

Isolation of Phyllosphere Bacterial Community Members in Forage Grasses

An Independent Honors Thesis

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ABSTRACT

As global warming continues, forage grasses that are critical to agricultural activity are exposed to increasing occurrences of drought, which brings about the question of how microorganisms can help crops resist and recover from drought conditions. There is much to be learned about the bacteria present on the leaf surface, or phyllosphere, of forage grasses. In particular, there is a current lack of understanding on how phyllosphere communities respond to drought. We hypothesize that different species of host plants have distinct phyllosphere communities, have different core members and some possess the ability to fix nitrogen in drought conditions. To investigate this topic, we took phyllosphere samples from *Festuca arundinacea*, *Dactylis glomerata* and *Lolium perenne* exposed to drought and control conditions over a 13-week period. Their DNA was isolated for gene marker analysis of the 16S rRNA and *nifH* gene, allowing us to determine isolate identity and nitrogen fixation potential. Using graphical analyses, the relative abundance of different phyllosphere community members, including core community members, was determined. Results show that host plant species determines the composition of the phyllosphere. In addition, the core community is less prevalent in drought conditions. Lastly, the *nifH* gene was not present in the bacteria isolated from the study.

INTRODUCTION

One of the most pressing issues facing the world is global climate change, which affects a wide array of industries, including agriculture. Due to global warming, worldwide crop production is facing unprecedented challenges. Currently, we are encountering increasing numbers of extreme weather patterns such as flooding or drought (Hashim and Hashim 2016). That puts crops that serve an important role in creating food sources around the world in danger. As a result, food scarcity is becoming an increasingly prevalent problem, along with diminished economic production in nations that rely on the agricultural industry (Ruwoldt 2013).

Pastures, which are used for a significant portion of agricultural activity, contain forage grasses that can be heavily impacted by the onset of drought. These pastures are critical for providing sustenance for grazing animals, including livestock. Based on recent findings, the effects of drought on forage grasses may be affected by microbes found on the leaf surfaces of these plants, the phyllosphere (Bechtold *et al.*, 2021).

To understand the significance of symbiotic phyllosphere microorganisms to crops under drought conditions, it is first important to investigate what organisms are present in the phyllosphere of forage grasses. Knowledge of phyllosphere composition provides important insight on what organisms could be involved in conferring drought-survival capabilities to host plants. Additionally, it is crucial to learn what genes these microbes possess that allow them to protect plants against drought. By knowing more information about the species and their genes involved in conferring drought tolerance, more research can be done on how to use these species as ways of fortifying plants against the effects of climate change.

In the current climate of environmental microbiology, the phyllosphere is highly understudied in comparison to other parts of the plant, such as the rhizosphere (the microbiome

of plant roots) (Vorholt, 2012). There is an extensive gap in knowledge on the significance of the phyllosphere in host plant health. As such, it is crucial to understand its implications as they may provide valuable insight on an entire group of plant symbionts.

Specifically, I look to understand what organisms are found in the phyllosphere of the perennial grass species *Festuca arundinacea*, *Dactylis glomerata* and *Lolium perenne*. These organisms are critical to the wellbeing of agriculture due to their role in feeding grazing animals. The effects of drought on their phyllosphere compositions were studied by comparing microorganisms found in both drought and control growing conditions.

The overall aim of my study is to better understand how the effects of different host plants and drought impact the phyllosphere. This could provide insight on how commensal bacterial communities respond to a shifting global climate, which could have implications on plant health and future methods of protecting crops. Additionally, understanding the differences in phyllosphere communities based on host plant could provide valuable insight on how to fortify them against drought.

The overall question being asked for this study is, ‘can microorganisms in the phyllosphere support forage grasses in resisting to or recovering from drought conditions?’ To help answer this broad question, it is important to first answer the questions (1) what microorganisms are present in the phyllosphere of forage grasses in drought and watered conditions? and (2) is there a core community present in the phyllosphere? Additionally, (3) can we isolate nitrogen-fixing bacteria? By collecting data to answer these specific questions, the overarching question of the study may be answered.

The hypotheses of the study are that (1) host plants have different relative abundances of bacteria and different responses to drought conditions and (2) different plant hosts have their

own distinct core communities. Moreover, we hypothesize that, (3) nitrogen-fixing bacteria can be isolated from the phyllosphere.

To prove my hypotheses, relevant data must be collected using an array of methods. Over a 13-week period, phyllosphere samples of forage grasses exposed to control and drought growing conditions were taken. We extracted DNA from bacterial isolates taken from the grass phyllosphere that can be used to undergo gene analysis. Sanger sequencing and BLAST searches were performed on DNA samples to learn what organisms are present. Additionally, using PCR amplification and gel electrophoresis, I looked for the presence of the marker gene *nifH* to see if these bacteria are potentially nitrogen fixing plant growth-promoting bacteria (PGPB). *nifH* is a gene that is known to be involved in bacterial nitrogen fixation (Kuypers *et al.*, 2018). Additionally, using RStudio, an analysis of the temporal core community was performed.

