indicate host-microbe coevolution [241, 253]. Tests for cophylogeny conducted on all samples collected on day
38 regardless of treatment using ParaFit (ParaFitGlobal=1.6024, p=0.001, permutations=999), and PACo (PACo=0.999, p=0.003) revealed significant global-fit cophylogenetic relationships.

The influence of drought stress on cophylogenetic signal was determined to understand if microbial response to drought was a stochastic process, and to look for evidence of a joint plant-microbial response. Results using Parafit from both control (ParaFitGlobal=1.136, p=0.001) and drought (ParaFitGlobal=1.296, p=0.001) showed evidence of cophylogeny. In the control treatment there were 414 significant associations between bacteria and plant hosts and 340 significant associations in drought treatment samples. Tanglegrams displaying significant associations between host and microbe phylogenies were created for control and drought treatments (Figure 3.5). Evidence of cophylogeny at the end of the experimental period was additionally detected using PACo for control (PACo=0.998, p=0.001) and drought (PACo=0.999, p=0.002) treatments.
Figure 3.5. Cophylogenetic relationship analysis was conducted for (A) control plants (n=27) and (B) drought stressed plants (n=30). Blue lines in this tanglegram represent significant associations between phyllosphere bacteria on the left and their plant hosts on the right measured using ParaFitGlobal, which were determined if either of the ParaFit F statistics were below 0.05. Numbers under host species identity indicate the number of significant associations that a host species has with the bacterial phylogenetic tree. The bacterial phylogenetic tree was constructed in QIIME2 using FastTree which infers approximately-maximum-likelihood phylogenetic trees. The maximum-likelihood tree for the grass host phylogeny was constructed in MEGA. Only five grass species were included because host sequence information was not available for the *Brachiaria* hybrid.

3.4.4 *nifH* Gene Abundance Varies Over Time and By Host Species

No significant trend in *nifH* gene abundance was observed as a result of drought treatment, but significant differences in abundance were observed between host species and over time (Figure 3.6). The temperate grasses displayed a decrease in abundance over time to varying degrees, but the tropical grasses did not. Ryegrass control samples were temporally stable, but drought samples significantly decreased between day 1 and day 38 (p.adj=0.004). Tall Fescue (p.adj=0.02) and Orchardgrass (p.adj=0.006) significantly decreased between sample day 1 and 38 regardless of treatment. Control samples of *B. brizantha* showed no significant changes over time, but *nifH* copy number significantly increased over time in drought samples (p.adj=0.001). *Brachiaria* hyb. showed no differences as a result of drought but significantly varied across days (Day1 compared to 26 (p.adj=0.001) and day 33 (p.adj=0.03)). *B. decumbens* significantly increased over time in both control and drought conditions (p.adj>0.001). The trends over time for *nifH* abundance closely matched the trends observed in average UniFrac distance over time for each host species (Figure 3.9).
Figure 3.6. Abundance of the nifH gene was significantly different between host species and changed over time. However, it was not significantly impacted by drought stress. nifH abundance was measured using qPCR and standardized to number of copies per gram of leaf material.

3.5 Discussion

Plant host species and provenance impact grass phyllosphere communities and their response to drought. Consistent with previous studies across multiple plant species, we found that host species was the most important factor influencing community assembly and that Alphaproteobacteria dominated communities [72–74, 122]. Additionally, our study revealed that communities changed over time and as a result of drought. We observed strong temporal patterns in which Gammaproteobacteria were replaced over time by Cytophagia, similar to studies in switchgrass that found Gammaproteobacteria were replaced throughout the growing season by Alphaproteobacteria [61]. While temporal replacement occurred on each host species, degree of replacement varied widely and between treatments. By the end of the experimental period, Cytophagia was the
dominant class on control plants while *Alphaproteobacteria* dominated drought plants. The persistent presence of *Alphaproteobacteria*, in particular *Sphingomonas* and *Methylobacterium*, throughout the experiment on control and drought stressed plants likely resulted from niche partitioning and their complementary metabolisms uniquely suited to the phyllosphere [58, 61]. In the phyllosphere, *Sphingomonas* survive on a wide range of substrates due to high abundance of TonB receptors, while *Methylobacterium* can grow on one-carbon compounds such as methanol, a byproduct of host cell-wall metabolism [58, 86]. Additionally, their flexible metabolisms allow for adaptation to changing nutrient availability as leaf conditions change. Not only are they able to survive the harsh phyllosphere environment, they can promote plant growth and stress tolerance. Inoculation of *Sphingomonas* onto soybean plants resulted in increased tolerance of drought conditions and *Methylobacterium* on leaf surfaces are able to fix nitrogen and increase plant biomass production [105, 198]. The observed persistence under stress conditions in combination with their functional benefits, could indicate coevolutionary adaptation to life in the phyllosphere. Furthermore, *Sphingomonas* and *Methylobacterium* should be explored as biofertilizers because of their widespread presence and observed drought tolerance.

Host species effect on community assembly increased over time. On day one of the experiment, host species accounted for 38% of community variability in the control samples compared to 57% on the last day. This likely results from host selection on community assembly; host species selection increases over time as communities successfully establish, as more bacteria land on the leaf surface through dispersal, and as
communities change in relation to plant development [61, 62, 67]. Interestingly, the effect of host species overtime was different between the native temperate grasses and the non-native tropical grasses. On the first day of the experiment, host species exhibited similar influence on microbial communities from temperate and tropical grasses. However, by the end of the experimental period, species explained 72% of the variability on temperate grass hosts but only 39% on tropical grass hosts. The difference in effect over time between the tropical and temperate grasses likely results from host-microbe evolutionary relationships that exist for the native temperate species but not for the non-native tropical species.

To understand if phyllosphere communities from temperate grasses experienced increased selection compared to tropical grasses, we determined how ecological distance changed over time for each host species. Since temperate grasses were grown in their native environment, we expected increased host selection compared to tropical grasses. While change over time accounted for similar amounts of variability in the tropical (27%) and temperate grasses (23%), the average distance of communities found on each unique host species decreased in temperate grasses but remained stable in tropical grasses. In other words, communities from tall fescue replicates were more similar to each other at the end of the experiment than they were at the beginning of the experiment, while communities from B. brizantha replicates maintained the same degree of similarity throughout the experimental period. These significant differences suggest deterministic assembly in the phyllosphere. In the temperate grasses, decreased distance within host replicates could result from increased selection caused by existing plant-microbe
relationships. However, since tropical grasses were not grown in an environment with their native microbiota, changes over time and between species were more likely a result of host physiology. Community distance over time was measured separately for each individual host species. Since the host species have varying phylogenetic relationships, an important analysis would be to assess how host phylogeny influences host selection over time in the phyllosphere communities. In our study, host phylogeny and host provenance could be confounding factors because the tropical species are more related to each other than the temperate host species are. Therefore, an important future study should be designed to account for both host species relatedness and provenance. This could be achieved by testing a larger number of unique host species across a broader phylogenetic range. It would be important to include hosts that are native to temperate regions but more closely related to the tropical hosts and vice versa. Microbial communities are host selected and understanding how host phylogeny relates to microbial communities will help us further understand phyllosphere community assembly processes.

Presence of phylosymbiosis under non-stressed conditions indicates host-species influences community assembly and that bacterial communities are more similar to each other on plant hosts that are more phylogenetically similar [254]. While phylosymbiosis could result from coevolution or cospeciation, it can also result from differences in host ecological niche, geographic locations, or host filtering in which related hosts have many shared traits [232, 255, 256]. Therefore, phylosymbiosis does not determine a specific mechanism. Presence of phylosymbiosis demonstrates that phyllosphere community
assembly is a deterministic process but what is driving it is still not fully understood. By growing plants in the same environment, we eliminated some of the confounding factors that might otherwise contribute to this relationship such as differences in soil, weather patterns, or biogeographic separation. Previous work across animal species concluded that when related hosts grown under identical conditions maintain distinct microbial communities, it is analogous to microbial markers of host evolutionary relationships [235]. An important caveat in interpreting the phylosymbiosis analysis is that only five host species were used. This low sample size can decrease how robust the analysis is and the strength of the conclusions. However, phylosymbiosis has been used across a range of studies looking at as few as three host species [235, 257–261].

Unsurprisingly, the cophylogenetic analysis revealed strong evidence of cophylogeny in the temperate grass species with hundreds of significant correlations compared to only dozens observed in the tropical grasses. Thus, cophylogenetic signal was stronger in the native temperate grasses than in non-native tropical grasses. The differences between tropical and temperate hosts further supports the idea that the host-species effect seen in the temperate grasses is a result of microbes adapting alongside their host.

### 3.5.1 Microbial Community Response: An Adaptation to Drought

Understanding how phyllosphere communities respond to drought in relation to their plant host is important for understanding how we can use bacteria as biofertilizers to promote plant health in the future. Interestingly, no difference in alpha diversity as a result of drought was observed even though drought caused changes in bacterial
community structures. Previous work found that phyllosphere community diversity but not composition was related to plant community productivity [111]. Therefore, shifts we are seeing in community structure but not in alpha diversity could indicate microbial communities act as a stress response trait. Important follow-up experiments to test this hypothesis could include parallel studies inoculating plants and gnotobiotic plants with different individual bacteria, synthetic communities, and active microbial communities washed from control and drought stressed leaf surfaces to understand if different inocula have different effects on plant health and growth. Inoculating individual microbes and synthetic communities allows us to correspond traits with specific microbes, while inoculating communities from plant washes can help us understand if communities from plants at different stages of stress change plant health outcomes and accounts for the fact that many microbes are not culturable. 

The forage grass species used in this experiment have varying degrees of drought tolerance resulting from the different strategies used under drought stress. Drought tolerance is well documented for the temperate grasses used in this study. Ryegrass is the most drought susceptible and under field conditions tall fescue is the most drought tolerant [191, 262]. However, when grown in pots, orchardgrass exhibits higher drought tolerance due to its abilities to take up water in low soil moisture conditions, promote membrane stabilization, and protect its meristem from dehydration [191, 263]. In the field, tall fescue has higher drought tolerance due to its ability to form deep root networks, which were limited by the depth of pots in which they were grown. The C4 tropical plants have greater water use efficiency due to their ability to maintain higher
photosynthetic rates under decreased water stress compared to the C3 temperate species [264, 265]. Additionally, they can form extensive root networks that enable high water uptake efficiency from soil [192]. These levels of known drought tolerance correlate with the changes we saw in microbial community structures. Ryegrass, the most drought susceptible, was the first to show signs of change, followed by orchardgrass and tall fescue. The more drought tolerant tropical grasses showed changes in microbial communities later than the temperate grasses. Even though previous studies found B. brizantha and B. decumbens to have similar drought tolerances, we observed changes in B. brizantha communities on day 33 but no significant changes as a result of drought in B. decumbens [192, 193]. What remains unclear is, if changes in microbial community structure are in direct response to drought or in response to changes in host physiology.

If changes in communities were in direct response to drought, we may expect to see a decrease in phylosymbiosis as selection imposed by host species decreased and communities became more similar to each other. Instead, we observed an increase in phylosymbiosis under drought stress indicating that selection on microbial communities is increasing. Conversely, total cophylogenetic associations decreased as a result of drought with less overall connections between host and microbe phylogenies. However, not all host species showed similar trends, indicating that microbial community response to drought, much like community assembly, is a deterministic process facilitated by changes in plant host physiology. Because strong evidence of phylosymbiosis and cophylogeny remain in spite of shifts in community structure, we propose that phyllosphere communities are a plant stress response trait.
Despite the strong host species effect on microbial communities during drought, we used machine learning to determine if there was a common response to drought across our host species. Determining if any microbes invariably survive under drought conditions across our range of hosts and have the potential to promote plant growth is important for determining prospective bacteria to test as biofertilizers. Our ML pipeline accurately predicted if a sample came from a drought stressed or control plant 87% of the time, confirming that there is a common response to drought despite divergent communities. No single bacterial ASV was responsible for model prediction, rather several ASVs provided minor predictive power. Of the top 8 predictors, 5 from the order Actinomycetales were slightly elevated in drought samples including Microbacterium. In the rhizosphere, Microbacterium can produce volatile compounds that promote plant health and growth [266] and help regulate plant response to drought stress by altering the metabolite profile to promote osmoregulation [266]. Thus, even though microbial communities are host specific, core functions exist in phyllosphere communities across plant hosts that enable microbial survival under harsh conditions while also offering functional support to their plant host.

3.5.2 Nitrogen Fixation: A Core Function

We used nitrogen fixation as one example of an important function microbes provide plants. Recent studies found phyllosphere communities input nitrogen into their ecosystems [99, 105, 267]. When we assessed our communities for nitrogen fixation potential, we found stable diazotroph presence on every host species. However, nifH abundance was not negatively impacted by drought. The occurrence of temporally stable
diazotrophs on every host species indicates it could be an important part of the functional core community, while the differential abundance between host species points to the evolutionary relationships between plants and their microbes.

When under drought stress, the most drought tolerant host species, *B. decumbens*, exhibited increased *nifH* abundance and a strong cophylogenetic relationship with the bacterial family Oxalobacteraceae accounting for 14 of the 47 significant relationships. Additionally, relative abundance of Oxalobacteraceae from the class Betaproteobacteria increased on *B. decumbens* under drought. Oxalobacteraceae are adapted to oligotrophic conditions and some genera are nitrogen-fixers (Figure 3.13) [268]. Because of the sustained presence of *nifH* across time and treatments in combination with their correlation to community structure, we propose nitrogen-fixation as a keystone function of phyllosphere communities. This further supports our hypothesis that microbial communities are a plant trait which help promote plant stress tolerance.

### 3.5.3 Conclusion

This study revealed phyllosphere community assembly is related to host evolutionary history. The strong evidence of phylosymbiosis in combination with increased selection and cophylogeny in the native temperate grasses compared to the non-native tropical grasses, suggests that microbial communities are impacted by the relatedness and provenance of their plant hosts. The conserved presence but differential abundance of important bacteria such as *Sphingomonas* and *Methylobacterium*, and the functional potential of nitrogen fixation during drought stress support the idea that microbial
communities are plant traits that promote plant growth and stress tolerance. Future studies should look at the effect of inoculating plants with the taxonomically and functionally important bacteria identified in this study that were also temporally and drought stable. Creating biofertilizers with ecologically important microbes that can establish and survive on both native and non-native plant hosts could be a way to promote plant health and tolerance to a changing climate across a broad range of plant hosts.

In chapter 2 and 3, similar questions were addressed about microbial community assembly dynamics and response to drought stress. In chapter 2, questions were addressed through two separate greenhouse experiments that looked at five different species of tropical and temperate grasses. In chapter 3, questions were addressed in one field study that looked at six species of tropical and temperate grass host species. The second greenhouse study looking at two temperate grass species and the field study were set up in a manner to have nearly identical experimental designs (same soil, plants grown in pots, drought imposed and regulated in the same manner, similar length of drought period), so that we could compare the results from the two studies to understand if assembly patterns changed between the different environments. In the next chapter, we compare the patterns of community assembly and response to drought that occurred on tall fescue and orchardgrass host species that were reported in chapter 2 and 3. This allowed us to assess differences in assembly patterns between the environments.
3.6 Data Availability Statement

The 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA758974.

3.7 Supplemental Materials

3.7.1 Transplanting Grasses

In June 2019, all individual plants were transplanted into 15x30cm pots filled with soil collected from natural grass fields in Amherst, MA. The top 15 cm of topsoil was collected, all rocks and roots were removed, and soil was immediately placed in pots and allowed to settle for two days before plants were transplanted. Soil nutrient contents were tested at the University of Massachusetts Soil and Plant Nutrient Testing Laboratory (Table 3.2). Before transplanting, roots and shoots were each trimmed to a uniform length of three inches. For the three temperate grass species, there were five replicates per plant species per treatment. For the three tropical grasses, four pots were used in the ‘control’ treatment and five were used in the ‘drought’ treatment. This resulted in 57 total pots.

3.7.2 Library Preparation

All phyllosphere community DNA samples underwent a two-step PCR amplification to attach barcodes and Illumina adaptor sequences. In the first PCR step, chloroplast excluding primers targeting the V5-V6 region of the 16S rRNA gene with linker sequences were used. The primer pair consisted of the forward primer (799F): 5’ACACTGACGACATGGTTCTACA AACMGGATTAGATACCCKG-3’, and the reverse primer (1115R): TACGGTAGCAGAGACCTTGGTCT AGGGTTCGCTCG
TTG-3’, where the underlined portion is the linker sequence followed by the primer sequence. The first PCR step was a 30 µl reaction containing 3 µl of 10X OmniKlentaq buffer (DNA Polymerase Technology, St. Louis, MO), 2.4 µl dNTPs (10 µM), 0.75 µl forward primer (5 µM), 0.75 µl reverse primer (5 µM), 0.5 µl bovine serum albumin (BSA), 0.2 µl OmniKlentaq LA, 3 µl DNA, and 19.4 µl molecular-grade water. The PCR reaction had an initial denaturation step at 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 60°C for 30 s, 68°C for 30 s, and a final elongation step for 3 min at 68°C. The product from this PCR was used as the DNA template for the second PCR step, which attached individual barcodes to each sample to allow separation of samples in downstream analyses. The second PCR step was a 20 µl reaction containing 10 µl of 2X Dreamtaq Mastermix (Thermo Fisher Scientific, Waltham, MA), 4 µl water, 2 µl DNA, and 4 µl of Access Array Barcode primer pools (Fluidigm, San Francisco, CA). The second PCR reaction consisted of an initial 5 min denaturation step at 95°C; 8 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final 7 min elongation step at 72°C. The amplicons were pooled and sequenced on Illumina MiSeq Platform, with 251 bp paired-end sequencing chemistry. Illumina PhiX was spiked-in (~ 25%) to account for the low base diversity. Sequencing was performed at the Genomics Resource Laboratory (University of Massachusetts-Amherst).

3.7.3 Quantitative PCR of Nitrogen Fixing Bacteria

The abundance of nitrogen-fixing bacteria and the corresponding effect of drought stress were determined using qPCR quantifying the \textit{nifH} gene. All samples were amplified in triplicate using the PolF(5’ TGC GAY CCS AAR GCB GAC TC 3’) and PolR primers(5’
ATS GCC ATC ATY TCR CCG GA 3’) in a 20 µl reaction containing 10 µl Luna qPCR mastermix (NEB, Ipswitch MA, USA), 1.5 µl forward primer (10 µm), 1.5 µl reverse primer (10 µm), 1 µl DNA, and 6 µl molecular-grade water. The qPCR reaction was carried out using 5 min of initial denaturation at 95°C, 40 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s, followed by a final 10 min elongation step at 95°C. Abundance of nifH genes was normalized to an average mass of leaf samples used during DNA extraction for each host species.