REVIEW OF LITERATURE

AGRICULTURE

An essential aspect of any nation's structure is how the country provides food for its citizens. Agriculture, the industry concerned with cultivating plants and domesticating animals, has been the key sector for producing sustenance for civilization since its advent. When the agricultural industry is performing well, a country can allocate food and resources for both consumption and trade. For some nations around the world, agribusiness is a critical component of the economy. In fact, the agricultural industry accounts for four percent of the globe's gross domestic product (GDP), and even upwards of 25 percent of the GDP of some developing countries (Agriculture and Food, 2020). Thus, when agriculture does not perform well, there is a great deal at stake.

Currently, the effects of global warming on agriculture are one of the world's most pressing issues. There is an array of problems that come with climate change, including the adverse effects it has on growth conditions for crops. Changes in precipitation patterns, extreme temperatures, and an increase in the prevalence of drought are some of the major factors driving issues with crop cultivation (USGCRP, 2017). Altered growing conditions can have a significant impact on crop yield, a measure of agricultural productivity (Alexandrov, *et al.*, 2000). Drought is a major contributor to weakened crop production (Ray, *et al.*, 2018). Lowered crop yields can have monumental consequences on a society, including limited food security and an increase in emigration. Since a large number of developing countries rely heavily on agriculture for sustenance and economic growth, the effects of climate change disproportionately affect less developed nations.

An important crop in agriculture that is affected by global climate change are forage grasses. These crops are a critical facet of the agricultural industry. Forage grasses are estimated to represent seventy percent of agricultural area (Capstaff, *et al.*, 2018). They are a highly prevalent crop due to their role in feeding livestock, and their economic significance is thus immense. Moreover, these crops are ecologically significant, as they prevent soil erosion, promote clean drinking water, and sequester carbon in the atmosphere (Li *et al.*, 2018).

Some of the most common temperate forage grasses include *Festuca arundinacea*, *Dactylis glomerata* and *Lolium perenne*. These grasses are all perennial grasses that play a role in agricultural production, especially in serving as food for grazing animals. Cattle, horses and sheep are some of the major grazing animals that rely on these grasses for nourishment. These animals are important facets of agriculture in many countries around the world. Therefore, the health of these grass species is vital for the wellbeing of livestock. Given the negative impacts of global climate change on crops, these forage grasses are susceptible to growth problems. Extreme weather patterns, such as drought, are one of the major dangers these forage grasses face. Understanding how drought impacts the growth of forage crops could provide ways of protecting them from drought conditions. Due to climate change, there is a predicted overall increase in the prevalence of drought in the future (Steiger *et al.*, 2019). As the prevalence of climate change will likely only increase in the coming years, it is imperative to find ways to mitigate its effects on agriculture.

THE PHYLLOSPHERE

To understand the mechanisms by which forage grasses grow and survive, it is critical to understand the symbiotic microorganisms that live on them. The phyllosphere is found on the

outer surface of plants, and it includes a variety of microbes, including bacteria, archaea and fungi (Vorholt, 2012). The phyllosphere includes the aerial parts of plants. As such, the phyllosphere is made up of the anthosphere, caulosphere, phylloplane and carposphere, which refer to the surfaces of flowers, stems, leaves and fruits, respectively (Vacher *et al.*, 2016). Plant leaves make up the vast majority of the phyllosphere. Thus, the microorganisms that are found on the leaves of plants make up a significant portion of phyllosphere microbes.

The organisms that inhabit the phyllosphere can vary because of certain factors, such as levels of available nutrients and water. Other environmental factors, such as pollution and radiation, also play a significant role in shaping the composition of the phyllosphere, in terms of organisms present and the quantity of bacterial cells found on the leaf. Unlike other regions of the plant, such as the rhizosphere (the root microbiome), the environment of the phyllosphere can vary greatly. As the prevalence of water and nutrients increases, the size of bacterial aggregates on leaf surfaces tends to also increase (Monier and Lindow 2004). Host plants have the ability to transfer metabolites to the leaf surface, where microbes can utilize them (Stone *et al.*, 2018). Additionally, the density of microorganisms on plant leaves varies based on the characteristics of the leaf; microbes tend to populate on the leaf surface where they are protected from environmental stressors. Thus, the highest density of microbes is generally found around leaf veins, between leaf cells and near stomata (Stone *et al.*, 2018). These areas of the leaf are also where nutrients on the leaf are most densely found. Moreover, these leaf structures may help anchor microbes to the leaf surface, which allows for biofilm formation.

Carbon sources are another key factor in determining how organisms in the phyllosphere fare in their environment. Microbes rely heavily on the carbon that comes from host plants. Sugars that are produced by photosynthesis, such as glucose, sucrose and fructose, are critical

nutrients required by many phyllosphere symbionts (Bringel and Couée 2015). Microbes found in the phyllosphere may also obtain supplementary forms of carbon from plants, such as nucleic acids, organic acids, and sugar alcohols. The availability of these nutrients throughout the day varies due to changes in sunlight levels as days progress. As a result, temporal variations in microbe density and distribution occur as carbon levels vary throughout the day.

In addition to carbon sources, nitrogen sources also play a significant role in affecting phyllosphere microbes. Microbes in the phyllosphere can obtain nitrogen by means of plant-produced amino acids that leach to the leaf surface. They can utilize various nitrogen sources, including ammonia and organic nitrogenous compounds, such as amino acids (Vorholt, 2012).

Phyllosphere microbes also metabolize volatile organic compounds (VOCs). VOCs are emitted by the host plant, and they may take the form of a variety of compounds. Some of the common VOCs that are released by plants include methanol, methane, and terpenes (Bringel *et al.*, 2015). Phyllosphere organisms can use these compounds as energy sources or for other metabolic purposes. Certain phyllosphere bacteria may produce their own VOCs that are utilized by the host plant.

The biochemical exchange that must occur between host and symbiont is crucial for the survival of phyllosphere organisms. Due to the lipidic and waxy cuticles on the host leaf, the exchange of key nutrients can be inhibited. Thus, there are several processes involved in this exchange, including excretion, wounding, guttation, exudation, leaching, and infiltration (Bringel and Couée 2015). These exchange processes allow the host to directly provide nutrients to phyllosphere bacteria. For example, guttation pushes fluid from inside the plant to the outside, where microbes can utilize the plant's nutrients.

While the microorganisms of the phyllosphere rely heavily on their host plant, the host plant is also significantly affected by the microbes that inhabit its leaf surfaces. For example, microbial symbionts synthesize biofilm layers on leaf surfaces. Biofilms are composed of extracellular polymeric substance (EPS), which helps prevent water from escaping through the plant's leaves (Stone *et al.*, 2018). EPS also shields plants from high levels of UV light exposure, which can be detrimental to plant health.

Additionally, phyllosphere bacteria produce plant hormones that can be used by the host via biogeochemical exchange. Auxins and cytokinins are common phytohormones involved in plant growth that can be modulated by phyllosphere microorganisms (Legein *et al.*, 2020). For example, biosynthesis of the auxin indole-3-acetic acid (IAA) has been observed in many genera of phyllosphere bacteria (Duca *et al.*, 2014). As this hormone plays a key role in cell elongation, cell division, and fruit development, its production can be highly beneficial to the host's growth.

Phyllosphere bacteria can also compete with pathogenic organisms that harm the host plant. Mutualistic bacteria on the leaf can compete with pathogens for nutrients and resources, produce antibiotics and activate an immune response by the host (Stone *et al.*, 2018). For example, phyllosphere microbes can induce the production of degradative enzymes in hosts that help lessen the severity of fungal infections (Fernanando *et al.*, 2007). Such mutualistic interactions are critical for the wellbeing and survival of plants, especially in conditions where pathogens are prevalent in the environment.

Moreover, the phyllospheres of many varieties of plants contain diazotrophic bacteria that fix atmospheric nitrogen into forms that can be utilized by plants (Grady *et al.*, 2019). Diazotrophs are bacteria that can perform biological nitrogen fixation (BNF), which is an essential component of the nitrogen cycle. Nitrogen is often a limiting nutrient for plants as they

cannot directly utilize N₂ from the atmosphere. Diazotrophs convert atmospheric nitrogen into organic compounds, such as ammonia and nitrates, that plants can use. BNF by these organisms is especially important since the only other way plants can obtain nitrogen is through nitrogen fertilizers. The issue with these nitrogen fertilizers is that they can be harmful to the environment. Nitrogen fertilizer use is associated with nitrate pollution in bodies of water and nitrous oxide pollution in the atmosphere (Byrnes 1990).

There are several genes associated with bacteria involved in nitrogen cycling, such as *nifH*, *nirS* and *nirK* (Kuypers *et al.*, 2018). In particular, *nifH* can be used as a marker gene to identify organisms as nitrogen-fixing bacteria. *nirS* and *nirK* are important genes for denitrification.

On a larger scale, microbial diversity in the phyllosphere can have major implications on entire ecosystems. There is evidence to suggest that phyllosphere microbial diversity is linked to terrestrial ecosystem productivity (Laforest-Lapointe *et al.*, 2017). Ecosystem productivity is defined as the rate of biomass generation. An array of different phyllosphere compositions leads to an increase in biomass for reasons that are not currently understood.

OTHER FACTORS AFFECTING PHYLLOSPHERE COMPOSITION

The structure of phyllosphere communities is determined by a number of factors, including the effects of dispersal. Dispersal events are when microorganisms are spread from one area to another through different mechanisms. Microbes can be transferred via dust particles, animals, and water. A plant's phyllosphere may be drastically affected by dispersal events, depending on when the event takes place in the plant's development (Maignien *et al.*, 2014).

Younger leaves have different phyllosphere compositions than those of older leaves. In young leaves, phyllosphere composition is heavily influenced by airborne bacterial colonizers (Maignien *et al.*, 2014). Young leaves generally have fewer phyllosphere microbes than their older counterparts (Freschet *et al.*, 2010). In a phenomenon known as priority effect, colonizing that occurs early on in a leaf's development could have important impacts on phyllosphere composition. The first microbes that colonize the leaf can grow freely with little or no competition, which allows them to dominate much of the space on the young leaf. Colonizing bacteria come from soil, seeds, nearby plants, insects, and animals, and this results in similar communities found between annual plants (Whipps *et al.*, 2008).