3.8 Supplemental Tables

Table 3.2. Soil physical-chemical analysis for experiments with tropical and temperate grasses. Macronutrient and micronutrient concentrations were determined using Modified Morgan extractables.

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Table 3.3. Taxonomic classification of ASVs using the Greengenes database. ASVs given are the 8 ASVs that contributed the most predictive power to our machine learning model predicting if microbial communities were from hosts under drought or control conditions.

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</table>

3.9 Supplemental Figures

![Pots were organized in a randomized block design. Half the plants were kept under rain shelters to induce drought and the other half were in the open and their soil moisture was maintained above 80% field capacity.](image-url)
Figure 3.8. Alpha diversity of phyllosphere communities did not significantly change as a result of drought stress. Alpha diversity was different between host species and varied over time. Alpha diversity was measured using Shannon Diversity Index, which takes into account both abundance and evenness of species present. We fit an interactive GLMM with treatment, time, and host species as fixed effects and sample ID as a random effect.

Figure 3.9. Average distance between samples from each host species were calculated for each sampling day using weighted UniFrac distance. Average distance within each host species significantly decreased in the communities from temperate hosts but had varying trends from each tropical host.
Figure 3.10. Bacterial community changes over time and as a result of drought on each species were determined using a PERMANOVA of weighted UniFrac distances (G-L) and visualized using NMDS (A-F). Images of control plants next to plants exposed to drought treatment at the end of the experimental period are given in figures M-R.
Figure 3.11. Machine learning using a random forest model resulted in high predictive power for determining if communities were from control or drought stressed plants. Any points occurring at levels higher than 0.5 in the area under the receiver operating characteristic curve (AUROC) indicates model prediction was better than by chance. Cross-validation and Test data (AUROC of 0.87) resulted in similar AUROC values indicating the model had high predictive power.

Figure 3.12. Two bacterial species were highly important for accurately predicting if a community came from a tropical or temperate host species on the last day of drought. *Methylobacterium organophilum* occurred at a higher relative abundance on tropical grasses compared to temperate grasses. *Sphingomonas mali* was present in low relative abundances on tropical grasses, but it was not detected on any temperate grass host.
Figure 3.13. Average relative abundance of genera belonging to the class Betaproteobacteria over the 38-day experimental period for each control and drought stressed plant host species. The host plant *Brachiaria decumbens* had a significant increase in the genus *Massilia* under severe drought conditions, which is known to have nitrogen fixation potential [3].
CHAPTER 4

COMMUNITY ASSEMBLY DYNAMICS AND STABILITY IN THE PHYLLOSHERE ARE SHAPED BY THEIR ENVIRONMENTAL SETTING

4.1 Abstract

Phyllosphere microbes can support growth and development of the plant host through protection from pathogens, production of plant hormones, and nutrient cycling among other things. Therefore, understanding phyllosphere community assembly and succession in different environments offers an opportunity to leverage these relationships to promote plant health. We conducted comparative studies in a greenhouse and parallel field environment looking at phyllosphere community assembly and response to drought over time on two forage grass species to understand how environmental setting, host species, and time impacted phyllosphere community structure and stability. Comparative high-throughput analyses of 16S rRNA amplicon data revealed vastly different community dynamics between the two environments. Microbial community profiles were markedly different at the class taxonomy level, with field samples dominated by Alphaproteobacteria and greenhouse communities dominated by Gammaproteobacteria. Moreover, host species had a greater effect on community structure in the field environment than in the greenhouse environment, while the impact of drought on community structure was stronger in the greenhouse than in the field. Additionally, network analysis revealed that microbial communities from field grown plants exhibited stronger selection and were more stress-tolerant than greenhouse communities. From this study, we concluded that differences in ecological processes associated with each
environmental setting played an important role in phyllosphere community dynamics and stability.

4.2 Introduction

The phyllosphere—the aerial surface of plants that serves as a habitat for microbes—is one of the most extensive microbial habitats on earth [40]. Microbes in the phyllosphere play an essential role in plant health by providing protection from pathogens [93], synthesis of plant hormones [110], production of key nutrients such as nitrogen [99], and protection from abiotic stress [213]. As a result, phyllosphere microbes are critical to ecosystem functioning [111]. This importance has led to a recent focus on understanding phyllosphere community assemblage and functioning with the goal of promoting agricultural and ecosystem productivity and sustainability.

By studying the phyllosphere, we can understand how community assembly relates to community stability and how these factors impact the resilience of plants and ecosystems under climate stress [39]. One way to examine how community assembly relates to stability is using network analysis. In microbial ecology networks, individual taxa are represented by nodes and correlations/interactions between taxa are visualized as edges [149, 269]. Relative community stability can then be inferred by comparing network properties. Using this method, complex networks with higher ratios of negative to positive interactions are considered more stable [147–149]. Positive relationships can indicate niche overlap and/or positive interactions such as mutualism. This can sometimes result in compounding dependent community relationships, meaning the
success of one taxon depends on the success of another and decreased abundance of one taxon will result in decreased fitness of another. In contrast, taxa with negative interactions could either occupy different niches so their fitness is not reliant on the associated taxa or negative associations such as competition [147, 149]. As a result, a higher negative to positive interaction ratio indicates a community a more stable community. Since plant host fitness is dependent on its associated microbiome, understanding factors that influence stable community assemblages will enable us to develop management practices that promote plant health by optimizing beneficial plant-microbe interactions [148].

The phyllosphere is a harsh environment subject to fluctuating temperatures, extreme weather, low nutrient and moisture availability, and UV radiation [42]. Because of this high stress environment, phyllosphere communities are similar at a broad taxonomic level and are consistently dominated by Actinobacteria, Bacteroidetes, and Proteobacteria phyla [111, 225]. Additionally, environmental variables influencing selection of microbial community structure include host species [72], geographic location [73], urbanization [74, 104], season [61], and climate stress (Aydogan et al., 2018; Bechtold et al., 2021). To further investigate these variables, studies aimed at understanding phyllosphere community dynamics have been carried out in both field and greenhouse environments. Greenhouses offer a more controlled environment in which growing conditions can be sufficiently replicated over different experiments. Although only a few studies have compared greenhouse and field conditions, the available studies have concluded that despite differences in phyllosphere communities between the two
environments, greenhouse studies are useful for assessing phyllosphere community assembly [187, 270].

Greenhouse studies have helped to develop models of phyllosphere community dynamics, but it remains unclear how similar community dynamics are between different environments. Our objective was to understand if microbial community assembly and succession on forage grass host species follow similar patterns and response to stress in different environments. To address this objective, we compared community assembly between the environments by exploring the following questions: (i) Is microbial community diversity similar? (ii) How do microbial communities change over time and are they influenced by plant host species? and (iii) Do communities respond to external stress such as drought in comparable manners? To answer these questions, we compared findings from studies of community assembly and response to drought on two forage grass species conducted in either a greenhouse or field environment. The plant replicates for each of these studies were planted at the same time and under the same conditions to eliminate differences that would otherwise arise from variations in soil and early microbial establishment. Additionally, drought was standardized in each experiment by controlling soil moisture content.

4.3 Materials and Methods

4.3.1 Study System

We conducted a metanalysis of our first two research aims (chapter 2 and chapter 3) to understand if we had similar results between the greenhouse and field environment. Six
host species were studied in total in the previous two experiments, but we focused only on tall fescue and orchardgrass because the experimental design between the two experiments such as DNA extraction method were the same for the temperate grass hosts but not the tropical host species. We outline the experimental design again here to demonstrate the similarities in the two experiments through side-by-side comparisons.

Two C₃ temperate forage grass species, *Dactylis glomerata* (orchardgrass) and *Festuca arundinacea* (tall fescue), were selected based on their widespread use in North American forage systems. Orchardgrass (Echelon) and tall fescue (Cowgirl) seeds were acquired from Albert Lea Seed Company (Albert Lea, MN, USA). Seeds were initially germinated in Pro-Mix commercial potting medium (Quakertown, PA, USA) and allowed to establish for at least three months. Individual plants were then removed from the Pro-Mix and transplanted into plastic pots containing soil collected from a pasture in Amherst, MA. The top 15 cm of topsoil were collected, plants, rocks, and roots were removed, and the pots were filled with the soil. A total soil nutrient analysis was conducted at the University of Massachusetts Soil and Plant Nutrient Testing Laboratory (Table 2.2). Three weeks before the start of the experiment, plants were trimmed to a uniform height (approximately 10 cm canopy height) and were not disturbed until the end of the experiment.

### 4.3.2 Greenhouse Experiment

Greenhouse experiments were conducted at University of Massachusetts-Amherst College of Natural Sciences Research and Education Greenhouse. Plants were maintained
in a greenhouse at 21/18°C (day/night temperatures), 16-hour photoperiod supplemented by 1000 W metal-halide and 50% 600 W high pressure sodium lights, and relative humidity of 50 to 60%. The experiment was initiated at approximately 5 months after germination (2 months following transplant into 13x23 cm pots containing field soil).

Plants were arranged on the greenhouse bench in a completely randomized block design, with two species, two watering treatments (control, drought), and five biological replicates per species-treatment combination for a total of 20 plants. Leaf tissues were harvested for measurement of bacterial communities at 1, 14, 23, and 36 days of treatment. All plants were watered every 2 to 3 days with standard tap water from the greenhouse facility. Drought plants received approximately 20% of the amount of water provided to control plants determined using soil moisture, which was measured using a MiniTrase TDR with a 20-cm stainless steel probe (Soilmoisture Equipment Corp., Goleta, CA, USA). Soil moisture of control plants was maintained at approximately 25 to 30% volumetric soil moisture content, whereas soil moisture of drought plants was reduced to approximately 3 to 5% by the end of the experiment. The experiment lasted 36 days, until plants were significantly stressed based on physiological parameters described below.

4.3.3 Field Experiment

Following 7 months of establishment in the greenhouse, plants were transported outside to the Crop and Animal Research and Education Farm in South Deerfield, MA in June 2019. Before transplanting the plants into 15x30 cm pots containing field soil, roots and
shoots were trimmed to a uniform length of 8 cm. Plants were then allowed to reestablish for two months in the field. Similar to the greenhouse experiment, this experiment consisted of 2 species, 2 watering treatments (control, drought), and 5 biological replicates per species-treatment combination for a total of 20 plants. Plants were organized in a completely randomized block design.

At the start of the experiment, drought treatment plants were placed under 3 meter high rain shelters made of greenhouse plastic, which allowed maximal airflow around the plants and high UV light penetration. The field experiment lasted 38 days (21 Aug to 27 Sept 2019). Plants in the control group were given supplemental water when necessary to maintain soil moisture at 25 to 30% volumetric soil moisture content as measured using a TDR as described in the greenhouse study. Plants in the drought group were given supplemental water only when necessary to maintain an even dry-down rate, with soil moisture content eventually reduced to approximately 3 to 5% by the end of the experiment. Physiological parameters were measured weekly, and bacterial communities were sampled at 1, 19, 26, 33, and 38 days of treatment as described in more detail below.

4.3.4 Plant Health Measurements
To understand the effects of drought on the plant host and to standardize plant responses among the greenhouse and field experiments, selected physiological parameters were assessed on a weekly basis as a measure of plant health in response to control and drought treatments. Plant water status was monitored based on leaf relative water content
(RWC) according to the methods of Barrs and Weatherley (1962), with modifications by DaCosta et al. (2004). Leaf cellular membrane stability was determined based on an electrolyte leakage assay [271]. For each plant replicate, three leaves were removed, rinsed with distilled water, and placed into tubes containing 50 mL distilled water. The tubes were placed on a shaker for 8 hours, and then initial conductivity (Ci) was measured using a conductivity meter (Orion 3-Star Conductivity Meter, Thermo Scientific, Beverly, MA). The tubes were placed in an autoclave to kill the leaf tissues, then placed on a shaker for 24 h before the final conductivity (Cf) was measured. The electrolyte leakage was calculated as Ci/Cf x 100. In weeks when bacterial communities were sampled, these physiological measurements were taken on the same day to directly correlate phyllosphere community data with plant health. At the end of the drought period, above ground biomass was measured by dividing plant material into four components: flowering stem, dead, mature, and young leaves. After determining fresh mass, samples were dried at 70°C for 5 days and dry mass was measured.

4.3.5 Bacteria Community Sampling

At each sampling time outlined above, bacterial community DNA was extracted from each individual plant replicate using the Nucleospin Plant II Extraction Kit (Machery-Nagel, Düren, Germany) following a modified protocol. Three whole leaves of each plant were aseptically removed from the plant and placed into a 15 ml conical tube with 1.5 ml of NucleoSpin Type-B beads and 4X volume of Buffer PL1. Tubes were vortexed horizontally for 5 min at room temperature. The lysate was incubated for 60 min at 65°C, placed in a NucleoSpin Filter tube, and centrifuged for 2 min at 11,000xg. The filtrate
was added to 4X Buffer PC and extraction continued following the manufacturer’s manual. Aydogan et al. found that vortexing whole leaf samples in tubes with lysis buffer and beads extracted important community members from biofilms with minimal co-extraction of plant DNA [84]. Extracted DNA samples then underwent a two-step PCR amplification to attach Illumina adaptor sequences and barcodes. The first PCR step used chloroplast excluding primers 799F (5’ACACTGACGACATGGTTCTACA AACMGG ATTAGATACCCKG-3’) and 1115R (5’TACGGTAGCAGAGACTTGGTCT AGGG TGCGCTCGTTG-3’) targeting the V5-V6 region of the 16S rRNA gene [111] with linker sequences to attach Access Array Barcodes (Fluidigm, San Francisco, CA, USA) [173]. The underlined portion is the linker sequence followed by the primer sequence. Amplicons were pooled, and the quality was assessed using Agilent 2100 Bioanalyzer DNA High Sensitivity assay, Qubit (dsDNA High Sensitivity assay) and library qPCR assay (NEB). The pooled library was spiked with ~ 20% PhiX control library (Illumina) to balance the low nucleotide base diversity. The final pool was sequenced on Illumina MiSeq Platform, using the v2-500 cycle kit, with 251 bp paired-end sequencing chemistry using Illumina recommended protocol at the Genomics Resource Laboratory (University of Massachusetts-Amherst).

4.3.6 Sequence Analysis
Using the QIIME2 [172] pipeline, paired-end reads were demultiplexed, merged, trimmed to 315 base pairs, and binned inferring amplicon sequence variants (ASVs). Taxonomic identities were assigned using the naïve Bayes sklearn classifier trained with the 799F/1115R region of the Greengenes 13_8 database.
The data from the greenhouse study contained 6,400 ASVs from 74 samples. All samples were rarefied to 4000 reads, which resulted in the loss of four samples due to low read depth. The remaining 70 samples ranged from 5,147 - 46,296 reads/sample for a total of 1,358,543 quality reads.

The data from the field study contained 9,207 ASVs from 99 samples. All samples were rarefied to 4000 reads, which resulted in the loss of five samples due to low read depth. The remaining 94 samples ranged from 5,499-116,158 reads/sample for a total of 2,281,843 quality reads.

4.3.7 Quantitative PCR of Nitrogen Fixing Bacteria

The abundance of nitrogen-fixing bacteria and the corresponding effect of drought stress from the two environments was determined using qPCR to quantify the \textit{nifH} gene. All samples were amplified in triplicate using the PolF (5′ TGC GAY CCS AAR GCB GAC TC 3’) and PolR primers (5′ ATS GCC ATC ATY TCR CCG GA 3’) [173] in a reaction containing Luna qPCR mastermix, 0.75 µm forward primer, 0.75 µm reverse primer, 1 µl DNA, and molecular-grade water. The qPCR reaction was carried out using 5 min of initial denaturation at 95°C, 40 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s, followed by a final 10 min elongation step at 95°C. Abundance of \textit{nifH} genes was normalized to the number of \textit{nifH} genes per gram of leaf mass.
4.3.8 Drought Treatment Occupancy

Using presence/absence data, similarities in ASV composition between control and drought treatment were conducted separately for each host species and each experimental location. This was performed using the ggvenn package [272] in R [175]. ASVs with less than 10 read counts were removed, and presence was considered if an ASV was present on at least one replicate host plant of a given condition.

4.3.9 Co-occurrence Networks

Network analyses were carried out in R using the packages igraph [273], Hmisc [274], and Matrix [275]. To determine co-occurrence patterns in the microbial communities, we used rarefied ASV tables with corresponding taxonomy. ASVs with less than 50 read counts were removed from the analysis. We used the Spearman correlation coefficient to determine if ASVs were significantly correlated and correlations with a p-value < 0.05 were retained. An additional selection criterion was applied so that samples were only retained if they had a minimum correlation strength of 0.75. By imposing the two selection criteria we can limit type I errors. Additionally, this selection method was chosen over methods that apply p-value adjustments to limit type II errors. The ASV table was then converted to an undirected adjacency matrix with weight representing the level of correlation. Isolated nodes were removed from the network and node size was weighted based on the calculation from the hub_scores() function in igraph, while numbers of connections were determined using the degrees() function. Positive interactions were plotted in blue and negative interactions were plotted in red.
4.3.10 Statistical Analyses

Differences in \textit{nifH} abundance were determined using a generalized linear model (GLM) comparing host species, treatment, and location. Generalized linear mixed models (GLMMs) were used to assess changes in alpha diversity. Separate models were created for Shannon Diversity Index, species richness (number of ASVs), and Faith’s Phylogenetic Diversity (PD). The fixed effects used in the models were drought treatment, host species, location, time, and sample ID. All models used gamma distribution with a log-link since abundance and alpha diversity are continuous variables bounded at zero. Models were run using the \texttt{lme4} package [176] in R and effects of each variable were determined using TukeyHSD (Tukey Honest Significant Differences).

To test if host species, drought treatment, and time had similar impacts on microbial community structure in the greenhouse and field, we conducted separate permutational analysis of variance (PERMANOVA) using weighted UniFrac distances on samples from each location. Results were visualized using non-metric multidimensional scaling (NMDS). PERMANOVA and NMDS were conducted using the \texttt{Vegan} package [179] and visualized using \texttt{ggplot2} in R [181]. Statistically significant differences in relative abundance of bacterial community groups were determined using analysis of variance (ANOVA) followed by TukeyHSD implemented in R.

4.4 Results

We compared bacterial community assembly and response to drought on orchardgrass and tall fescue maintained under modified field conditions to grasses of the same cultivar
and seed lot grown in a nearby greenhouse. Plants from the field and greenhouse experiments were potted in soil collected from the same pasture, allowing us to isolate soil chemical, physical and microbial legacy effects from the phyllosphere effects. Plant response to drought showed similar trends between the two experimental environments. However, plants in the field showed slightly more stress than plants grown in the greenhouse. Drought stress resulted in a significant decrease in relative water content (orchardgrass Greenhouse: p<0.001; Field: p<0.001; tall fescue Greenhouse: p<0.001; Field: p<0.001) (Figure 4.1A). On the last sampling day when drought effect was strongest, all plants showed a significant decrease in plant cellular membrane stability as indicated by an increase in leaf electrolyte leakage (orchardgrass Greenhouse: p=0.02; Field: p<0.001; tall fescue Greenhouse: p<0.001; Field: p<0.001) (Figure 4.1B).

Additionally, total above ground plant biomass showed a significant decrease under drought conditions in all treatments except tall fescue plants grown in the greenhouse, which showed a decreasing trend (orchardgrass, Greenhouse: p=0.04; Field: p=0.007; tall fescue, Greenhouse: p=0.1; Field: p=0.03) (Figure 4.8).

Despite similar trends in plant response to drought between the two experimental environments, microbial community response varied greatly. Greenhouse control plants had a significantly higher alpha diversity than plants grown in the field, as measured by bacterial richness, Faith’s PD and Shannon diversity (Figure 4.2, Figure 4.9). Additionally, alpha diversity levels were impacted differently by drought stress in the two environments. At the end of each experiment, alpha diversity on field grown plants was indistinguishable between the treatments, while greenhouse grown plants under drought
stress exhibited a significant decrease in alpha diversity compared to control plants (orchardgrass: p=0.01; tall fescue: p=0.003).

Figure 4.1. Plant health measurements displayed similar trends as a result of drought stress in the field and the greenhouse. (A) Leaf relative water content (RWC) decreased as a result of drought and (B) electrolyte leakage increased under drought stress.

Figure 4.2. Alpha diversity of the phyllosphere community was significantly impacted by drought in the greenhouse but not in the field. The diversity measure shown here was calculated using Faith’s phylogenetic distance (PD). Asterisks indicate significant differences between the control and drought treatment (significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).
Field and greenhouse phyllosphere communities were dominated by similar classes of bacteria but at significantly different abundances (Figure 4.3, Table 4.3). In field grown samples, Alphaproteobacteria were the dominant taxa with an overall average relative abundance of 32%, while the greenhouse plants were dominated by Gammaproteobacteria with an abundance of 43%. Conversely, Gammaproteobacteria in the field samples were present at 24% and Alphaproteobacteria in the greenhouse had an abundance of 9%. Additionally, drought stress in the greenhouse group resulted in a significant increase in Gammaproteobacteria (All: p.adj<0.001; orchardgrass: p.adj=0.02; tall fescue: p.adj<0.001), but no significant difference was detected in the relative abundance of Gammaproteobacteria under drought in the field (All: p.adj=0.421; orchardgrass: p.adj=0.925; tall fescue: p.adj=0.892). The genera that make up these classes have different profiles and patterns of community succession depending on their environment (Figure 4.10). From the class Actinobacteria, the genus Curtobacterium was consistently found in field samples, but not in greenhouse samples. No genera from Gammaproteobacteria showed any trend in the field grown samples, but drought samples from the greenhouse conditions had high relative abundances of the aphid symbiont Buchnera, indicating increased potential for invasion of the phyllosphere communities by bacteria from the aphid microbiome. Finally, in the Alphaproteobacteria class, Methylobacterium and Sphingomonas were consistently found in high abundance in the field, whereas in the greenhouse Agrobacterium was present in high abundance.
Figure 4.3. Average relative abundance of the dominant phyllosphere bacteria collected over the experimental period. Asterisks on the right indicate significant differences between the treatments based on relative abundance of all sample days and treatments. Green asterisks indicate significant differences between the greenhouse and field environment for orchardgrass, and yellow asterisks indicate significant differences of phyllosphere communities for tall fescue. Changes in class by environment were tested using an ANOVA followed by a post hoc Tukey multiple comparison test. Additional comparisons for treatments and days are given in Table S1. Significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 4.4. (A) Sphingomonas and (B) Methylobacterium had higher relative abundance in the field compared to the greenhouse. Average relative abundance of each genus was plotted over time and by watering treatment for each species in the greenhouse and in the field.
Methylobacterium and Sphingomonas are found at high relative abundance across many phyllosphere studies. Due to their prevalence, we further compared how the relative abundance of each genus responded to drought, and changes over time between the two environments for each host species; we found different community dynamics between the two environments (Figure 4.4). In orchardgrass, Methylobacterium occurred at a significantly lower overall abundance in the greenhouse compared to the field (p.adj < 0.001). Additionally, how abundance of Methylobacterium changed over time was different between the two experiments. At the start of the study, there was no significant difference in relative abundance of Methylobacterium between the environments but by the end of the experiment, field samples had a significantly higher relative abundance compared to greenhouse samples (p.adj=0.04). However, neither field nor greenhouse samples were significantly impacted by drought stress. Similarly, relative abundance of Methylobacterium from tall fescue hosts were significantly lower in the greenhouse than in the field (p.adj < 0.001) and were not affected by drought in either experiment. The phyllosphere ubiquitous genus Sphingomonas showed no community changes over time or as a result of drought in the greenhouse experiments for both host species and occurred at an overall lower abundance in the greenhouse compared to the field on orchardgrass (p.adj < 0.001) and tall fescue (p.adj < 0.001). Additionally, relative abundance significantly increased over time on orchardgrass (p.adj < 0.001) and on tall fescue (p.adj < 0.001) in the field experiments.
Table 4.1. Phyllosphere community structure on temperate grasses was driven by different variables in the greenhouse compared to the field environment. Impact of each variable on community structure was determined using a PERMANOVA on weighted UniFrac distance measures. Significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

<table>
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<th>Variable</th>
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<td>6.029</td>
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<td>0.003**</td>
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</table>

To understand if we could replicate similar trends in community assembly and response to stress in the greenhouse, we conducted PERMANOVAs on weighted UniFrac distances (Table 4.1). Time, host species, and drought treatment significantly influenced community structure under both the greenhouse and field conditions, but the extent of this influence varied between the experiments. Sampling day had the biggest impact in both experiments and had a similar influence on community structure (Greenhouse: $R^2=0.180$, $p=0.001$; Field: $R^2=0.175$, $p=0.001$). However, under control conditions, there was greater bacterial species turnover in the field compared to the greenhouse samples. This was determined by measuring the difference in community composition between the first sampling day and the last sampling day in the field ($F=12.840$, $R^2=0.382$, $p=0.001$) and in the greenhouse ($F=3.094$, $R^2=0.192$, $p=0.016$). In addition, microbial communities were substantially more impacted by drought stress in the greenhouse experiment ($R^2=0.044$, $p=0.003$) than in the field experiment ($R^2=0.021$, $p=0.032$), while host species had a greater impact on community structure in the field experiment ($R^2=0.141$, $p=0.001$).
compared to the greenhouse experiment ($R^2=0.029$, $p=0.014$) (Table 4.1) (Figure 4.5) (Figure 4.11). Furthermore, under control conditions in the greenhouse experiment, communities were not distinguishable between host species ($F=0.900, R^2=0.030$, $p=0.506$), but were distinguishable in the field control plants ($F=7.184, R^2=0.133$, $p=0.001$) indicating drift has a stronger effect on the greenhouse community while host selection is stronger in the field.

![Figure 4.5. Host species was a stronger driver of community structure in the field environment (A) compared to the greenhouse environment (B). Nonmetric multidimensional scaling (NMDS) ordination was based on weighted UniFrac distances of all 94 field samples and 70 greenhouse samples. Points are colored based on host species (green for orchardgrass; yellow for tall fescue). Ellipses represent 95% confidence intervals around samples.](image)

To further explore impacts of drought, we conducted occupancy analyses comparing the number of unique and shared ASVs for each treatment found on orchardgrass and tall fescue in both the greenhouse and field experiments (Figure 4.6E-H). In the field experiment, orchardgrass had 40% shared ASVs between control and drought treatment plants, while tall fescue had 45% shared ASVs. In contrast, orchardgrass grown in the
greenhouse only had 15% shared ASVs between the treatments while tall fescue had 8% shared ASVs.

Network analysis conducted on the last day of each experiment showed increased community connectedness in the field compared to the greenhouse (Figure 4.6A-D). Under control conditions, field communities had 88 nodes and 310 edges, for an average of 7 degrees per node. Of the 88 nodes, 23 had more than 10 degrees indicating high connectivity within the community. Bacterial families with high connectivity included *Cytophagaceae, Methylobacteriaceae, Oxalobacteraceae, Pseudomonadaceae,* and *Sphingomonadaceae.* Additionally, field control communities had a negative to positive interaction ratio of 0.58. Under control conditions in the greenhouse, however, there were 68 nodes with only 151 edges, for an average 4.4 degrees per node. Of these nodes, only 2 from the family *Pseudomonadaceae* had more than 10 degrees. The network from greenhouse control communities also had a lower negative to positive interaction ratio than was observed in the field. This indicates that networks from field communities under control conditions were more complex which suggests they are more stable than networks from greenhouse communities. We therefore expect more community stability in the field than greenhouse environments, which we found when looking at communities under drought stress.

While drought resulted in decreased connectivity in both the field and greenhouse experiments, the communities from greenhouse plants experienced a much greater decrease in connectivity than the communities from the field grown plants. In the field,
drought plants had 83 nodes and 224 edges, for an average of 5.4 degrees per node. 14 nodes had more than 10 degrees from families including *Cytophagaceae*, *Methyllobacteriaceae*, and *Sphingomonadaceae*. In the greenhouse, there were only 18 nodes with 20 edges, for an average of 2.2 degrees per node. A node from the family *Nocardioidaceae* had 4 degrees, which was the highest number from drought plants grown in the greenhouse. Communities from the field environment were able to maintain a complex network under stress conditions, but communities from the greenhouse experienced a collapse in their network indicating these communities were less stable and less resistant to drought stress.

**Figure 4.6.** Network and occupancy analysis revealed more stable phyllosphere communities in the field compared to the greenhouse. (A-D) Network analysis showed that field communities were more complex than greenhouse communities, had a higher ratio of negative to positive interactions, and were more able to withstand drought stress. A connection indicates a significant interaction (p<0.05) between two ASVs determined by calculating the Spearman correlation. Node size corresponds to the number of connections, or degrees. (E-H) Occupancy analysis using presence/absence data showed higher overlap between control and drought treatment plants in the field compared to the greenhouse. Value is the percent overlap between the treatment groups on each species.
The abundance of the *nifH* gene per leaf was determined for each host species in the greenhouse and field for the last experimental day when drought affect was strongest. We expected similar *nifH* abundances between the environments because we hypothesized that plant hosts would select for similar functions between the environments even if community composition varied. However, *nifH* abundance was significantly lower in the greenhouse experiment than in the field experiment for each host species (Figure 4.7). Additionally, there were no detected differences between the two host species in the greenhouse experiment. However, under field conditions tall fescue had a significantly higher *nifH* gene copy number per gram of leaf than orchardgrass indicating host selection is stronger in the field than in the greenhouse.

**Figure 4.7. Abundance of the *nifH* gene was significantly different between environments.** *nifH* abundance was measured using qPCR and standardized to number of copies per gram of leaf material. Significance levels are assigned as *p* > 0.05 (not significant); *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001.
4.5 Discussion

We compared microbial community assembly and response to drought on two species of forage grass in a greenhouse and field setting. Our results showed vastly different community profiles, patterns of community assembly and succession, and levels of stability between environments. Consequently microbial communities in the greenhouse environment displayed less drought tolerance than communities from field grown plants despite field plants showing slightly higher levels of physiological stress. This was true of both orchardgrass and tall fescue regardless of the two plant species having different stress tolerance levels and physiological responses to drought.

Host species’ effect on community structure was different between the two environments. In the greenhouse, host species only explained 3% of the variability in community structure, while it explained 14% of the variability in the field. One explanation is that communities experienced reduced competition in the greenhouse environment, so host selection was less pronounced. This could be compounded by dispersal limitation reducing the influx of new microbes into the enclosed environment, which results in communities drifting apart. Future work measuring dispersal patterns between the two environments could help us understand if dispersal limitation is contributing to differences in community assembly patterns. Phyllosphere communities coevolve with their native hosts, resulting in beneficial interactions [72, 225]. We compared one beneficial interaction prominent in the phyllosphere, nitrogen fixation [104, 105, 276], to understand if important functions provided by the phyllosphere were represented in the greenhouse. However, we found that the abundance of bacteria capable of nitrogen
fixation was minimal in the greenhouse. Based on our findings, we hypothesize that dispersal limitation in this greenhouse study reduces the chances that beneficial microbes reach their host, which could hinder the development of competitive advantages typically found under harsh field conditions. Ultimately, the stable environment provided by the greenhouse impacted stochastic and deterministic community assembly, thereby reducing microbial diversity and the robustness of potentially beneficial interactions.

Dispersal of microbes in the phyllosphere can originate from the atmosphere, insects, soil, and plant seeds [61, 67, 71, 277]. In our study, all host plants regardless of experimental designation were initially planted together in a greenhouse and then transplanted into soil from the same pasture before drought experiments began. Because primary succession of the plants’ phyllospheres was the same and they were planted in soil from the same temperate grassland, we expected similar community profiles at the start of the experiments. However, even at the broad taxonomic level of class, community profiles were different. While this could indicate that soil is not an important microbial source, it could also result from differences in environmental selection. The phyllosphere is a harsh environment subject to severe temperature fluctuations, changes in weather, and UV radiation. In the greenhouse, these stresses on the phyllosphere environment are limited as temperature is maintained and weather effects such as wind and rain are nonexistent. Additionally, the lack of wind, rain, and insects limits dispersal of new microbes in this controlled environment, which we observed through higher species turnover in the field compared to the greenhouse.
Higher levels of dispersal can increase the effective community size, or the community size affected by competitive interactions and ecological drift. Increasing the effective community size changes drivers of community assembly by increasing the number of competitive interactions, which results in higher levels of selection and decreased levels of ecological drift [278]. Thus, the harsher environmental conditions and increased dispersal in the field environment resulted in increased competition in communities from the field environment compared to communities from the greenhouse environment. Furthermore, competition within the phyllosphere community is predicted to lead to a decrease in diversity and an increase in stability [147, 279]. While diversity can decline due to a disappearance in less competitive strains, stability can increase with metabolic niche differentiation and spatial separation. [279–281]. This correlation between increased stability and decreased diversity was observed within our own experiment. Greenhouse communities under controlled conditions displayed higher alpha diversity and were temporally unstable in contrast to the field communities, which not only exhibited lower alpha diversity levels and less variability between days but also less variability within each day.

Greater community stability was further demonstrated in the field environment compared to the greenhouse by using network analysis. The network analyses pointed to decreased stability in the greenhouse communities compared to the field communities because of a more complex network, as well as a higher ratio of negative to positive interactions in the field communities [147, 149]. This finding is associated with higher resilience to environmental stress, as positive relationships indicate high niche overlap and/or positive
interactions between taxa such as mutualism while negative relationships are indicative of divergent niches and/or negative interactions including competition [147, 149]. Negative interactions can stabilize communities, for reasons such as taxa independence from one another. However, positive interaction can destabilize communities through positive feedback loops where the abundance of one community member increases the abundance of all those connected to it [147, 149]. Therefore, environmental stress that decreases one member of the positive feedback loop can decrease the presence of several others in that loop, thus destroying the whole network. Simultaneously, microbes in different niches, represented as negative interactions, would not be negatively affected. The high network complexity and higher ratio of negative to positive correlations led us to conclude that communities from the field environment were more stable than communities from the greenhouse environment in this study.

Lending support to the above conclusion, the less stable greenhouse phyllosphere communities were more susceptible to drought than the field communities. This was determined using several different metrics. First, alpha diversity significantly decreased as a result of drought in the greenhouse communities but not in the field communities. Second, drought had a stronger effect on community structure in the greenhouse compared to the field. Finally, occupancy analysis revealed a higher overlap of ASVs between control and drought communities in the field compared to those of the greenhouse. Networks from the control conditions in the greenhouse had both a lesser network complexity and a lower ratio of negative to positive associations compared to the network from field communities. As a result, the environmental disturbance had a
cascading effect on the greenhouse community causing its network to collapse, while the higher ratio of negative to positive associations in the field resulted in a decrease in stability but an overall preservation of its network.

Network instability in the greenhouse resulted in fewer microbial hubs, which are important for network complexity as network complexity can indicate community stability [282]. Moreover, these hubs are essential to shaping microbial community structure on plant hosts as they represent taxa connected to many other species in a network [148, 283]. In the field, several of the hubs were from the genera *Methylobacterium* or *Sphingomonas*. These genera were found in high relative abundance in the phyllosphere in our field study as well as in several other studies [50, 58, 61, 63, 163]. They are involved in important processes that promote plant health, growth, and stress tolerance including increased drought tolerance, nitrogen fixation, plant growth promotion, and protection from pathogens [93, 105, 197]. Their ability to survive across many host species under harsh phyllosphere conditions likely results from their flexible metabolisms. Members of the genus *Methylobacterium* utilize one-carbon compounds such as methanol, which is commonly available in the phyllosphere as a byproduct of cell-wall metabolism [58]. Additionally, members of the genus *Sphingomonas* are able to utilize a wide range of substrates due to the presence of TonB receptors. In our findings, *Sphingomonas* and *Methylobacterium* were present in the greenhouse but at a low relative abundance, which could be due to different selection pressures in the greenhouse or dispersal limitations causing plant-specific microbial species within these genera to not reach or establish appropriate niches on the plant. We propose that *Sphingomonas* and
Methylobacterium are important for promoting plant health and growth by not only providing the plants with important functions but for promoting microbial community stability and stress tolerance.