In addition to dispersal, microbial evolutionary phenomena, including evolutionary diversification, genetic drift, and selection, affect phyllosphere composition (Vacher *et al.*, 2016). Diversification occurs because of abiotic stressors that lead to mutations in bacteria. These abiotic stressors could include reactive oxygen species (ROS) or high levels of UV light. Mutations diversify the gene pool of different species, leading to variability in bacterial genotypes. Genetic drift is a product of a loss of genetic diversity due to stochastic events, such as flooding. Due to such events, certain genes or species in the phyllosphere may become more or less prevalent. The last type of evolutionary event that affects phyllosphere composition is selection. Certain microbial traits are more advantageous in a given environment than others, so genes for these traits are selected for. For example, microbes that are able to resist UV damage from an environment with high levels of UV light are more likely to survive than those without these capabilities. As such, more fit bacteria will outcompete those that do not possess survival advantages.

The host plant genotype also plays a significant role in determining the organisms present in the phyllosphere. Individual plants of the same species have highly similar phyllosphere community compositions (Redford *et al.*, 2010). There is evidence that plant genotypes determine leaf colonizers, leading to the early establishment of the phyllosphere's community (Whipps *et al.*, 2008). Additionally, there are evolutionary associations between phyllosphere bacteria and their host plants (Kembel *et al.*, 2014).

As such, the microbial communities that are present in the phyllosphere of *F. arundinacea*, *D. glomerata* and *L. perenne* will likely remain consistent between individuals. However, given that these three grasses are of a different genus, their microbial communities are poised to be distinct from one another. Similarities in their microbial composition may be due to the fact that these three grasses are grown in comparable climate conditions. The climate that a plant is grown in influences the microbial composition of the plant (Vokou *et al.*, 2012). For the climate that these three plant species are grown in, it can be expected that *Proteobacteria*, *Actinobacteria* and *Firmicutes* will likely be common phyla in their phyllosphere communities (Moore-Colyer *et al.*, 2018). Some of the organisms that will also likely be present in the phyllosphere of these forage grasses are diazotrophs, as they are important for nitrogen fixation for the host.

EFFECT OF DROUGHT ON THE PHYLLOSPHERE

Drought causes a number of issues in the growth and development of plants. Drought is an extreme weather phenomenon in agriculture that is defined as a prolonged lack of water in soil (Staniak *et al.*, 2015). Water stress leads to a lower rate of plant cell division and photosynthesis, as well as an inability to maintain osmotic pressure within cells. Severe water

stress can cause permanent damage to the physiology of the organism, along with plant death. To combat drought conditions, plants synthesize abscisic acid (ABA) and proteins associated with mitigating the effects of limited water. In addition, different species have other varying mechanisms of combating drought. Within an organism's ontogeny, plants may acclimate to drought using stress avoidance techniques. Avoidance is when plants can prevent the loss of water using a number of mechanisms, such as producing barriers that stop water from escaping the plant (Staniak *et al.*, 2015).

Symbiont microorganisms can also help host plants resist the effects of drought. They can perform this by inducing physical and chemical changes in hosts. Extensive studies have been conducted on how rhizosphere bacteria confer drought resistance. For example, rhizosphere bacteria produce EPS that causes soil to stick to roots, which is important for obtaining water from soil (Sandhya *et al.*, 2009). Another way in which they protect plants in drought is by synthesizing phytohormones that allow the plant to survive despite environmental stress. Plants that are primed with rhizosphere plant growth-promoting rhizobacteria (PGPR) fare better in drought conditions than plants that are not provided with these microorganisms (Kasim *et al.*, 2013). Currently, there is a lack of studies pertaining to the effects of phyllosphere plant growth promoting bacteria (PGPB) on drought resistance in crops. There is still some evidence to suggest that phyllosphere bacteria may have a beneficial effect on plants in drought.

One of the effects of drought is nitrogen limitation, as observed by a lower carbon to nitrogen ratio in leaves exposed to drought (Stone *et al.*, 2018). Diversity and richness of nitrogen-fixing bacteria in the phyllosphere increases in drought, which indicates phyllosphere nitrogen-fixers play an important role in conferring drought tolerance to the host plant through mitigating the effects of low nitrogen levels. One study found that in wild grass species,

biological nitrogen fixation (BNF) by certain bacteria can contribute over 30% of a plant's nitrogen demand (de Morais *et al.* 2012). The *nifH* gene is important for BNF functioning in bacteria. The *nifH* gene codes for a dinitrogenase-reductase, which is utilized by nitrogen-fixing bacteria to carry out BNF (Li *et al.*, 2019). It should be noted that the mere prevalence of *nifH* in the genomes of symbiotic bacteria does not necessarily mean nitrogenase is actively being expressed because nitrogenase is regulated both before and after translation (Ueda *et al.*, 1995). *nifH* abundance merely correlates with dinitrogenase-reductase activity in the phyllosphere. Microbes in the phyllosphere may also confer drought resistance and recovery by means of affecting plant hormone levels. Certain phyllosphere bacteria produce abscisic acid (ABA). ABA controls the closing of stomata, and this closure is important for drought resistance, as this prevents water from escaping the plant (Sussmilch *et al.*, 2017). PGPR have been found to confer drought tolerance to plants by producing other phytohormones, such as gibberellins, auxins and cytokinins.