4.6 Conclusion

In this study, community assembly dynamics and subsequent stability of the phyllosphere microbiome varied based on the environmental setting. Given that plant host identity plays a critical role in community selection and the fact that all plants were planted together in soil from the same field, we expected to observe similar community profiles at higher taxonomic levels. However, our findings disputed our hypothesis. In summary, we found that community profiles were different between each environment even at the taxonomic class level, functional profiles determined using the nifH gene were different between the experimental environments, and selection by plant host species was greater in the field than in the greenhouse. These differences resulted in more stable microbial communities in the field environment compared to communities from the greenhouse environment, which exhibited a stronger susceptibility to drought. The observed differences in community dynamics could result from (1) dispersal limitation in the greenhouse reducing the chances that commensal microbes reach their host, (2) decreased competition in the greenhouse, (3) different selection pressures between the two environments, and (4) an absence in the competitive advantages normally observed in phyllosphere microbes under harsh native field conditions due to the more stable environment provided in the greenhouse. Based on our findings that environmental setting has an important role in community dynamics and stability, further exploration
into what ecological assembly processes result in community differences and how these processes relates to plant health is warranted. Future studies should directly measure differences in dispersal rates between the environments and could use ecological null modelling processes to quantify deterministic and stochastic assembly processes in each environment.

In the first three chapters we focus on community assembly dynamics and response to drought. These studies allowed us to understand that communities from different plant hosts show varying degrees of change in response to drought stress. We wanted to further understand the ecological importance of changes in microbial communities and develop a better understanding of community stability by moving beyond resistance to also look at community resilience and begin to measure functional stability. In chapter 5, we assess community stability by looking at resistance to water-reduction stress, resilience by looking at recovery after the water-reduction period is over, and at functional stability over the entire chronosequence, by measuring rate of nitrogen fixation and bacterial community growth over time.
4.7 Data Availability Statement

The 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA727101 for grasses grown in the greenhouse and PRJNA758974 for grasses grown in the field.

4.8 Supplemental Tables

Table 4.2. Soil physical-chemical analysis for experiments with tropical and temperate grasses. Macronutrient and micronutrient concentrations were determined using Modified Morgan extractables.

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Table 4.3. Comparison of relative abundance of the most dominant classes of bacteria on (A) tall fescue and (B) orchardgrass between greenhouse and field environments. Comparisons were made for all samples, samples collected on the last day of each drought, control samples from the last day and drought samples of the last day. Comparing the last day allows us to determine response to drought by comparing the treatments when the drought effect was strongest. Changes in class by environment were tested using an ANOVA followed by a post hoc Tukey multiple comparison test. Significance levels are assigned as P > 0.05 (N.S., not significant); *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

<table>
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<tr>
<td>Cytophagia</td>
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<td>Deinococcus</td>
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<td>N.S.</td>
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Total aboveground plant biomass showed similar decreasing trends as a result of drought stress in both greenhouse and field experiments. Biomass was destructively measured at the end of the experimental period by removing all above ground plant material, drying it at 70 °C and then determining mass.

**Figure 4.8.** Total aboveground plant biomass showed similar decreasing trends as a result of drought stress in both greenhouse and field experiments. Biomass was destructively measured at the end of the experimental period by removing all above ground plant material, drying it at 70 °C and then determining mass.
Figure 4.9. Differences in microbial community dynamics between the field and greenhouse environments were determined by the effect of environment, drought, and time on alpha diversity. Alpha diversity was measured using (A) richness or total number of ASVs, (B) Shannon diversity index, and (C) Chao1.
Figure 4.10. Comparison of genera from three of the dominant classes found in the greenhouse and field environments to understand differences in community structure. Graphs depict the average relative abundance of bacteria from (A – C) 20 plants (10 control and 10 drought) sampled at 5 separate time points over 38 days grown in the field and (D – F) 19 plants (9 control and 10 drought) sampled at 4 separate time points over 36 days grown in the greenhouse. * F indicates family level resolution in graph C and F.
Figure 4.11. Host species had a stronger influence on community structure in the field compared to the greenhouse, and greenhouse communities were more influenced by drought stress. NMDS ordination was plotted using weighted UniFrac distances on samples collected from the last day of drought treatment when drought effect was strongest.
CHAPTER 5

HOST SPECIES IDENTITY AND ECOLOGICAL ASSEMBLY PROCESSES MODERATE THE DEGREE OF RESISTANCE AND RESILIENCE OF PHYLLOSHERE COMMUNITIES

5.1 Abstract

Leaf surface microbiomes have the potential to influence agricultural and ecosystem productivity. Understanding how stable these microbial communities are during disturbance events is important for predicting how plant-microbe interactions will influence ecosystem processes in a changing climate. We explored the stability of phyllosphere communities found on three dominant species of forage grass hosts: *Dactylis glomerata, Lolium perenne,* and *Festuca arundinacea.* Resistance was assessed during a ten-week period in which plants received no water and resilience was assessed during a subsequent three-week rewatering period. Community stability was assessed on each host species by determining when compositional or functional changes were observed during the disturbance period and whether or not communities were able to return to non-stressed levels during the recovery period. Additionally, ecological modeling was performed to understand community assembly processes over a growing season and determine if they are disrupted during disturbance events. Degrees of stability were observed between the three host species, which related to host species phylogeny and drought tolerance. This related to the dominant assembly processes observed on each grass host over time and as a result of disturbance. *L. perenne,* the most drought susceptible host, had the least stable microbial communities and was governed by dispersal limitation, undominated processes, and variable selection. *F. arundinacea,* the most drought tolerant host species, showed slightly more community stability than *L.*
perenne, and was governed by undominated assembly processes and dispersal limitation. *Dactylis glomerata*, the least related of the three host species, showed the most compositional stability and was governed by undominated assembly processes and homogenizing dispersal. Since phyllosphere communities are composed of a few dominant taxa and many rare taxa, they are prone to ecological drift which likely accounts for the strong degrees of dispersal limitation observed. This study revealed how plant-microbe relationships play an important role in ecological assembly processes and microbial community response to environmental disturbance in the phyllosphere.

5.2 Introduction

Microorganisms are an important source of biodiversity and are ubiquitous in nature. Across diverse environments, microbes contribute to ecosystem functionality through their involvement in biogeochemical cycling [284]. Climate extremes, such as drought, heat waves, and flooding, are expected to increase in frequency and severity due to anthropogenic climate change [285]. Because of microbial community importance in ecosystem functioning, understanding how microbial community functional and compositional profiles respond to disturbances will help us predict community and ecosystem response to future climate conditions [131, 132].

Disturbances are events that either alter the environment thus affecting the inhabiting communities or are events that directly change a community through processes such as mortality or change in relative abundance of certain members [132]. Depending on their duration, disturbances can be classified as pulses or presses [133]. Pulses are distinct,
short-term events, while presses are continuous, long-term events [132]. How communities respond to disturbance is known as stability, which can be broken down into two measurable states: resistance and resilience [132, 134]. Resistance is the degree to which communities remain unchanged as a result of disturbance and resilience is the ability of a community to recover to pre-disturbance conditions [132]. Stability can be determined by looking at community composition, function, or the interrelatedness of the two metrics. Disturbance does not always result in the same degree of change in terms of community composition and function. For example, communities that display a high degree of functional redundancy could develop into a stable functional profile while their compositional profile still changes, resulting in net neutral effects on ecosystem functioning [134].

One important but understudied microbial ecosystem is the phyllosphere, or aerial surface of plants [42]. Phyllosphere communities must withstand harsh, ever-changing conditions (UV radiation, precipitation, rapidly fluctuating temperatures) and are subject to frequent and unpredictable dispersal events (insects, herbivores, wind, precipitation). Despite these conditions, previous studies in the phyllosphere found predictable temporal changes and patterns of seasonal succession in phyllosphere communities [60–62]. Additionally, microbes in the phyllosphere promote host fitness and ecosystem functioning through phytohormone and nutrient production, increased stress tolerance, and protection against pathogens [40, 93, 99, 111, 286], but little work has been done to understand the stability of phyllosphere communities during and after environmental disturbances. Recent studies have estimated that plants and bacteria are the two leading sources of
biomass on earth [287]. Therefore, understanding how they interact has important implications for understanding ecosystem functionality in the face of changing climates. We focus on the phyllosphere of grasslands because this biome is globally important in agriculture and has potential to help with climate change mitigation [6, 16]. As an external stressor we chose water-reduction disturbance because grasslands around the world are expected to experience an increase in frequency and severity of drought as a result of climate change [151, 152, 155, 156].

By understanding how changes in community composition and functionality relate to ecological processes that drive community assembly over time, between environments, and during disturbance, we can develop a better and more holistic understanding of community stability. Community assembly is the result of deterministic and stochastic processes shaping microbial communities [117]. Deterministic selection is itself comprised of two processes: variable and homogenous selection. Variable selection occurs when turnover is greater between communities than is expected by chance resulting in significantly different communities. Homogenous selection is when turnover occurs at a lower rate than expected by chance resulting in more similar, or convergent, communities [116, 117, 289, 290]. Stochastic changes are composed of two dispersal processes: dispersal limitation and homogenizing dispersal. Dispersal limitation occurs when low levels of movement between communities causes them to drift apart, which can occur over space or time [116, 117, 288, 290]. Important to note, communities separated over time that are characterized by dispersal limitation are experiencing ecological drift and not an inability to mix [289]. Homogenizing dispersal occurs when communities
have a high dispersal rate resulting in similar community structures [116, 288]. Finally, community assembly can be a result of multiple competing processes or weak selection and dispersal resulting in no dominant process. Under these conditions, community turnover is described as undominated [116, 288].

The goal of this study was to understand phyllosphere bacterial community resistance and resilience to water reduction disturbance in different grass host species during the course of a growing season. Since phyllosphere inhabitants are adapted to life in an extreme habitat, we hypothesized that phyllosphere communities would exhibit high levels of compositional and functional stability, but the degree of stability would directly relate to known drought tolerance of host species. Here we looked at compositional stability using two different measurements: rate of nitrogen fixation and changes in bacterial biomass. We further investigated community succession and stability by exploring community assembly processes using ecological null models. Due to the harsh nature of the phyllosphere, we expected microbial communities to be dominated by deterministic processes: homogenous selection within each host species under control conditions, but variable selection under disturbance conditions. We found that community stability and assembly processes are directly related to plant host phylogeny. Different degrees of stability were observed within each host species, which related to the dominating assembly processes on each host species.
5.3 Material and Methods

5.3.1 Experimental Design

Experiments were conducted in western Massachusetts at the University of Massachusetts Research and Education Farm in a silty loam soil (28.8% sand, 64.3% silt, 7.0% clay) (soil analysis conducted at University of Massachusetts Amherst Soil and Plant Nutrient Testing Laboratory- Table 5.4). Seeds for three native temperate grass species, *Festuca arundinacea* (endophyte free Tall Fescue cultivar ‘Cowgirl’), *Dactylis glomerata* (Orchardgrass cultivar ‘Echelon’), and *Lolium perenne* (Ryegrass cultivar ‘Sierra’), were acquired from Albert Lea Seed Company (Albert Lea, MN, USA). Seeds from each grass species were planted in Summer 2019 in a pattern of 10 plots that were 2 x 3 m rectangles and organized in a randomized block design resulting in 30 total plots. A 1.5 x 2.5 m area at the center of each plot was selected as the research area to limit edge effects. A 3 m border of Kentucky bluegrass was planted around the plots to limit growth from other species into the plots. From May-June 2020 plots were mowed to a height of 20 cm to stimulate growth prior to the experimental period and in June 2020 plots were fertilized with an all-purpose 10-10-10 fertilizer (NutrienAg, Loveland, CO, USA). The border around the plot field and the 50 cm distancers between the plots were mowed weekly to a height of 2.5 cm.
In July 2020, treatment plots were covered with rainout shelters, which were modified 3.7 m W x 9.2 m L x 3 m H greenhouse cold frames (Growspan, CT, USA). Each cold frame was constructed to cover 5 plots (Figure 5.1) and was covered with Thermal AC Greenhouse film (Greenhouse Megastore, CT, USA). This specific greenhouse film was chosen for its high light transmission, it allowed more of the UV spectrum to pass through the film than conventional greenhouse films, and for its drip control which prevented condensation from dripping onto the experimental plots. Each greenhouse frame was modified so that greenhouse film could be rolled up and down the sides and ends. This design allowed for maximal airflow through the plots and prevented heat from being trapped under the shelters. Drought conditions began on July 13, 2020 and lasted for 10 weeks until rain shelters were removed on September 19, 2020. Plots were rewatered for the subsequent 3 weeks to allow for a recovery period.
Drought plants received no water during the 10-week drought period. In addition to any seasonal rain, control plants were given supplemental water as needed to maintain soil moisture at approximately 25 to 30% volumetric soil moisture content. Soil moisture was measured using a MiniTrase TDR with a 15-cm stainless steel probe (Soilmoisture Equipment Corp., Goleta, CA, USA). Soil moisture of drought plants was reduced to approximately 15 to 18% by the end of the drought period and returned to the original 25-30% during the recovery period. Physiological and bacterial parameters were measured every week throughout the 13-week experimental period, described in more detail below.

5.3.2 Plant Health Measurements

Physiological parameters were assessed on a weekly basis as a measure of plant health in response to control, drought, and recovery treatments. Plant water status was monitored based on leaf relative water content (RWC) according to the methods of Barrs and Weatherley (1962), with modifications by DaCosta et al. (2004). Leaf cellular membrane stability was determined based on an electrolyte leakage assay [271]. Leaves were removed from plants, rinsed with distilled water, and placed into tubes containing 50 mL of distilled water. The tubes were placed on a shaker for 8 hours, and then initial conductivity (Ci) was measured using a conductivity meter (Orion 3-Star Conductivity Meter, Thermo Scientific, Beverly, MA, USA). The tubes were placed in an autoclave to kill the leaf tissues, then placed on a shaker for 24 hours before the final conductivity (Cf) was measured. The electrolyte leakage was calculated as Ci/Cf x 100. Relative chlorophyll content was measured using a FieldScout CM 1000 NDVI chlorophyll meter.
(Spectrum Technologies, Inc, Aurora, IL), which assesses chlorophyll content by measuring the normalized difference vegetative index (NDVI).

Proline is an amino acid found in plants that helps them respond to stress [291]. Proline accumulates under various stresses including water deficit, low temperature, and UV radiation. To help plants survive these stress conditions, proline can act as an osmolyte involved in osmotic adjustment and can help stabilize sub-cellular structures [292]. Proline levels in leaves were determined using the ninhydrin-based colorimetric assay as modified by Abraham et al. using samples that were flash frozen in liquid nitrogen [293]. Proline concentration was determined for samples collected at the end of the drought period (week 10), and at the end of the recovery period (week 13).

Plant growth was measured nondestructively every two weeks using a quadrat system. A quadrat, that did not touch the grass blades to ensure microbes were not disrupted, was used to estimate the area of coverage within a plot. Additionally, average height of grass was measured using the grazing stick method to measure height at several locations within each plot by placing the stick vertical to the ground and recording the average height of the plants at that spot. At the end of the drought period, above ground biomass was destructively measured by dividing plots in half. Half of the plot remained untouched for the recovery experiment and the remainder was destructively harvested. Above ground plant material was cut to 1.5 cm above the soil surface, fresh and dry biomass were measured. Root samples were taken from 18 plots (3 from each species and treatment) to understand rooting differences as a physiological response to drought stress.
From within each plot, 3 root samples were taken to a depth of 60 cm. The samples were divided into three segments: top 20 cm, middle 20 cm, and bottom 20 cm. Soil was gently removed from the roots, roots were dried at 70°C, and mass of the dry root samples was measured.

5.3.3 Bacteria Community Sampling

Each week, bacterial community DNA was extracted using the Nucleospin Plant II Extraction Kit (Machery-Nagel, Düren, Germany) following a modified protocol. Three whole leaves of each plant were aseptically removed and placed into a 50 ml conical tube with 1.5 ml of NucleoSpin Type-B beads and 1.6 ml of Buffer PL1. Tubes were vortexed horizontally for 5 min at room temperature. The lysate was incubated for 60 min at 65°C, placed in a NucleoSpin Filter tube, and centrifuged for 2 min at 11,000xg. The filtrate was added to 1.6 ml Buffer PC and extraction continued following the manufacturer’s protocol. Extracted DNA samples then underwent a two-step PCR amplification to attach Illumina adaptor sequences and barcodes. The first PCR step used chloroplast excluding primers 799F (5’ACACTGACGACATGGTCTACA AACMGGA TTAGATAC CCKG-3’) and 1115R (5’TACGGTAGCAGAGACTTGCTTGCTCGT AGGGTTGCG CTC GTTG-3’) targeting the V5-V6 region of the 16S rRNA gene [111] with linker sequences to attach Access Array Barcodes (Fluidigm, San Francisco, CA, USA) [173]. The underlined portion is the linker sequence followed by the primer sequence. Pooled amplicons were separated into two sequencing runs that shared 10% of the same samples. The quality was assessed using Agilent 2100 Bioanalyzer DNA High Sensitivity assay, Qubit (dsDNA High Sensitivity assay), and a library qPCR assay (NEB, Ipswich, MA,
USA). The pooled libraries were spiked with ~ 20% PhiX control library (Illumina) to balance the low nucleotide base diversity. Each pool was sequenced on Illumina MiSeq Platform, using the v2-500 cycle kit, with 251 bp paired-end sequencing chemistry using Illumina recommended protocol at the Genomics Resource Laboratory (University of Massachusetts-Amherst).

5.3.4 Sequence Analysis

Using the QIIME2 [174] pipeline, paired-end reads were demultiplexed, merged, trimmed to 315 base pairs, and binned inferring amplicon sequence variants (ASVs). Taxonomic identities were assigned using the naïve Bayes sklearn classifier trained with the 799F/1115R region of the Greengenes 13_8 database.

The data from the greenhouse study contained 8,292 ASVs from 403 samples. All samples were rarefied to 1500 reads, which resulted in the loss of 15 samples due to low read depth. The remaining 388 samples ranged from 1950-44,878 reads/sample for a total of 4,984,830 quality reads. Because of the large number of samples, sequencing had to be performed in 2 separate runs. 15 samples were the same between the two different sequencing runs to ensure no sequencing bias was introduced. The samples were assessed to determine if alpha diversity values (Shannon, Pielou’s evenness, Chao1, Observed Richness) and community composition were the same between the sequencing runs. After determining no differences existed between the results of the sequencing runs, sequences were combined and the analysis progressed as one dataset.
5.3.5 Statistical Analysis

Generalized linear mixed models (GLMMs) were used to assess changes in bacterial biomass, alpha diversity (Shannon Diversity Index, observed species richness (number of ASVs), estimated species richness (Chao1), and Faith’s Phylogenetic Diversity (PD)), \textit{nifH} abundance, and rate of nitrogen fixation. The fixed effects used in the models were treatment, host species, and sampling time; sample ID was incorporated as a random effect to account for resampling over time. All models used gamma distribution with a log-link since the data consists of continuous variables bounded at zero. Models were run using the \texttt{lme4} package [176] in R and effects of each variable were determined using TukeyHSD (Tukey Honest Significant Differences). Differences in relative abundance of bacterial community groups was determined using analysis of variance (ANOVA) followed by TukeyHSD implemented in R.

To understand how communities on different host species were changing over the season, if they were impacted by water-reduction stress, and if they were subsequently able to recover, we conducted permutational analysis of variance (PERMANOVA) using Bray-Curtis distances. Results were visualized using non-metric multidimensional scaling (NMDS). PERMANOVA and NMDS were conducted using the Vegan package [179] and visualized using ggplot2 in R [181]. Paired PERMANOVAs were conducted by subsetting host species and/or sampling day and then using the Bonferroni correction to adjust the p-values.
5.3.6 Ecological Null Modeling

Ecological null modeling was performed to explore community assembly in the phyllosphere over time, on different host species, and how these processes are influenced by water-reduction stress and recovery. This was done using the Stegen framework [288] by calculating $\beta$-nearest taxon index ($\beta$NTI) and Raup-Crick (Bray-Curtis) ($RC_{BC}$) to understand contributions from deterministic and stochastic processes, respectively. Using the approach by Danczak et al., $\beta$-mean nearest taxon distance ($\beta$MNTD) was first calculated for every pairwise comparison and for 999 random distributions using the `comdistnt` function in the picante package [294]. $\beta$NTI was then calculated by comparing the observed $\beta$MNTD values to the randomization values. Once $\beta$NTI was calculated, pairwise comparisons between communities were made to understand phylogenetic turnover and to distinguish between stochastic and deterministic processes. If $|\beta$NTI| was greater than 2, differences in the communities were a result of deterministic processes. If, however, $|\beta$NTI| was less than 2, communities underwent a second null modeling step using $RC_{BC}$ to determine if assembly was a result of stochastic processes.

Deterministic and stochastic processes can be further classified. If $\beta$NTI $>$ 2, community assemblages are a result of variable selection, or when differences in communities are more different than is expected by random chance. Alternatively, if $\beta$NTI $<$ -2, communities are significantly more similar than is expected by random chance resulting from homogenous selection. When $\beta$NTI did not indicate strong deterministic assembly processes ($|\beta$NTI| $<$ 2), $RC_{BC}$ was calculated to determine how dispersal and drift influenced community assemblages. $RC_{BC}$ was calculated by creating null communities.
based on the microbial communities from 9999 permutations for each pairwise comparison, which were then used to calculate a null distribution of Bray-Curtis values which was compared to the observed Bray-Curtis values. The results are normalized from +1 to -1 and represent deviations of the observed values compared to the null values. RC\textsubscript{BC} > 0.95 suggest community assembly is influenced by dispersal limitation, which occurs when communities are significantly different from each other due to their inability to mix over time and/or space, which results in drift. RC\textsubscript{BC} < -0.95 indicates homogenous dispersal in which communities are similar to each other due to their ability to mix in their environment. However, if |RC\textsubscript{BC}| < 0.95, differences in communities are considered undominated, or in other words cannot be explained by a single process. Since RC\textsubscript{BC} can only be used to distinguish stochastic processes, instances when |\beta\textsubscript{NTI}| > 2 were not included in the RC\textsubscript{BC} calculation.

5.3.7 Community Function Analysis

5.3.7.1 Bacteria Biomass Sampling. The mass of three leaves placed in 50 ml conical tubes was measured, before 10 ml of phosphate-buffered saline (PBS) was added, incubated at room temperature for 1 hour, then vortexed horizontally (vortex-adaptor, Qiagen, Germantown, MD, USA) at full speed for 10 min. The PBS leaf wash was collected and samples were then fixed with fresh paraformaldehyde to a final concentration of 3.7% and stored at 4°C. Samples were filtered onto black polycarbonate membrane filters (pore size 0.2 µm, 25 mm diameter) (Steriltech Corporation, Kent, WA, USA), stained using 0.1% acridine orange for 3 min, and analyzed with epifluorescence microscopy by counting 20 fields using SimplePCI (Hamamatsu, Japan) [171, 172].
5.3.7.2 Nitrogen Fixation Rate. To determine the rate of biological nitrogen fixation, stable isotope probing was conducted at 6 different time points. Three samples were taken during the drought period (week 6, 7, 10) and one each week during recovery (week 11, 12, 13). Rate of nitrogen fixation was determined by measuring incorporation of the stable isotope $^{15}$N into the leaf tissue. Leaf cuts of known area were incubated in an artificial atmosphere containing 80% $^{15}$N (Sigma-Aldrich, USA) and 20% O$_2$ (Airgas, USA) for 48 hours under ambient light and temperature. Corresponding control samples were incubated under normal atmosphere to determine natural $^{15}$N abundance. After incubation, samples were dried at 70 °C, weighed, finely ground, and 1-2 mg of plant powder were weighed in tin capsules. Samples were sent to University of Vienna to determine $^{15}$N incorporation using a continuous-flow isotope ratio mass spectrometer.

Nitrogen fixation rates were determined using the following equation where $N_{\text{leaf}}$ is foliar N concentration, $M_r$ is molecular weight of $^{15}$N, and $t$ is incubation time [99]:

$$N_2-\text{Fix} = N_{\text{leaf}} \times \frac{\text{at}\%^{15}\text{N}_{\text{sample}} - \text{at}\%^{15}\text{N}_{\text{control}}}{100} \times 10^3 / M_r / t$$

Bacterial DNA samples corresponding to each stable isotope probing timepoint were used to determine the absolute quantity of nitrogen fixing bacteria per leaf area for each of the grass species and treatment using qPCR of the $nifH$ gene. All samples were amplified in triplicate using the PolF (5′ TGC GAY CCS AAR GCB GAC TC 3’) and PolR primers (5′ ATS GCC ATC ATY TCR CCG GA 3’) [242] in a reaction containing Luna qPCR mastermix (NEB, Ipswich, MA, USA), 0.75 µm forward primer, 0.75 µm reverse primer, 1 µl DNA, and molecular-grade water. The qPCR reaction was carried out using 5 min of initial denaturation at 95°C, 40 cycles of 95°C for 30s, 60°C for 30s,
and 72°C for 30s, followed by a final 10 min elongation step at 95°C. Abundance of \textit{nifH} genes was normalized to the number of \textit{nifH} genes per gram of leaf mass.

5.4 Results

5.4.1 Plant Response to Drought

After 10 weeks of water reduction stress, soil moisture significantly decreased in all three host species (orchardgrass \textit{p} \textit{adj}<0.001; ryegrass \textit{p} \textit{adj}<0.001; tall fescue \textit{p} \textit{adj}<0.001) and recovered to control plot levels after three weeks of rewatering (Figure 5.2A). Soil moisture content in tall fescue plots was significantly higher under water reduction than in both orchardgrass (\textit{p} \textit{adj}<0.001) and ryegrass (\textit{p} \textit{adj}=0.003), which were not significantly different from each other. Leaf relative water content (RWC), measured weekly to determine cellular water deficit, showed significant differences between hosts, but did not result in significant changes within each host species under water-reduction stress (Figure 5.2B). To further understand if plants were stressed, relative chlorophyll content was determined weekly (Figure 5.2C). Host species had significantly different relative chlorophyll content (\textit{p} \textit{adj}<0.001), which changed over time within each species (\textit{p} \textit{adj}<0.001). However, only tall fescue was significantly impacted by the water-reduction treatment (\textit{p} \textit{adj}=0.018). Plant cellular membrane stability, determined by measuring electrolyte leakage, was significantly higher during the first week of the experiment for each host species (\textit{p} \textit{adj}<0.001) with periodic spikes, but showed no significant difference as a result of treatment (Figure 5.2D). Furthermore, proline content in leaves, measured as an additional plant stress indicator in week 10 (end of water reduction period) and week 13 (end of recovery period), showed no significant difference as a result of water reduction or time, but was significantly higher in ryegrass compared
to orchardgrass (p adj <0.001) and tall fescue (p adj <0.001) (Figure 5.2E). Root depth, measured at the end of the water-reduction period (week 10), showed no significant difference as a result of species or treatment within any of the three sampling depths (Figure 5.2F).

Figure 5.2. Plant hosts showed few measured changes during the 10-week water reduction period despite significant decrease in soil moisture in each of the 3 host species (A). Plant measurements taken throughout the experimental period include (B) relative water content, (C) relative chlorophyll content, and (D) electrolyte leakage. (E) Proline content was measured at the end of the water reduction period (WRP) and at the end of the recovery period (RP). (F) Root mass was measured at three different depths at the end of the experimental period before re-watering occurred.
5.4.2 Alpha diversity

Community richness (number of unique ASVs observed within a community) increased over the experimental period on all host plants regardless of treatment (Figure 5.3A). However, observed species richness was significantly different between the three host species, with ryegrass communities displaying the highest observed species richness and orchardgrass the lowest observed species richness. Similar trends in diversity were observed for both observed and estimated species richness (Figure 5.10). Despite observed species richness increasing over the experimental period, community evenness (measured using Pielou’s evenness and defined as the number of each individual ASV) either showed no significant changes over time (orchardgrass and tall fescue) or showed a slight decrease over the experimental period (ryegrass) (Figure 5.3B). Additionally, orchardgrass showed significantly lower community evenness compared to ryegrass and tall fescue, which showed no difference from one another. The corresponding relationships between richness and evenness relate to microbial species dominance within the communities. Species dominance was higher in communities from orchardgrass hosts and increased on all host species over time.
Figure 5.3. Microbial community diversity was measured for each species and treatment over the experimental period using (A) observed species richness and (B) evenness. Community evenness on tall fescue hosts was the only alpha diversity metric to show a significant response to water reduction (p=0.001). Differences in diversity levels between host species is represented by the lower-case letters at the top of each plot. Significant differences are represented by different letters and no difference is represented by the same letter. Trends over time were determined using linear models. $R^2$ values are represented at the bottom of the plots for significant trends. N.S., not significant.

Figure 5.4. Relative abundances of bacterial classes were significantly different between the plant host treatments and had different responses to water reduction. Communities from tall fescue and ryegrass hosts were more similar to each other than they were to orchardgrass. The red triangle separates the water reduction period from the recovery period. Legend column A represents classes from orchardgrass hosts that were significantly different from both ryegrass and tall fescue. B represents classes that were significantly different between ryegrass and fescue in both control and water reduction treatment. C represents classes that were different between ryegrass and tall fescue water reduction treatment but not the control treatment. Significance levels are assigned as $p > 0.05$ (not significant); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. 

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**Table 5.1:** Summary of statistical analyses for microbial community diversity. 

<table>
<thead>
<tr>
<th>Species</th>
<th>Richness</th>
<th>Evenness</th>
<th>$R^2$ Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Drought</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Table 5.2:** Classification of bacterial classes. 

<table>
<thead>
<tr>
<th>Class</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrospira</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroflexi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deinococci</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenericutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Figure 5.5:** Temporal changes in microbial community composition across different treatments. 

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**Figure 5.6:** Impact of water reduction on microbial community structure. 

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**Figure 5.7:** Synergistic effects of host species and water reduction on microbial community diversity.
Throughout the experimental period, orchardgrass communities were dominated by bacteria from the class Gammaproteobacteria (57.0%) (Figure 5.4). Additionally, dominance increased throughout the experimental period (p=0.05); orchardgrass communities were composed of 53.7% Gammaproteobacteria during week 1 compared to 65.5% in week 13 (week 1:13 p<0.001). Comparatively, Alphaproteobacteria was the dominant class in communities from ryegrass (27.6%) and tall fescue (44.2%). Dominance of Alphaproteobacteria increased to different degrees within each of these host species. In ryegrass, Alphaproteobacteria increased from 23.3% to 34.1% (p=0.001), while in tall fescue it increased from 23.5% to 50.4% (p<0.001). Alphaproteobacteria and Gammaproteobacteria were not significantly impacted by water-reduction on any of the three hosts species. Other dominant classes of bacteria showed different trends over time and under stress based on host species; relative abundances and trends of bacterial classes were more similar on tall fescue and ryegrass compared to orchardgrass.

5.4.3 Compositional Response

Phyllosphere community structures were impacted by host species, time, and watering treatment. The strongest driver of phyllosphere community structure was plant host species (R²= 0.59, p=0.001; PERMANOVA on Bray-Curtis distances) (Table 5.1) (Figure 5.10). Sample time (R²=0.04, p=0.001) and watering condition (R²=0.03, p=0.001) were also significant drivers. PERMANOVAs were conducted on each individual week to understand how host species influenced community assembly throughout the growing season and determine if and when water-stress affected community structure. While host species was always a significant driver of community
differences, its contribution to community structure increased over the course of the growing season (Figure 5.5). In the first week of the experimental period, host species explained 32% of the variability compared to the last week of the experiment in which it explained 41% of the observed variability (Figure 5.5E).

Despite almost no differences observed in plant traits between control plants and water-reduced plants, phyllosphere communities showed significant responses to the water-reduction treatment. Significant differences between treatments were first observed in week 3 ($R^2=0.05$, $p=0.031$) and increased during the water reduction period (week 10: $R^2=0.11$, $p=0.003$). During the recovery period, treatment continued to have a significant but decreasing impact on community structure (week 13: $R^2=0.07$, $p=0.01$), indicating communities were not fully able to recover from water-reduction stress during the re-watering period (Figure 5.5E).

**Table 5.1.** Phyllosphere community structure was most impacted by host species and was also changed over time (week) and as a result of water-reduction treatment. Impact of each variable on community structure was determined using a PERMANOVA test on Bray-Curtis distance measures.

<table>
<thead>
<tr>
<th></th>
<th>F value</th>
<th>$R^2$</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>369.59</td>
<td>0.594</td>
<td>0.001***</td>
</tr>
<tr>
<td>Treatment</td>
<td>39.46</td>
<td>0.032</td>
<td>0.001***</td>
</tr>
<tr>
<td>Week</td>
<td>52.01</td>
<td>0.042</td>
<td>0.001***</td>
</tr>
<tr>
<td>Species*Treatment</td>
<td>12.71</td>
<td>0.020</td>
<td>0.001***</td>
</tr>
<tr>
<td>Species*Week</td>
<td>9.94</td>
<td>0.016</td>
<td>0.001***</td>
</tr>
<tr>
<td>Treatment*Week</td>
<td>3.54</td>
<td>0.003</td>
<td>0.029*</td>
</tr>
<tr>
<td>Species<em>Treatment</em>Week</td>
<td>1.52</td>
<td>0.002</td>
<td>0.194</td>
</tr>
<tr>
<td>Residuals</td>
<td></td>
<td>0.290</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.5. Bacterial communities from each host species became more distinct over time and were significantly impacted by water reduction stress. NMDS ordination was plotted using Bray-Curtis distances for (A) the beginning of the experimental period (week 1), (B) early water reduction period (week 3), (C) late water reduction period (week 9), and (D) end of the recovery period (week 13). (E) PERMANOVAs were conducted for each corresponding period to understand community stability by determining when communities under disturbance showed signs of change and if they were able to recover from the disturbance event.
While communities from each plant host were influenced by time and water-reduction stress, the degree each community was affected and able to recover was different based on plant host species (Table 5.2). Ryegrass communities had the largest impact as a result of water-reduction treatment ($R^2=0.083$, $p=0.001$), were the first to show changes as a result of disturbance (week 3 $R^2=0.2325$, $p=0.031$), and community composition remained significantly different after the recovery period (week 13: $R^2=0.31$, $p=0.024$). The next most susceptible communities were from tall fescue hosts ($R^2=0.076$, $p=0.001$), which first showed differences in community structure as a result of drought stress in week 4 ($R^2=0.29$, $p=0.021$), and remained significantly different through the entire recovery period (week 13: $R^2=0.25$, $p=0.036$). The more distantly related host species, orchardgrass [295], was the least affected by water-reduction treatment ($R^2=0.052$, $p=0.001$), did not show significant differences as a result of water-reduction stress until week 7 ($R^2=0.30$, $p=0.027$), and communities showed full recovery after disturbance (week 13: $R^2=0.16$, $p=0.48$). These results indicate that phyllosphere communities show varying degrees of stability, and this variation relates to the phylogeny and known drought tolerances of the hosts.
Table 5.2. The effect of water-reduction stress on phyllosphere community composition was different based on host species. Ryegrass communities had the strongest response to water-reduction and were the first to show changes as a result of treatment (week 3). Tall fescue communities were more stable than ryegrass communities showing a lower overall response to stress and showed changes as a result of stress later than ryegrass communities (week 4). Orchardgrass communities were the most stable, which was determined by the lowest overall impact of stress, the last to show significant changes as a result of water reduction (week 7), and was the only host species in which communities fully recovered to non-stressed composition.