Additionally, phyllosphere bacteria can confer host plant resistance to pathogens in drought conditions. Drought may make the environment more favorable for certain pathogens to survive and reproduce, leading to the increased potential for crop infection. Mutualistic bacteria on the leaf can compete with pathogens for nutrients and resources, produce antibiotics and activate an immune response by the host (Stone *et al.*, 2018). For example, phyllosphere microbes can induce the production of degradative enzymes in hosts that help lessen the severity of fungal infections (Fernando *et al.* 2007).

APPLICATIONS OF PHYLLOSPHERE STUDIES

Different plant microbial communities, such as the rhizosphere, have been largely explored, but the phyllosphere has been relatively untouched (Laforest-Lapointe *et al.*, 2019). It is imperative to understand more about the microorganisms that inhabit this region of the plant. Studies on phyllosphere communities can provide knowledge on biodiversity and population dynamics in a mostly unexplored area of the plant (Redford *et al.*, 2010). Such work could yield information on how microbial communities function in an environment that frequently changes temporally and throughout the year. Moreover, phyllosphere studies can help researchers understand more about host-microbe interactions, which has a number of uses in agriculture.

Learning more about the phyllosphere and how it impacts plant health in harsh conditions can provide insight on how to deal with the imminent climate change challenges facing the world today and in the future. Phyllosphere studies allow researchers to understand how plants can survive at the microbial level. Understanding the phyllosphere at a deeper level can have major implications to different fields in agricultural science. For example, knowing what microbial communities are best suited for preventing infection to crops during drought could better crop yields. As the prevalence of drought increases, the need for information on how to protect the world's food sources becomes even more important. With the need to double the world's food supply by the year 2050, novel approaches to facing agricultural problems must be researched (Food 2009).

Overall, understanding the phyllosphere is not only important to the field of microbiology, but to many other realms of science. The phyllosphere could serve as the key to facing the agricultural problems that plague the world. As such, applications of phyllosphere studies have the potential to provide sustenance to people for years to come.

MATERIALS AND METHODS

Field Design and Plant Growth

Plants were grown in western Massachusetts at the University of Massachusetts Research and Education Farm. The soil composition of the plants was 28.8% sand, 64.3% silt and 7.0% clay, as tested by the University of Massachusetts Amherst Soil and Plant Nutrient Testing Laboratory. All seeds used for planting were provided by the Albert Lea Seed Company. The three species used for the experiment were the native temperate grass species *Lolium perenne* (Ryegrass ‘Sierra’), *Festuca arundinacea* (Tall Fescue ‘Cowgirl’) and *Dactylis glomerata* (Orchardgrass ‘Echelon’). In the summer of 2019, seeds from these species were planted in 6x10 ft plots that were organized based on growing conditions (Table 1). There were 10 total plots designated for each species. A 10 ft border of Kentucky bluegrass was planted around the growing plots to prevent other species from entering the plots.

Drought	Ryegrass	Ryegrass	Orchard	Fescue	Ryegrass
Control	Fescue	Orchard	Fescue	Ryegrass	Orchard
Drought	Ryegrass	Fescue	Orchard	Orchard	Fescue
Control	Orchard	Fescue	Ryegrass	Orchard	Ryegrass
Control	Orchard	Ryegrass	Fescue	Ryegrass	Fescue
Drought	Fescue	Orchard	Ryegrass	Fescue	Orchard

Table 1. The setup of forage grasses grown in the field. Growing plots are organized in rows by drought or control conditions. There are 10 plots for each species, resulting in a total of 30 plots.

Seeds were planted on July 1, 2020, and sample collection lasted for a total of 13 weeks. After planting the seeds, half of the growing plots were covered with rainout shelters to simulate drought conditions for the experiment. Shelters were constructed using cold frames and Thermal AC Greenhouse film. These shelters allowed for high levels of light transmission and airflow. Drought conditions commenced on July 1, 2020 and ended on September 19, 2020, for a total of 10 weeks. During this time, drought plots were not watered. The drought plots were watered for the remaining 3 weeks of the experiment to stimulate a recovery period. Soil moisture of drought plants was brought down to approximately 15-18% by the end of the drought period, and it returned to 25-30% during the recovery period. Control plots had a soil moisture level kept at approximately 25-30% volumetric soil moisture content. These plots were watered by means of rainfall and supplemental water, as needed. A MiniTrase TDR was utilized for soil moisture measurements. Leaf samples and bacteria community samples were taken each week for the 13-week experimental period.

Relative Water Content Analysis

To monitor plant water status, leaf relative water content (RWC) was measured for each week of the experiment. RWC is an indicator of plant health during the study. RWC analysis was performed based on the methods of Barrs and Weatherley (1962), with modifications by DaCosta et al. (2004). First, approximately 10 whole leaves from different tillers were placed in a covered Petri dish and weight was recorded. Petri dishes were filled with distilled water and refrigerated overnight. Leaf samples were then dried with a paper towel and weight was recorded (turgid weight). Samples were then placed in envelopes and dried at approximately 70°C for several

days, and weight was recorded. RWC was calculated using the formula: $RWC\% = [(Fresh\ weight - dry\ weight) / (Turgid\ weight - dry\ weight)] * 100$.

Bacterial Community Sampling

Each week, three whole leaves were taken from each growing plot, and bacterial community DNA was extracted with the Nucleospin Plant II Extraction Kit. The leaves, along with 1.5 mL of Nucleospin Type-B beads and 1.6 mL of Buffer PL1, were placed into a 50 mL conical tube. The tube was vortexed for 5 minutes at room temperature. The lysate was incubated at 65°C for 1 hour, put into a NucleoSpin Filter tube, and centrifuged at 11,000 x g for 2 minutes. 1.6 mL of Buffer PC was added to the resulting filtrate. The remaining steps of DNA extraction followed the kit's provided protocol.

A two-step PCR amplification of the resulting extracted DNA was performed to attach Illumina adaptor sequences and barcodes. For the first PCR step, chloroplast excluding primers 799F(5' ACACTGACGACATGGTTCTACA AACMGGATTAGATACCKG-3') and 1115R (5' TACGGTAGCAGAGACTTGGTCT AGGGTTGCGCTCGTTG-3') targeting the V5-V6 region of the 16S rRNA gene were utilized (Laforest-Lapointe et al. 2017). Underlined portions are the linker sequences, and they were used to attach Access Array Barcodes. Amplicons were mixed to form 2 pools that share 10% of the same samples. An Agilent 2100 Bioanalyzer DNA High Sensitivity assay, Qubit and library qPCR assay were then used to determine quality. The pooled libraries were spiked with ~20% PhiX control library. Lastly, both pools were sequenced with the Illumina MiSeq Platform using the Illumina recommended protocol at the University of Massachusetts Amherst Genomics Resource Library.

DNA Sequence Analysis

The QIIME2 (Bolyen et al., 2019) pipeline was used for sequence analysis. Paired-end reads were demultiplexed, merged together and trimmed down to 315 base pairs. Amplicon sequence variants (ASVs) were then inferred from binning. Taxonomic identities were then found using the Greengenes 13_8 database of the Bayes sklearn classifier. 8,292 ASVs were found from 403 samples. Samples were rarefied to 1,500 reads, which led to 15 samples being lost.

Isolate Library Preparation and Analysis

On the last day of drought conditions, leaves from all three host species and both growing conditions were collected. Leaves were washed in 10 mL of phosphate buffered saline and vortexed for 5 minutes. 100 μ L of 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} dilutions of cell washes were then inoculated onto R2A and M125 media. Distinct colonies from these plates were chosen based on their visual appearance and isolated on individual plates. Specifically, if a colony appeared physically distinct from other colonies and was the one of its kind for a given host species or treatment, then it was chosen for streaking. After obtaining isolated colonies, glycerol freezer stocks were made for each colony. These were inoculated into TSA broth. 500 μ L of bacterial culture was mixed with 500 μ L of glycerol and stored at -80°C .

To prepare samples for Sanger sequencing, phenol-chloroform extractions and ethanol precipitation of DNA were performed, based on the methods of Barker (1998). 500 μ L of TE-saturated phenol-chloroform was added to 500 μ L of each DNA sample. The mixtures were vortexed for 20 seconds and centrifuged for 5 minutes at room temperature. The resulting aqueous layer was extracted, and an equal volume of chloroform was added. Vortexing and centrifugation were performed twice more. 45 μ L of 3 M sodium acetate, pH 4.8 were added to

450 μ L of this product and mixed. 1 mL of 100% ethanol was added and then incubated at -20°C overnight. The samples were centrifuged at 12,000 rpm for 20 minutes, and the supernatant was aspirated. The pellet of DNA was washed with cold 70% ethanol and let to dry. Lastly, it was resuspended in 500 μ L of TE, pH 8.0.

Sanger sequencing of the 16S rRNA gene was performed using the 27F primer. Some of these colonies were used for Sanger sequencing with 799F and 1115R primers. Samples were sent to GENEWIZ for sequencing.

The resulting Sanger sequencing data was optimized with Four Peaks, which removed poor quality reads from the sequence data. Next, BLAST on the NCBI database was used to determine the identity of isolates present (See Appendix B) (National 1988). The sequences with a percent identity of greater than 96.5% were noted, and graphs showing the relative abundance of genera over the course of the study were created using RStudio (RStudio 2020).

The data obtained from these techniques were graphically analyzed using RStudio. A core community was identified using such analysis. The criterion for this temporal core was that these organisms needed to be present in at least 90% of samples for all 13 weeks of the study. Additionally, graphs showing host species colony forming units (CFUs) and relative water content were constructed using the ggplot2 and tidyverse packages in RStudio (see Appendix A) (Wickham 2016) (Wickham *et al.*, 2019).

RESULTS

To determine the RWC of host plants throughout the study, leaf samples were taken once a week. Fresh and dry weights of leaves were measured, and the formula $RWC\% = [(Fresh\ weight - dry\ weight) / (Turgid\ weight - dry\ weight)] * 100$ was used to calculate RWC across host plants across for both control and drought conditions (Figure 1). The RWC peaked at week 8 for all three species and in both control and drought conditions.