<table>
<thead>
<tr>
<th></th>
<th>Orchardgrass</th>
<th>Ryegrass</th>
<th>Tall Fescue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F value</td>
<td>R²</td>
<td>Adjusted p</td>
</tr>
<tr>
<td>Week</td>
<td>18.065</td>
<td>0.121</td>
<td>0.001***</td>
</tr>
<tr>
<td>Treatment</td>
<td>7.753</td>
<td>0.052</td>
<td>0.001***</td>
</tr>
<tr>
<td>Week * Treatment</td>
<td>1.291</td>
<td>0.009</td>
<td>0.202</td>
</tr>
<tr>
<td>Residuals</td>
<td>0.818</td>
<td></td>
<td>0.789</td>
</tr>
</tbody>
</table>

Individual Weeks

<table>
<thead>
<tr>
<th>Week</th>
<th>F value</th>
<th>R²</th>
<th>Adjusted p</th>
<th>F value</th>
<th>R²</th>
<th>Adjusted p</th>
<th>F value</th>
<th>R²</th>
<th>Adjusted p</th>
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<tbody>
<tr>
<td>Week 1</td>
<td>0.787</td>
<td>0.101</td>
<td>1</td>
<td>1.475</td>
<td>0.156</td>
<td>0.093</td>
<td>0.989</td>
<td>0.123</td>
<td>1</td>
</tr>
<tr>
<td>Week 3</td>
<td>1.326</td>
<td>0.142</td>
<td>0.774</td>
<td>2.328</td>
<td>0.225</td>
<td>0.031*</td>
<td>0.984</td>
<td>0.110</td>
<td>1</td>
</tr>
<tr>
<td>Week 4</td>
<td>1.816</td>
<td>0.185</td>
<td>0.258</td>
<td>3.696</td>
<td>0.316</td>
<td>0.024*</td>
<td>2.895</td>
<td>0.286</td>
<td>0.021*</td>
</tr>
<tr>
<td>Week 7</td>
<td>3.497</td>
<td>0.304</td>
<td>0.027*</td>
<td>4.397</td>
<td>0.386</td>
<td>0.027*</td>
<td>2.927</td>
<td>0.268</td>
<td>0.033*</td>
</tr>
<tr>
<td>Week 10</td>
<td>1.932</td>
<td>0.194</td>
<td>0.027*</td>
<td>2.637</td>
<td>0.248</td>
<td>0.024*</td>
<td>2.596</td>
<td>0.270</td>
<td>0.024*</td>
</tr>
<tr>
<td>Week 13</td>
<td>1.464</td>
<td>0.155</td>
<td>0.48</td>
<td>3.546</td>
<td>0.307</td>
<td>0.024*</td>
<td>2.359</td>
<td>0.252</td>
<td>0.036*</td>
</tr>
</tbody>
</table>

5.4.4 Ecological Null Modeling

To better understand the assembly processes that lead to the observed community differences, ecological null modeling was performed using β-nearest taxon index (βNTI) and Raup-Crick index (Bray-Curtis) (RC_BC). Both processes generate randomized communities that are compared to the observed communities to determine if change in community structure is more or less similar than what would occur due to random chance. βNTI is first used to determine if assembly processes are deterministic and then further differentiates deterministic processes into variable and homogenous selection (Table 5.3) [296]. Significant βNTI values (|βNTI|>2) indicate changes in community structure result from deterministic processes, while nonsignificant values indicate changes result from stochastic or undominated processes. Communities that do not have a
significant βNTI value then undergo a second null modeling process using \( RC_{BC} \). The \( RC_{BC} \) index determines if assembly process are stochastic or unable to be attributed to stochastic or deterministic assembly processes (undominated). If \( |RC_{BC}| > 0.95 \), assembly stems from stochastic processes which can be divided into dispersal limitation (such as drift) \( (RC_{BC} > 0.95) \) or homogenizing dispersal \( (RC_{BC} < -0.95) \). When \( |RC_{BC}| < 0.95 \) assembly processes are considered undominated and cannot be attributed to an individual process.

**Table 5.3.** Definition of terms and associated outcomes from the ecological models.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Process</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable Selection</td>
<td>Microbial populations diverge due to selective pressures ie differences in physical conditions.</td>
<td>Deterministic</td>
<td>( \text{BNTI} &gt; 2 )</td>
</tr>
<tr>
<td>Homogeneous Selection</td>
<td>Microbial populations converge due to selective pressures causing similar community compositions ie similarities in physical conditions.</td>
<td>Deterministic</td>
<td>( \text{BNTI} &lt; -2 )</td>
</tr>
<tr>
<td>Dispersal Limitation</td>
<td>Microbial populations separated by time or space are unable to mix resulting in ecological drift driving community divergence.</td>
<td>Stochastic</td>
<td>(</td>
</tr>
<tr>
<td>Homogenizing Dispersal</td>
<td>Microbial populations are freely interacting resulting in microbial exchange between communities. Mixing between populations results in more similar communities between samples.</td>
<td>Stochastic</td>
<td>(</td>
</tr>
<tr>
<td>Undominated</td>
<td>Occurs when a single assembly process is unable to explain the variation because both deterministic and stochastic processes are influencing assembly.</td>
<td>Both</td>
<td>(</td>
</tr>
</tbody>
</table>

Results from the ecological null modelling align with the results from the PERMANOVA; each species is governed by different processes which correspond with their phylogenetic relatedness and stability during water-reduction stress. Community assembly processes were compared between communities from the control treatment to the water-reduction treatment for each species to understand how stress influenced
community assembly each week (Figure 5.6). Furthermore, assembly processes were
assessed for each host species and treatment over time by comparing communities
sampled each week to the communities sampled in the first week, which allowed us to
understand how community structure changed over the experimental period (Figure
5.13). Additionally, communities from each week were compared to communities
sampled the previous week, allowing us to understand small scale changes over time by
assessing assembly processes from week to week (Figure S5.14).

While all three host species displayed strong levels of undominated processes, indicating
no individual assembly process can explain the observed differences, different degrees of
deterministic and stochastic assembly processes were observed across host species
(Figure 5.12). Community assembly on orchardgrass was marked by homogenizing
dispersal and undominated processes (Figure 5.6A-B). When comparing control to water-
stressed communities within a given week, homogenizing dispersal accounted for 32% of
community assembly, suggesting species exchange between these communities (Figure
5.7). Additionally, in both time series comparisons, homogenizing dispersal had a greater
impact on community assembly in the control treatment than in the water-reduction
treatment, thus control plants are more similar over time than the water-reduction plants.
Not surprisingly, homogenizing dispersal was stronger when comparing week to week
(Figure 5.14A) than when comparing from each week to week 1 (Figure 5.13A)
indicating communities are getting more different as time progresses
Figure 5.6. Community assembly processes were different on each of the different hosts species, but all three hosts had high levels of undominated processes. Boxplots show the βNTI values (A, C, E) and RC_{BC} values (B, D, F) comparing communities from the control treatment to communities from the water reduction treatment for each sampling week. Dashed red lines represent the significance cutoffs for βNTI (|βNTI|>2) and RC_{BC} (|RC_{BC}| > 0.95). RC_{BC} values that fall between the dashed red lines represent undominated processes.
Community assembly on ryegrass was influenced by variable selection, dispersal limitation, and undominated processes. When comparing communities between treatments within each week, variable selection (23%) and dispersal limitation (47%) drive assembly processes (Figure 5.7). Additionally, an increase in deterministic processes began to occur in week 4 as differences between ryegrass communities resulting from treatment intensified (Figure 5.6C). When comparing assembly processes from each week to the start of the experimental period, dispersal limitation accounted for 40% of assembly in the control communities and 40.6% in the drought communities (Figures 5.13B, 5.13D), demonstrating ecological drift occurred over time. Furthermore, variable selection was responsible for 20% of community assembly in the water-reduction treatment compared to only 2% in the control treatment over time. Variable selection began to influence community assembly on the water-reduction treated hosts starting in week 4, indicating changes in the host environment (water reduction) are driving community differences. When comparing community assembly from week to week, dispersal limitation accounts for 36% of assembly processes in the control community compared to only 14% in the water-reduction communities suggesting drift has more influence on assembly from week to week in the control communities than the communities from the water reduction treatment (Figures 5.14B, 5.14D).

On tall fescue hosts, water-reduction within a given week was characterized mostly by undominated processes, but dispersal limitation (22%) and variable selection (6%) did have moderate impacts on community assembly (Figures 5.6E-F, 5.7). Community assembly processes from tall fescue hosts varied when comparing communities to the
start of the experiment and when comparing from week to week. Assembly processes were more influenced by dispersal limitation the longer the experiment progressed (Figure 5.13C). However, when comparing week to week, community development was a result of mostly undominated processes (Figure 5.14C). Variable selection similarly influenced communities from control and water-reduction treated plants over the course of the experiment (15% and 13%, respectively) (Figure 5.13D), but had a greater influence on water-stressed communities from week to week (4% and 15%, respectively) (Figure 5.14D).

**Figure 5.7.** Treatment effects on community assembly processes for each of the host species. While undominated processes were prevalent on each host species, the defined processes varied. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.
Mantel tests were used to correlate results from the ecological null modeling over time and with host species. To understand relationships between community assembly and time, sampling day was transformed into a Euclidian distance matrix which was correlated to both the βNTI and RC$_{BC}$ distance matrices. This was done separately for each host species and treatment. βNTI was correlated with time for communities from the water-reduction treatment on ryegrass ($R=0.116$, $p<0.001$) and orchardgrass ($R=0.061$, $p=0.05$). RC$_{BC}$ values were significantly correlated with time for every host species and treatment (Table 5.14). Phylogenetic host distances were used to correlate community assembly processes with host relatedness. βNTI values were correlated to communities from the control treatment ($R=0.06$, $p<0.001$) but not from the water reduction treatment. However, RC$_{BC}$ values were significantly correlated to host phylogeny for both control ($R=0.41$, $p<0.001$) and water-reduction treatments ($R=0.39$, $p<0.001$).

5.4.5 Measurement of Functional Stability

Microbial biomass in the phyllosphere was determined through direct cell counts using epifluorescence microscopy (Figure 5.8). Ryegrass had a significantly higher bacterial biomass per leaf mass than orchardgrass ($p<0.001$) and tall fescue ($p<0.001$). Biomass was not affected by water treatment on any of the host species but was influenced by time. In tall fescue and orchardgrass, samples taken on the first day of the experiment had significantly higher biomass levels than the subsequent sample days which were stable from week 5 until week 13. Bacterial biomass from ryegrass hosts had more temporal variability and showed an overall decreasing trend over the course of the experiment,
with only week 12 (p=0.005) and week 13 (p=0.009) showing a significant decrease from week 1.

Figure 5.8. The effects of host species and treatment on bacterial biomass over time. The total number of bacterial cells per gram of leaf material from each host species was calculated by washing bacterial cells off the surface of leaves, counted using epifluorescence microscopy, and compared to leaf areas calculated using ImageJ. Each watering treatment is represented by 5 biological replicates for each host species on each sampling day. After the first sampling day, orchardgrass and tall fescue showed stable bacterial biomass, but ryegrass had more day-to-day variation.

Ability of phyllosphere bacteria to fix nitrogen was determined by measuring the rate of nitrogen fixation using stable isotope probing. Bacterial communities from ryegrass had a significantly higher rate of nitrogen fixation per gram of leaf material than was found on tall fescue (p<0.001) and orchardgrass (p<0.001) (Figure 5.9A). Within each host species, no difference was seen as a result of treatment or over time. To test if rate of nitrogen fixation related to potential of nitrogen fixation, nifH gene copy number was determined using qPCR. nifH abundance was not significantly affected as a result of treatment or time (Figure 5.9B). However, gene abundance per gram of leaf material was different between all three host species. Tall fescue had the highest number of nifH genes.
while orchardgrass had the lowest. Rate of nitrogen fixation compared to \textit{nifH} gene abundance was evaluated using a linear regression but was not found to be a significant relationship ($R^2=-0.007$).

![Figure 5.9](image)

**Figure 5.9.** Rate of nitrogen fixation (A) and abundance of the \textit{nifH} gene (B) were significantly different between host species, but were not affected by the water reduction disturbance. Nitrogen fixation rate was measured using stable isotope probing. The abundance of \textit{nifH} genes was measured using qPCR and standardized to number of copies per gram of leaf material. Significant differences between host species, determined by linear models with TukeyHSD post-hoc analysis, is represented by lowercase letters in the upper left corner of each plot. Different letters indicate statistical differences.

### 5.5 Discussion

Many phyllosphere studies show that host species selection is an important driver of community assembly, but few studies have been conducted across grass species [60, 61, 72, 75–77, 122]. Despite grass leaves being close to the soil, which could provide a constant source of microbial colonizers, phyllosphere communities in our study could be differentiated based on host species identity. Ryegrass and tall fescue are more related to each other than they are to orchardgrass [295], which was closely mirrored in microbial community similarities: they had similar diversity levels and were more similar to each
other compositionally than they were to orchardgrass. Like in many phyllosphere
studies, all communities in our study were dominated by Proteobacteria [61, 75, 76, 84,
297]. However, orchardgrass was dominated by Gammaproteobacteria while ryegrass
and tall fescue were dominated by Alphaproteobacteria. In addition to compositional
differences at a high taxonomic level (class) between host species, community succession
throughout the growing season followed different patterns between the host species.
From this, we concluded that successional changes are plant-host species dependent. To
further understand differences in community assembly, we investigated if response to
disturbance remains host species dependent or if a common disturbance drives
communities to be more similar to each other.

Microbial community stability has many definitions, but we used the Shade et al.
definition that stability is composed of resistance (degree a community is unaffected by
disturbance) and resilience (rate a community returns to undisturbed condition) [132]. We
used water-reduction as a disturbance event because it is a climate stress grasslands are
experiencing with increased frequency and severity [151, 155, 221]. Community stability
can relate to compositional or functional stability, which can exhibit similar trends under
stress but do not always do so [134]. We assessed compositional stability over time for
each host species by looking at community profiles using the 16S rRNA gene.
Additionally, we looked at a few measures of community function by measuring the \textit{nifH}
gene abundance and rate of nitrogen fixation as well as measuring microbial biomass,
which allowed us to assess changes in bacterial growth rates. Resistance was measured
by determining if and when a community showed significant differences (alpha diversity,
beta diversity, or functionality) from the control community. Resilience was assessed by determining if communities were able to recover to a state indistinguishable from the control community. Because of the observed community succession over the course of the experiment, resistance and resilience were assessed in relation to control communities measured the same day as opposed to comparing to pre-disturbance communities.

Microbial community composition was significantly affected by water-reduction stress despite almost no measurable effect on plant hosts. All three hosts showed stable alpha diversity measurements during disturbance, but showed varying degrees of compositional stability. Host species phylogeny had a strong influence on phyllosphere community stability levels with known drought tolerance of the host species exhibiting a lesser effect. Ryegrass and tall fescue, which are more closely related to each other than they are to orchardgrass, had more similar levels of microbial community stability compared to orchardgrass. Additionally, tall fescue communities were more stable than ryegrass communities which is consistent with the higher drought tolerance exhibited by tall fescue [298]. However, orchardgrass communities showed much greater stability than tall fescue communities despite having a slightly lower drought tolerance [298]. These differences could relate to overall physiology and stress survival strategies of the hosts. Even though plants did not show visible signs of stress and our measurements showed minimal differences between the treatments, plants undergo biochemical changes before visual signs of stress are observed [299]. Furthermore, orchardgrass and tall fescue have different drought tolerance strategies. Tall fescue primarily relies on a deep root system whereas orchardgrass has better water extraction efficiency from soil and exhibits
dehydration tolerance in its tissues [190]. These differences are seen to a small degree within our plant health measurements. Tall fescue maintained a higher soil moisture level than orchardgrass, which could be attributed to tall fescue plants accessing water at a much greater soil depth and orchardgrass extracting more water from the surface soil. Additionally, chlorophyll content was higher under water-reduction treatment compared to control treatment in tall fescue plots but not in orchardgrass plots. This is consistent with previous studies in tall fescue that found an increase in chlorophyll content under mild drought stress could result from decreasing cellular growth compared to chlorophyll turnover [300]. A more in-depth comparison of plant traits and metabolic profiles under normal and stressed environments could provide further insight into the differences observed between microbial communities from different hosts and if these changes help mitigate negative effects associated with stress.

To understand what ecological processes may be underpinning compositional stability in the phyllosphere, ecological null modeling was used to determine the influence of deterministic and stochastic processes on community assembly under healthy and disturbed conditions. Since the phyllosphere is considered a harsh environment, we expected deterministic processes would dominate community assembly. Instead, we found high levels of stochastic processes and processes that could not be well defined as stochastic or deterministic (undominated assembly), with only low levels of deterministic assembly processes. Across host species, treatment, and sample day, dispersal limitation accounted for 44% of assembly processes, variable selection accounted for 11%, and homogenizing dispersal for 3%. Furthermore, within these quantifiable processes,
different trends were observed within each host species which related to their varied levels of community stability.

Ryegrass was marked by high levels of dispersal limitation over time and showed higher levels of variable selection between control and stressed samples than the other host species. Variable selection occurs when communities are more different than is expected by chance [301] and these changes between communities can result from selection pressures caused by ecological disturbances [132]. The compositional instability observed on the drought sensitive ryegrass hosts could result from disturbance driving deterministic selection processes [302]. Despite our plant analysis revealing little effect on plant host as a result of disturbance, finer scale responses may be taking place at the metabolic level resulting in different selection pressures.

Tall fescue had the highest levels of undominated processes when comparing control to disturbed samples within a given week and when comparing subsequent weeks. This could result from strong competing assembly processes, where both deterministic and stochastic processes are occurring and thus potentially cancelling out the signal of each process. However, when comparing communities to the first week, dispersal limitation accounted for 59% of assembly processes in the control hosts. This trend was also observed across time in ryegrass in which dispersal limitation results in 40% of control and 41% of disturbed assembly processes. In all instances, we see dispersal limitation increase as time from sample week one increases. This indicates that microbial communities are unable to interact due to temporal separation. Furthermore, when
communities are unable to mix, ecological drift over time causes species to become significantly different from one another [117, 288, 290].

Orchardgrass was the only host species with high levels of homogenizing dispersal. This was true both across time and when comparing control and disturbed communities. Homogenizing dispersal suggests low rates of microbial community turnover as a result of dispersal mixing the microbial communities [301]. Additionally, orchardgrass had the lowest levels of dispersal limitation indicating communities were not separated by space or time. The interconnectedness of the communities on orchardgrass could account for the increased resistance and resilience and lower community richness observed on this host species.

Due to the harsh nature of the phyllosphere, we expected microbial communities from control plants to exhibit homogenous selection within each host species, and when subjected to disturbance expected to find variable selection as the dominant process as has been demonstrated in other systems [302–306]. While we did observe some increase in variable selection under disturbance conditions, we found community assembly processes resulted mostly from dispersal limitation and undominated processes. Similar results were found in a serpentinizing aquifer system for which the authors concluded that the low diversity system dominated by a few taxa and many variable low abundance species made the system prone to ecological drift [290]. Previous work has shown that environments exerting strong selection forces could result in low biomass and low diversity communities characterized by a few abundant taxa and many rare taxa which
are susceptible to large variation in growth and death rates thus resulting in drift [117, 290, 307, 308]. Since phyllosphere communities are comprised of a few abundant taxa and many rare taxa [39], we propose they are similarly prone to drift despite strong environmental selection pressures, but that levels of drift relate to degrees of stability.