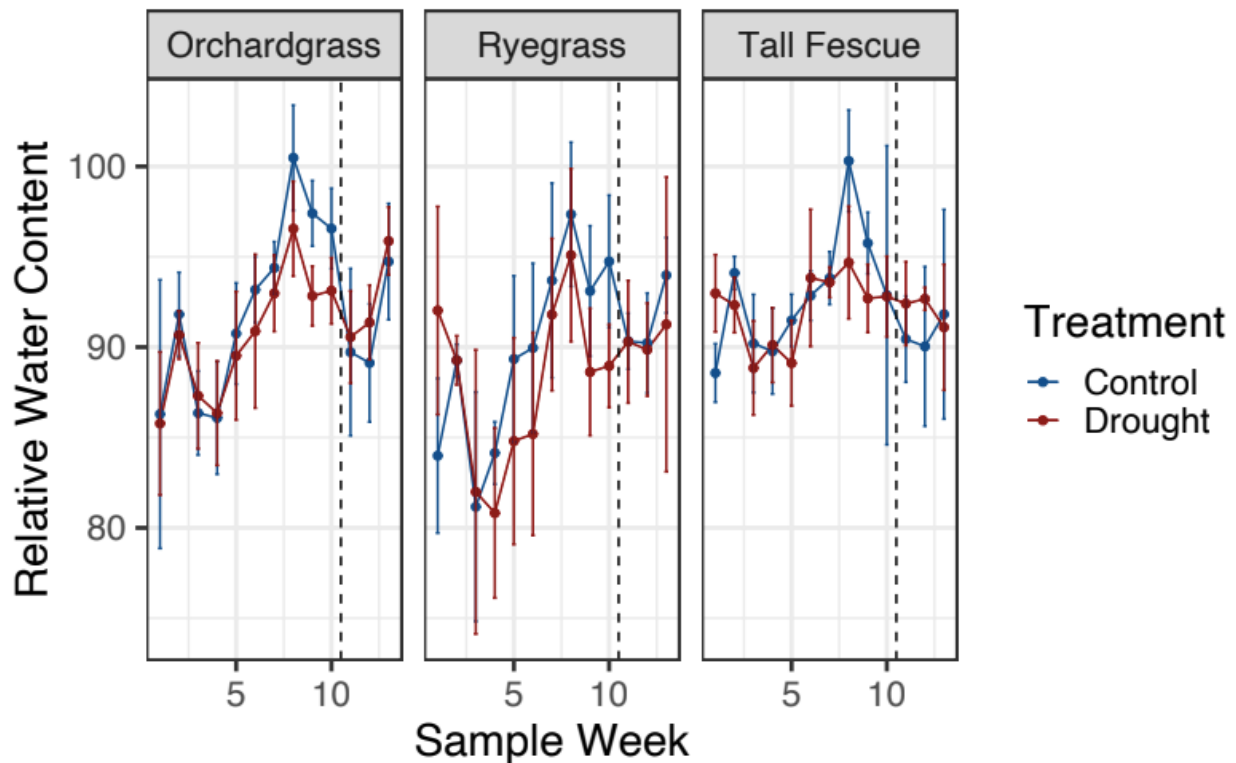


Figure 1. Relative water content of orchardgrass, ryegrass and tall fescue for 13 weeks in both control and drought conditions. The dashed line indicates the start of the recovery period after 10 weeks.

Gel electrophoresis of the 16S rRNA gene from 57 bacterial isolates was performed using the 27F primer and a 1 kb DNA ladder from New England Biolabs. Bands formed at 1465 bp for all lanes, except the negative control (Figure 2).