Undominated processes, which were a major driver of assembly in the phyllosphere communities, frequently result from weak selection, weak dispersal, diversification, or drift [116, 288]. Previous studies concluded that undominated processes can also result from counteracting forces that make it hard to distil an individual process [289, 290]. The extreme nature of the phyllosphere as a microbial habitat along with inconsistent dispersal events could result in counteracting deterministic and stochastic processes that resulted in the undominated processes observed between host species, over time, and during environmental disturbance.

In addition to compositional stability, functional stability was determined for two different functions: growth and nitrogen fixation. Bacterial biomass, used to understand cell growth, exhibited higher levels at the beginning of the experiment, which decreased and remained steady in orchardgrass and tall fescue, but showed more variability over time in ryegrass. The high biomass on day one of the experiment corresponds with a higher level of electrolyte leakage in all host species. Electrolyte leakage measures membrane permeability and is thus an indicator of cell death, therefore increased permeability leads to leaching of compounds which could be utilized by microbes and thus account for the observed biomass differences [309]. Despite the temporal
differences, treatment had no effect on cellular biomass indicating stable growth rates during disturbance. Growth rates are tied to resource use efficiency, which can have important implications for community stability. Communities with fast growers (copiotrophs) are more resilient but have less resistance, while slower growing microbes (oligotrophs) which have higher resource use efficiency have increased resistance [132]. This dynamic was observed in a study looking at resistance and resilience of grassland soil communities in response to drought. This study found the slow growing fungi were more resistant while the faster growing bacteria were more resilient [141]. The phyllosphere communities in our study were dominated by Alphaproteobacteria and Gammaproteobacteria, which are frequently characterized as oligotrophs [310]. These two classes of bacteria likely support community stability as they made up over half of the bacterial species found on each host species and showed no significant differences under drought stress.

Nitrogen fixation was measured throughout this experiment to first assess if it was occurring in phyllosphere communities and then to understand whether it was a stable process. Nitrogen fixation has been extensively studied in the rhizosphere and leaf-litter, but few studies have directly measured it as a process in the phyllosphere and none on grass hosts [98, 99, 104, 311]. Bentley and Carpenter found that phyllosphere microbes could account for 10-25% of the nitrogen content found in their study species, indicating phyllosphere microbes are likely an important and underrecognized global source of nitrogen [98]. In our study, we determined that rate of nitrogen fixation is occurring at a stable rate over time and under mild disturbance, but occurs at different rates depending
on host species. To further understand nitrogen fixation in the phyllosphere, we compared the rate of nitrogen fixation to the abundance of nifH genes to determine how potential relates to activity. Previous studies in leaf litter found nifH gene abundance was closely related to nitrogen fixation rates in free-living bacteria [312]. However, in our temperate grass system, nifH abundance did not relate to nitrogen fixation rate even though both rate and abundance were species specific and showed no significant variation over time or as a result of disturbance. Further studies investigating leaf uptake of phyllosphere available nitrogen and the microbes involved in nitrogen fixation will be important for understanding phyllosphere contribution to the nitrogen cycle and if phyllosphere microbes can be used as a biofertilizer to replace chemical nitrogen fertilizers. Both rate and abundance were species specific and showed no significant variation over time or as a result of disturbance indicating functional stability.

5.6 Conclusion

In conclusion, phyllosphere compositional stability in this study was highly related to plant host species phylogeny and, to a lesser extent, known stress tolerances. However, under mild disturbance levels, all host species showed functional stability for nitrogen fixation rate and changes in bacterial biomass. These discrepancies could be a result of functional redundancy within the community or could relate to the nature of phyllosphere communities being prone to ecological drift. Phyllosphere communities are composed of a few dominant taxa and many rare taxa that are constantly in flux. This results in a few taxa that are stable and many transient taxa. We propose that the stable, dominant taxa are responsible for most of the measurable functionality of the community which would
explain why we see variability within and between days but not in response to disturbance. Phyllosphere community assembly and stability is a result of complex interactions of ecological processes that are differentially imposed by host species. Given the vast expanse of phyllosphere ecosystems, it is important to continue to understand assembly processes in relation to community functionality and understand how these functions relate to host species growth and stress response.
5.7 Supplemental Tables

Table 5.4. Soil physical-chemical analysis for experiments with tropical and temperate grasses. Macronutrient and micronutrient concentrations were determined using Modified Morgan extractables.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil pH</td>
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</tr>
<tr>
<td>Macronutrients (ppm)</td>
<td></td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>17.8</td>
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<tr>
<td>Potassium (K)</td>
<td>109</td>
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<tr>
<td>Calcium (Ca)</td>
<td>936</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>161</td>
</tr>
<tr>
<td>Sulfur (S)</td>
<td>8.7</td>
</tr>
<tr>
<td>Micronutrients (ppm)</td>
<td></td>
</tr>
<tr>
<td>Boron (B)</td>
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</tr>
<tr>
<td>Manganese (Mn)</td>
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<tr>
<td>Zinc (Zn)</td>
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<tr>
<td>Copper (Cu)</td>
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<td>Iron (Fe)</td>
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<td>Aluminum (Al)</td>
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<tr>
<td>Lead (Pb)</td>
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<tr>
<td>Cation Exch. (meg/100 g)</td>
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</tr>
<tr>
<td>Exch. Acidity (meg/100 g)</td>
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</tr>
<tr>
<td>Soil Organic Matter</td>
<td>3.7</td>
</tr>
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</table>

Table 5.5. Mantel tests were performed on BNTI and $RC_{BC}$ values to correlate changes in community structure over time with each host species and treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\beta$NTI</th>
<th></th>
<th>$RC_{BC}$</th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Mantel Statistic R</td>
<td>p-value</td>
<td>Mantel Statistic R</td>
<td>p-value</td>
</tr>
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<td>N.S.</td>
<td>0.25</td>
<td>1e-04</td>
</tr>
<tr>
<td>Orchardgrass Drought</td>
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<td>0.05</td>
<td>0.22</td>
<td>1e-04</td>
</tr>
<tr>
<td>Ryegrass Control</td>
<td>0.028</td>
<td>N.S.</td>
<td>0.26</td>
<td>1e-04</td>
</tr>
<tr>
<td>Ryegrass Drought</td>
<td>0.116</td>
<td>8e-04</td>
<td>0.45</td>
<td>1e-04</td>
</tr>
<tr>
<td>Tall Fescue Control</td>
<td>-0.055</td>
<td>N.S.</td>
<td>0.41</td>
<td>1e-04</td>
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<tr>
<td>Tall Fescue Drought</td>
<td>0.043</td>
<td>N.S.</td>
<td>0.40</td>
<td>1e-04</td>
</tr>
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</table>
5.8 Supplemental Figures

Figure 5.10 Alpha diversity was calculated using several different metrics including (A) Chao1, (B) Faith’s PD, and (C) Shannon Diversity Index. Trends from the observed and estimated richness measures were similar. Observed ASV richness and evenness had different trends over time resulting in different trends when measuring Shannon diversity index.
Figure 5.11. Host species had the strongest impact on community structure. NMDS ordination was plotted using Bray-Curtis distances for every sampling day.
Figure 5.12. Heatmaps representing pairwise all comparisons for BNTI (A) and RC_{BC} (B). (C) Contribution of each process to community assembly was calculated for all host species, treatments, and sampling days by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.
Figure 5.13. Boxplots show the BNTI values (A-B) and RC_{BC} values (C-D) comparing communities from each sampling week to communities samples in week 1 for both the control and water reductions treatment for each host species. Dashed red lines represent the significance cutoffs for BNTI (|BNTI|>2) and RC_{BC} (|RC_{BC}| > 0.95). (D) Bar plots show the overall contribution of each process to community assembly process differences between treatments on each of the host species. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.
A. Orchardgrass  

B. Ryegrass  

C. Tall Fescue  

D.  

Figure 5.14. Boxplots show the BNTI values (A-B) and RC_{BC} values (C-D) comparing communities from each sampling week to communities sampled in the previous week for both the control and water reductions treatment for each host species. Dashed red lines represent the significance cutoffs for BNTI (|BNTI|>2) and RC_{BC} (|RC_{BC}| > 0.95). (D) Bar plots show the overall contribution of each process to community assembly process differences between treatments on each of the host species. The percentage of contribution was calculated by dividing the number of significant pairwise comparisons for each process by the total number of pairwise comparisons.
CHAPTER 6
CONCLUSION AND FUTURE WORK

6.1 Importance

Grasslands are an essential component of global food security which act as the foundation for ruminant milk and meat production. Yet, they are at risk of degradation due to human activity. As grassland conditions decrease or are converted to urban environments, new land must be cleared to maintain current agricultural outputs resulting in further ecosystem degradation, which leads to increased rates of greenhouse gas emissions. Increasing pasture productivity and lifespan of current grasslands facing the threat of climate change is crucial for supporting a growing human population.

Plant-microbe interactions that occur on the leaf surface, or phyllosphere, are an understudied and underutilized resource. Phyllosphere bacteria aid plants by improving plant resistance to pathogens, producing plant hormones, and increasing available nitrogen. Strategically supporting these bacteria could offer new sustainable farming practices. This dissertation explored community assembly and response to drought of phyllosphere bacterial communities from six different grass species that are prominent in temperate and tropical forage systems.

Figure 6.1. Microbe-microbe and plant-microbe interactions that occur on the surface of leaves have the potential to influence plant and ecosystem health.
6.2 Phyllosphere Assembly and Stress Response in the Greenhouse

Two separate greenhouse studies were conducted on temperate and tropical grasses to understand the composition of phyllosphere communities from five grass species, how they respond to drought stress, and if there are similar trends across grass species and environments. Five grass species were selected for their frequent use in forage systems, variations in native climate zone, taxonomy, drought tolerance levels, and pathways of carbon fixation. Some similarities in bacterial communities and their response to stress were observed. Bacterial community abundance and diversity decreased while relative abundance of potentially pathogenic bacteria increased, indicating reduced bacterial community health. Additionally, differences in microbial communities in relation to plant traits became more defined under stress conditions. Finally, we found that some bacteria are indicators of mild drought conditions which may act as potential plant symbionts suggesting they are targets for biofertilizers designed to promote agricultural systems under climate stress.

6.3 Phyllosphere Assembly and Stress Response in the Field

In the second research project, six forage grass species (including all five species used the greenhouse) were grown under identical conditions in pots in a modified field experiment. In this study, we found that microbial community structure is strongly linked not only to plant host identity but also to plant host phylogeny. Evidence of phylosymbiosis revealed that community similarity directly related to plant host phylogenetic distance. Furthermore, we found that phyllosphere communities are dynamic, showing strong temporal changes regardless of treatment, but the temperate
grass hosts appeared to have stronger selection over the course of the growing season than the tropical grass hosts. This could relate to temperate grass hosts being found in their native environment with microbes that have adapted to survive on them. Microbial community response to drought stress was different between the host species, which related to native environment, known drought tolerance within that environment, and drought tolerance strategies. Finally, in addition to looking at community composition, we explored the effect of host species, time, and drought on the abundance of one important functional gene, \textit{nifH}. While \textit{nifH} gene abundance was not significantly impacted by drought stress, it did show temporal differences and differences between host species.

6.4 Comparing Phyllosphere Assembly and Stress Response in Different Environments

In this study, we compared patterns of community assembly and response to drought between the field and greenhouse studies for two temperate grass species (orchardgrass and tall fescue) in order to understand if similar trends were observed across environments. We expected to observe similar trends in community structure and response to drought, i.e. similar dominant classes of bacteria, similar levels of host influence, and similar changes in community structure under drought stress. However, we found large discrepancies between the two environments. Greenhouse grown plants were dominated by Gammaproteobacteria while field grown plants were dominated by Alphaproteobacteria; host species had a much smaller effect on community structure in the greenhouse plants compared to the field plants, but were more influenced by drought
stress; and field grown plants had a significantly higher abundance of the nifH gene. Additionally, network analysis revealed that microbial communities from field grown plants exhibited stronger selection and were more stress-tolerant than greenhouse communities. We concluded that field grown plants had a more stable phyllosphere community than greenhouse grown plants, which likely related to competitive differences found in each of the environments.

6.5 Phyllosphere Community Resistance and Resilience

The fifth chapter explored the resistance and resilience (stability) of phyllosphere communities on three species of temperate forage grasses. Resistance was determined by withholding water for a 10-week period to see when and how communities responded to the disturbance event. Resilience was measured by rewatering the plants over a three-week period to determine if community composition and functionality returned to match undisturbed communities. Phyllosphere community stability was most strongly related to host phylogeny with secondary influence based on known drought tolerance of the host species. Despite strong selective forces on community assembly, such as from host species, assembly over the course of the experiment was greatly influenced by stochastic processes. One important focus of studying microbial community assembly under different environmental conditions or stresses is to be able to predict microbial community composition and functionality under different conditions. Creating these predictions can help provide many different ecological and agricultural services that are needed to deal with a changing climate. Stochastic community assembly can make this predictive process more difficult as these processes result from random interactions.
However, understanding the factors in a given environment that lead to stochastic assembly can help in the predictions of community composition and functionality. For instance, in these phyllosphere communities, stochastic processes were largely in the form of ecological drift, which result from communities dominated by a few dominant taxa and many rare taxa. In these phyllosphere communities, many of the dominant taxa vary in abundance with time but are stable in terms of presence across time and under disturbance. Stable dominant community members paired with the observed stability of our two measured functions could demonstrate that core members are responsible for functionality. Therefore, while drift is influencing composition, it may only have small impacts on functionality as the organisms most affected are temporally unstable microbes that frequently land on the plant surface but are never able to establish.

6.6 Synthesis

Across the experiments conducted in this dissertation, there were unifying features and takeaways. First and foremost, we found that phyllosphere communities on the leaf surface are dynamic and are strongly selected for by their host species. In addition to host species always having a significant impact on community structure, we also found that selection increased throughout the experiments driving communities to become more distinct from each other over time. Next, we found that microbial communities were impacted by drought stress. We tested different degrees of stress and found that even under very mild drought stress (assessed by host response), microbial communities were affected. Microbial communities responded to drought stress before measured plant traits showed response to stress. We found that community response to drought is linked to
host species identity and the hosts’ response to stress. The most drought tolerant host species showed the least effect from drought. This was shown as a comparison of the last sample day in every single experiment, and it was shown in relation to when communities began to change as a result of stress. Additionally, we found that these changes in community structure correlated with plant traits and stress response strategies.

Host species from their native environment had greater cophylogenetic relationships than the species grown in foreign environments. This indicates greater evolutionary relationships between the native hosts and their microbial communities than observed on the foreign hosts. This finding is unsurprising but confirms the idea that phyllosphere microbes have a shared evolutionary history with their host and demonstrates that deterministic processes are important in microbial community colonization. Furthermore, understanding and comparing community succession of plants in a foreign environment to a native environment helps us better understand community assembly, but also has important agricultural implications. Understanding microbial establishment and community dynamics between plants and microbes that have not previously interacted will be useful in creating broad application biofertilizers.

Despite these observed trends across experiments, we found that environment played an important role in community assembly and response to drought. Plants grown in the field had much higher degrees of community stability under drought stress compared to greenhouse grown plants. We concluded that the decreased stress tolerance we observed was an effect of being grown in the greenhouse and not merely a result of different
environments. Previous work has shown that geographic location plays an important role in microbial community assembly, but in those studies community profiles at the class level were not as dramatically different from each other.

Nitrogen fixation potential, measured as \textit{nifH} abundance, was detected at various levels across host species and environments. Within each of these studies, \textit{nifH} abundance was significantly different between host species, but was not impacted by drought stress. Furthermore, in our last study we also found nitrogen fixation activity was differentially present across host species but was also not significantly impacted by drought stress. Unfortunately, we found that \textit{nifH} abundance was not a good proxy for nitrogen fixation activity.

These findings raise a few important questions:

(1) Are microbial communities responding to stress before the plant host?
(2) Can bacterial communities act as a stress response trait for the host plants?
(3) Do plant hosts select for certain microbial functions, which would be conserved across geographic distances?
(4) Are functional profiles stable over time and under drought conditions?
(5) How do microbial functions support plant growth and are they changing in a way to promote survival of the plant under drought conditions?
(6) Is nitrogen that is fixed on the leaf surface utilized by the grasses?
If microbial response to stress promotes plant response to stress, potential ways microbial functionality could facilitate this is by producing plant hormones involved in osmotic adjustment such as abscisic acid, jasmonic acid, and salicylic acid.

6.7 Future Studies

In our work we have found clear trends in phyllosphere community assembly that have potentially important implications for plant health, agriculture, and ecosystem productivity. The next steps for being able to utilize phyllosphere communities to promote ecological functions will be to have a better understanding of the functional profiles of phyllosphere bacteria and how they directly interact with and support plant health.

6.7.1 Metagenomics

Future studies should focus on understanding functions and benefits microbial communities provide to their plant host, how those change under stress, and if those changes promote host survival under stress. The first step to understanding the functional profiles of plants would be to perform metagenomics on the communities. All of the DNA from these experiments has been saved and would be an important place to start. By doing metagenomic analysis of the samples collected in these studies, we would be able to answer several unexplored questions including understanding how functional profiles are different between plant hosts species, determine if functional profiles are temporally stable, and understand how and when certain functions change as a result of drought. By looking at the functional profiles we can look for important functions that aid
microbial establishment and survival in the harsh phyllosphere environment. We can also identify functions that may be involved in promoting plant health such as production of certain nutrients or phytohormones.

For the analysis of the phyllosphere metagenome, 19 samples from the 2019 field experiment have been sent to Joint Genome Institute for sequencing and we are still waiting for the complete sequencing data. Samples were selected from ryegrass and orchardgrass species from both control and drought treatment collected on the last day of the experiment when drought effect was strongest. This small subset of samples will not fully explore questions related to community functionality, response to stress, and stability over time in the phyllosphere of grasslands. However, it will give us an initial glimpse into functional capabilities and response to stress of the phyllosphere communities, which will help guide future questions related to community function.

6.7.2 Artificial Communities

To better understand microbe-microbe and microbe-plant interactions, future work should aim to develop synthetic communities and test how inoculation onto axenic plants influences productivity and stress tolerance. We currently have two phyllosphere isolate libraries that would serve as a starting point for developing synthetic communities. The isolates were collected during the two field experiments from both drought stressed and control plants of each of the species. Species identifications have been assigned to all isolates in the library collected during the 2020 field season (library A), however only 32 of the 139 isolates have been identified in the 2019 library (library B). Species identity
for the remaining isolates should be determined and identities compared to the other isolate library. 15 of the identified isolates from library A (Appendix B) were found to be in the core community (present on greater than 90% of samples from one species and treatment). Whole genome sequencing should be performed on these isolates to gain an understanding of what functions promote their survival in the phyllosphere and what important functions could they be providing to plant hosts. Some important functions might include: nitrogen fixation, biofilm formation, and production of important plant hormones Indole-3-acetic acid (IAA) and nitric oxide (NO). These functions have the potential to promote plant health and stress tolerance. Nitrogen is an important nutrient and essential component of protein, so by promoting nitrogen available to the plants we can increase biomass production and nutrient quality. Biofilms found on plant leaves help maintain available nutrients, prevent pathogen invasion, and limit desiccation. IAA is important for growth and development of plants while NO is a signaling molecule involved in many defense responses including stomatal closure, root development, and germination.

After identification of functionally important isolates, axenic and greenhouse plants would then be inoculated with single microorganism cultures either as seedlings (~1 week after germination), mature plants (~3 months after germination), or as a seedling with re-inoculation as a mature plant. Effect of microbes could be assessed by looking at changes in total biomass and nutrient profile compared to non-inoculated controls after a certain period of growth. Additionally, establishment and survival of inoculums should be assessed through 16S rRNA sequencing.
Microbe-microbe interactions could be assessed by creating artificial communities comprised of different isolates to understand how they interact with one another. Bacterial candidates could be evaluated for their compatibility with each other and for their ability to grow at different levels of nutrient availability. By growing the different candidates together on different media dilutions we could assess their interactions, look for bacteria important in suppressing pathogen growth, and select groups of bacteria for inoculation experiments by finding bacteria that will not inhibit one another and be able to grow under several different nutrient levels including low levels that are expected during times of stress such as drought.

Finally, artificial microbial communities could be inoculated onto seedlings, mature grass plants, and onto plants at both stages. After inoculation, seedlings would be allowed to grow until maturity at 3 months and could then be subjected to drought stress, continue growing, or reinoculated. For mature plants, microbial communities should be given three weeks to establish post-inoculation before being subjected to drought based on previous inoculation studies [160]. Studies should be comprised of four experimental groups groups: 1&2) inoculated grass under conventional watering conditions or under drought conditions, 3&4) uninoculated grass under conventional watering conditions or under drought conditions. Plant health should be assessed throughout the drought to determine if there is a difference between inoculated plants and the uninoculated controls. At the end of the drought, biomass and leaf nutrients could be measured to determine if inoculated plants produce more biomass without compromising the nutrient quality and
to determine if timing and frequency of inoculation matters. Throughout the drought, microbial community composition should be assessed regularly to understand how amendment of artificial communities influences phyllosphere community assembly and to determine if microbial community dynamics are different in an artificial community compared to a control community. Creating artificial communities helps us deepen our understanding of phyllosphere communities by understanding microbe-microbe and plant-microbe interactions. By understanding these dynamics and understanding how specific microbes interact with the plant host, we can develop microbial targets and methods to utilize microbes as biofertilizers to promote sustainable agriculture production.

6.7.3 Nitrogen Fixation

In this dissertation, we found that nitrogen fixation happens in the phyllosphere but do not yet understand how much nitrogen phyllosphere microbes contribute to the plant. One way to assess this would be look at total microbial input of nitrogen incorporated into the plant and separate it by rhizosphere input and phyllosphere input using stable isotope probing. One approach to achieved this could be to use stable isotope probing in greenhouse mesocosms. In this whole plant system, plants would be in an airtight system that allowed flow of nitrogen between the phyllosphere and the root zone. The rhizosphere input would be determined by creating an airtight chamber around the roots and soil only, and the phyllosphere input would be determined by creating an airtight chamber that encompassed only the above ground leaf material. These airtight environments would be composed of 79.95% heavy labelled nitrogen (N\text{15}), 20% oxygen,
and 0.05% carbon dioxide, which will be regulated by sensors and gas supply systems attached to the system that can adjust the inputs as necessary. A corresponding control experiment should be established that has the same setup but uses non-labelled nitrogen (N\textsuperscript{14}) to assess and compare the levels of naturally occurring N\textsuperscript{15}. After a set incubation time, leaf and root material will be harvested from each experiment and the control, and after leaves and roots are surface sterilized, nitrogen incorporation could be determined using isotope-ratio mass spectrometry. This study would help understand what percentage of nitrogen used by plants originates from the phyllosphere compared to the rhizosphere, allowing us to conclude if the phyllosphere is a good target for nitrogen biofertilizers.

6.7.4 Concluding Thoughts

As climate change alters weather patterns and intensifies stress on agricultural systems, we need to find sustainable ways to increase crop production while decreasing the number of intact ecosystems converted to agricultural land each year. I focus on grasslands because they are important for global food stability and as a vast ecosystem have serious global climate implications. I propose that targeted enhancement of the phyllosphere will help promote agricultural production in the face of climate stress allowing for more stress-resistance and increased pasture production. This also reduces the need for further ecosystem conversion. To achieve this, we must better understand how bacteria promote plant growth. These future studies will build on the foundation of microbial community assembly processes and response to stress by helping to elucidate the role phyllosphere bacteria play in promoting plant growth and in the plant-associated
nitrogen cycle. Understanding the roles of phyllosphere bacteria in plant health and global biogeochemical cycles will allow us to leverage plant-microbe relationships to promote sustainable farming practices and reduce greenhouse gas emissions.
Figure A1. Drought experiments were conducted either under greenhouse, modified field, or field conditions. Community diversity and composition were assessed using several different univariate and multivariate statistics, microbial biomass was assessed using fluorescence microscopy, and rate of nitrogen fixation was measured using stable isotope probing.
APPENDIX B

ISOLATE LIBRARIES

B.1.1 2020 Isolate Library Preparation

To create isolate libraries, leaves collected from control and drought stressed plants were collected from each host species (orchardgrass, ryegrass, tall fescue) on the last day of the drought before the recovery period began. Leaves were washed in 10 ml of phosphate buffered saline and shaken in a vortex adaptor for 5 min in order to remove leaves from the leaf surface. 100 ul of four dilutions of cell washes were inoculated onto R2A media. Colonies were assessed for each species and treatment and were isolated if they displayed unique visual appearances. In other words two colonies could be isolated if they displayed identical morphologies if they came from different host species or different treatments. After isolation, glycerol freezer stocks were made for each colony. Colonies were identified using sanger sequencing of the 16S rRNA gene using primer 27F and a subset of those underwent sanger sequencing with a PCR fragment using 799F and 1115R primers. This allowed us to directly compare isolates to our MiSeq community data to understand isolates distributions over time, under drought, and between species.
B.1.2 Library A: 2020 Isolate Analysis

Table B1. Identification of isolates from the 2020 library using Sanger sequencing of the 16S rRNA gene using primer 27F. Identity was assigned using BLAST on the NCBI database.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Number of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium fabrum</td>
<td>1</td>
</tr>
<tr>
<td>Agrobacterium rubi</td>
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</tr>
<tr>
<td>Bacillus pumilus</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus zhangzhouensis</td>
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<td>Brevundimonas vesicularis</td>
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<td>Curtobacterium luteum</td>
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</tr>
<tr>
<td>Curtobacterium pusillum</td>
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<tr>
<td>Microbacterium testaceum</td>
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<tr>
<td>Pantoea allii</td>
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</tr>
<tr>
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<td>Pseudomonas parafulva</td>
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<tr>
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<td>Sphingomonas parapaucimobilis</td>
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<tr>
<td>Sphingomonas sanguinis</td>
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<tr>
<td>Sphingomonas yahuuchiae</td>
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Table B2. Identification of isolates using Sanger sequencing of the 16S rRNA gene using a PCR product from the 799F and 1115R primers. Figure letter corresponds with the species abundance plot created using MiSeq community data in Figure B1.

<table>
<thead>
<tr>
<th>Figure Letter</th>
<th>Genus</th>
<th>ASV ID</th>
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</tr>
<tr>
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<td>B.</td>
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<td>k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus</td>
</tr>
<tr>
<td>C.</td>
<td>Bacillus</td>
<td>k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus</td>
</tr>
<tr>
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<td>Curtobacterium</td>
<td>k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Microbacteriaceae; g__Curtobacterium; s__</td>
</tr>
<tr>
<td>D.</td>
<td>Curtobacterium</td>
<td>k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Microbacteriaceae; g__Curtobacterium; s__</td>
</tr>
<tr>
<td>D.</td>
<td>Curtobacterium</td>
<td>k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Microbacteriaceae; g__Curtobacterium; s__</td>
</tr>
<tr>
<td>E.</td>
<td>Deinococcus</td>
<td>k__Bacteria; p__[Thermi]; c__Deinococci; o__Deinococcales; f__Deinococcaceae; g__Deinococcus; s__</td>
</tr>
<tr>
<td>F.</td>
<td>Leucobacter</td>
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<tr>
<td>G.</td>
<td>Methylobacterium</td>
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</tr>
<tr>
<td>I.</td>
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<tr>
<td>J.</td>
<td>Pseudomonas</td>
<td>k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas</td>
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<tr>
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</tr>
<tr>
<td>L.</td>
<td>Sphingomonas</td>
<td>k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas; s__</td>
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<tr>
<td>M.</td>
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<td>Xanthomonas</td>
<td>k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Xanthomonadaceae; g__Xanthomonas</td>
</tr>
</tbody>
</table>
**Figure B1.** Relative abundance species distributions of isolates sequenced using the same primers used in the MiSeq community sequencing project. Identity was assigned by comparing isolate sequence to the representative sequence database created in the MiSeq analysis.
B.2 Library B: 2019 Isolate Library

To create isolate libraries, leaves collected from control and drought stressed plants were collected from each host species (*Brachiaria brizantha, Brachiaria decumbens, Brachiaria hyb.*, orchardgrass, ryegrass, tall fescue) on the last day of the drought experiment. Leaves were washed in 50 ml of phosphate buffered saline and shaken in a vortex adaptor for 5 min in order to remove leaves from the leaf surface. 100 ul of four dilutions of cell washes were inoculated onto 4 different media: R2A, 1:10 dilution R2A, TSA, and Kings Medium B. Kings Medium B is used to detect Pseudomonas species. Colonies were assessed for each species and treatment and were isolated if they displayed unique visual appearances. In other words two colonies could be isolated if they displayed identical morphologies if they came from different host species or different treatments. After isolation, glycerol freezer stocks were made for each colony. A subset of colonies were identified using sanger sequencing of the 16S rRNA gene using primer 27F.

Table B3. Identification of isolates from the 2019 library using Sanger sequencing of the 16S rRNA gene using primer 27F.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Number of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium rubi</em></td>
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<tr>
<td><em>Curtobacterium flaccumfaciens</em></td>
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<td><em>Curtobacterium luteum</em></td>
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<td><em>Pantoea agglomerans</em></td>
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</tr>
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<td><em>Erwinia tasmaniensis</em></td>
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<td><em>Frigoribacterium faeni</em></td>
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</tr>
<tr>
<td><em>Microbacterium oleivorans</em></td>
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<td><em>Pantoea allii</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Pantoea ananatis</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Pseudomonas savastanoi</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Sphingomonas melonis</em></td>
<td>2</td>
</tr>
</tbody>
</table>
APPENDIX C

TURFGRASS STUDY

C.1 Overview

A study looking at drought tolerance and recovery of different turfgrass cultivars was organized by the US Golf Association (USGS) and the National Turfgrass Evaluation Program (NTEP) to identify cultivars with low water use requirements and high drought resistance. This information can inform users and government officials about turfgrasses that can be used as home lawns or on golf courses that have minimal watering requirements. The study was setup at 10 locations across the United States in Fall 2016 with five of the sites conducting the experiment under rainout shelters. The DaCosta lab conducted and oversaw one of the 10 studies that took place at the university of Massachusetts’ Joseph Troll Turf Research Center in South Deerfield, MA. In this study, 19 cultivars of tall fescue (*Schedonorous arundinaceus* Schreb.), 15 cultivars of Kentucky bluegrass (*Poa pratensis* L.), and 1 cultivar of perennial ryegrass (*Lolium perenne* L.) were grown in triplicate in 3ft x 3ft plots organized in a randomized block design. Cultivars were grown under a large rainout shelter that covered all three blocks. Drought stress was imposed by halting watering of the plots. Cultivars were assessed for their drought tolerance by determining length of time with no precipitation until a set level of drought stress was met. Drought stress was determined through visual quality rating, presence of wilt, and percent green cover. Once the drought stress threshold was met, plants were rewated to assess their rate and ability to recover. Plots were initially given 6 gallons of water and then were continuously given 4 gallons of water each week to ensure they were well watered. Multiple droughts could be imposed over the course of
one growing season. More detailed study and methods description can be found in a paper published by Morris and Kenna in 2017 [313].

In 2020 and 2021, we collaborated with the DaCosta lab to study the phyllosphere bacterial communities on these turf grass cultivars under the different stress conditions. In 2020, we selected eight cultivars to look at through the course of one drought experiment. Six of the cultivars were tall fescue and 2 were Kentucky bluegrass (Figure C1A). The cultivars were chosen based on their performance in the previous year’s drought study; we tried to select cultivars that were either top or bottom performers. In this experiment, each cultivar underwent three sampling times. The first was a pre-drought sample, a drought sample, and sample after recovery. The pre-drought and post-recovery samples were taken on the same day for every cultivar. However, the drought sample was taken on different sampling days because the drought threshold was assessed for each individual plot, so not every plot reached that threshold on the same day. In 2021, 12 cultivars were selected (6 tall fescue and 6 Kentucky bluegrass) (Figure C1B). Five cultivars from each species were exposed to two drought and recovery periods throughout the summer, while one cultivar from each species acted as a well-watered control. In experiments from both years, communities were assessed using 16S rRNA amplicon sequencing and bacterial biomass was determined using fluorescence microscopy to perform direct cell counts.
C.2 Goals

The goal of these studies was to address the following questions:

1. Are turfgrass phyllosphere communities influenced by drought stress and does the degree of stress relate to the known/measured drought tolerance of the cultivar host?
2. In addition to host species level differences, are there cultivar level community differences?
3. Are phyllosphere communities able to recover from drought stress?
4. Are they more drought tolerant after repeated drought exposure?
5. Are there temporal trends that can be observed between the sampling years?
6. Is community composition and response to drought similar on turfgrass cultivars compared to forage grass cultivars?
**Figure C.1.** Cultivars used in the (A) 2020 and (B) 2021 phyllosphere study and their location in the randomized block design. Xs indicate cultivars that were not sampled.
C.3 Preliminary Results

C3.1 2020 Results

**Figure C.2.** Alpha diversity showed decreasing trends as a result of drought for several cultivars but only two cultivars showed significant declines in alpha diversity as a result of drought. Significant relationships are represented by capital letters above boxplots, where different letters indicate significant differences. In cultivars where treatment did not result in significant differences, no letters are displayed.

**Figure C.3.** NMDS and PERMANOVA based on weighted UniFrac distance metrics shows that community structure changes as a result of drought, but is most influenced by host species.
Figure C.4. Taxa barplots at the (A) class level show that (B) Alphaproteobacteria and (C) Gammaproteobacteria are the most dominant genera. The two Kentucky bluegrass cultivars are given in blue labels and the 6 tall fescue have pink labels. Differences in community structure and response to drought are seen between the two host species and the different cultivars within each host species. Of note from Alphaproteobacteria (B), the genus *Agrobacterium* showed an increase in abundance during recovery while the genus *Sphingomonas* was susceptible to drought and showed little signs of recovery. In Gammaproteobacteria (C), *Enterobacter* increased in abundance during drought while *Xanthomonas* and *Pseudomonas* showed sharp increases during recovery.
C3.2 2021 Results

Figure C.5. Alpha diversity was measured for each cultivar using the Shannon Diversity Index. Kentucky bluegrass cultivars had a stronger response to drought with several of the cultivars decreasing under drought stress. Many tall fescue cultivars showed no response or only shifted during the first drought period. One explanation for the stronger shift during the first drought period could be due a priority effect in which microbes that survived the first drought period alter community profiles resulting in communities more tolerant to the second drought. For each host species, five cultivars (DRT) were exposed to repeating drought and recovery periods and one cultivar was a control irrigated over the entire experimental period (IRR). Sample Day 1 corresponds to pre-drought, Sample Day 2 to the end of the first drought period, Sample Day 3 to the end of recovery period 1, Sample Day 4 to the end of drought 2, and Sample Day 5 to the end of the final recovery period.
Figure C.6. NMDS and PERMANOVA based on weighted UniFrac distance metrics shows that community structure is different between host species but become more similar after several drought and recovery periods. Additionally, differences between irrigated controls and drought stressed plants measured on the same day had significantly different community structures, but their structures were not different on pre-drought or recovery days indicating that communities are resilient to drought stress.
Figure C.7 Taxa barplots at the (A) class and for dominant genera including (B) Actinobacteria, (C) Alphaproteobacteria, and (D) Gammaproteobacteria for each tall fescue cultivar. Gammaproteobacteria shows an increase in relative abundance during the drought days (2 and 4) which correspond with increases in *Enterobacteriaceae* family or *Erwinia* genus, while *Agrobacterium* from the Alphaproteobacteria class appear to have increased relative abundance on non-drought days (1, 3, 5). Other notable changes include a decrease in *Rhodococcus* (B) and an increase in *Sphingomonas* (C) over time.
Figure C.8 Taxa barplots at the (A) class and for dominant genera including (B) Actinobacteria, (C) Alphaproteobacteria, and (D) Gammaproteobacteria for each Kentucky bluegrass cultivar. Similarities in the abundance profiles compared to tall fescue include increased Gammaproteobacteria, specifically *Enterobacteriaceae* and *Erwinia*, during drought stress. Notable difference include overall higher abundance of *Methylobacterium* (C) and a large increase on the last sampling day.
C.4 Next Steps

The analysis from this study is still in its initial stages. Statistical analyses looking at the differences in diversity levels and taxon abundances between treatments and species needs to be completed. Additionally, we need to quantify the resistance and resilience of each cultivar's community and then compare that to drought tolerance data of each cultivar. This will help us understand if the degree of microbiome stability relates to drought tolerance levels of plant hosts. Furthermore, comparisons of results between years will help us understand if temporal trends exist which allows us to understand and predict temporal stability. Understanding temporal trends can be useful for understanding factors influencing community assembly, which gives insight into important bacteria and lays groundwork for understanding how to manipulate microbial populations to support plant health.


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