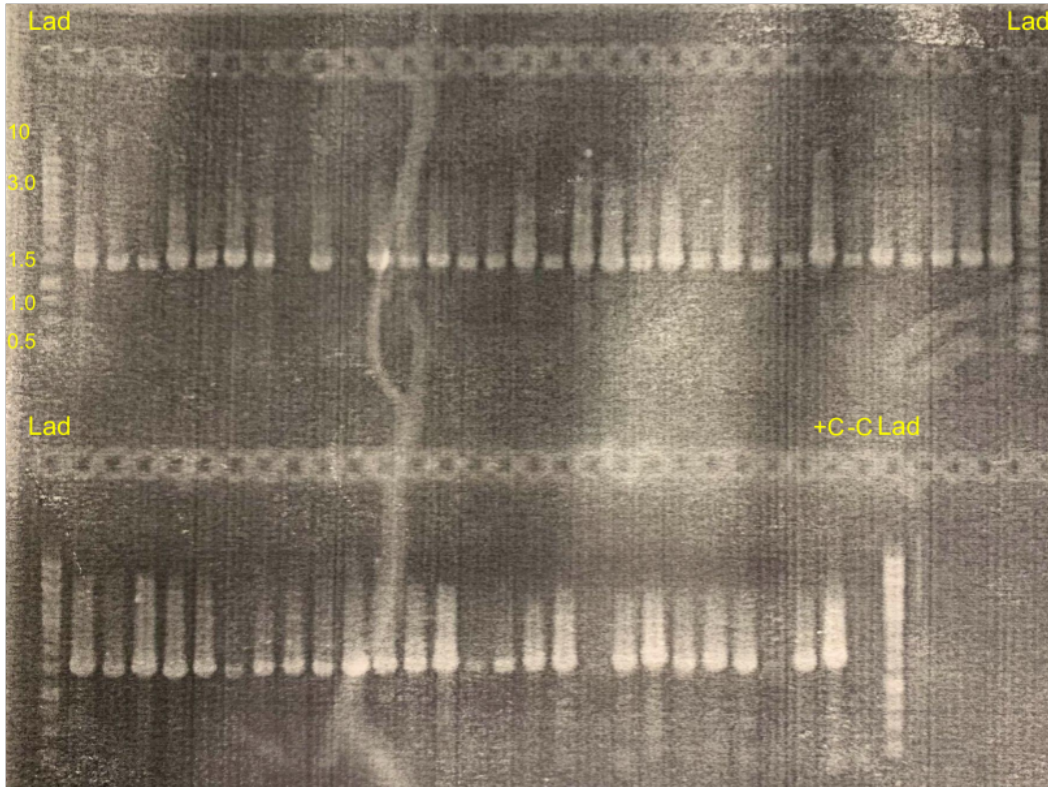


Figure 2. Gel electrophoresis of the amplification of the 16S rRNA gene from 57 bacterial isolates. All samples and the positive control (+C) had bands at 1465 bp, except for the negative control (-C). Four ladders (Lad) are present with kilo base pair lengths labeled.

100 μ L of 10^{-4} dilutions of all bacterial samples were inoculated onto R2A plates and the number of colony forming units per gram (CFUs/g) of leaf were determined (Figure 3). The samples were diluted in 10 mL of phosphate buffered saline. Ryegrass grown in control conditions had significantly more CFUs/g than orchardgrass grown in control conditions. CFUs/g in both conditions for tall fescue were not significantly different from each other nor other host plants.

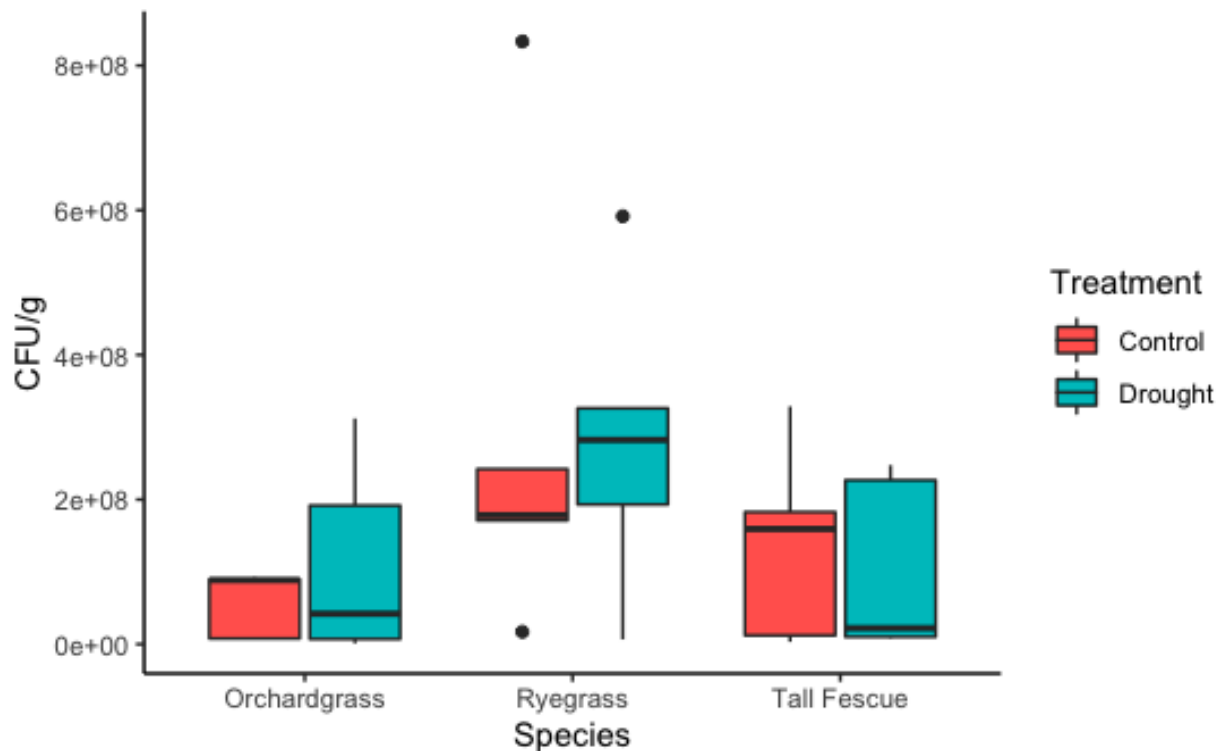


Figure 3. Boxplot showing colony forming units per gram of leaf (CFUs/g) in orchardgrass, ryegrass and tall fescue under drought and control conditions. There is a significant difference between orchardgrass exposed to control conditions and ryegrass exposed to control conditions.

Using RStudio, graphs showing the relative abundance of genera found in the phyllosphere as compared to the entire phyllosphere community were formed (Figure 4). In general, there were significant differences between the genera represented in each host plant. The species that are found in the phyllosphere of tall fescue and ryegrass are often distinct from those found in that of orchardgrass. For example, there are *Actinomycetales*, *Agrobacterium* and *Deinococcus* found in both tall fescue and ryegrass but are generally not found in orchardgrass (Figure 4a, 4b, 4e).

RStudio was used to create graphs showing the relative abundance of classes that made up the core community, as compared to the entire phyllosphere community (Figure 5). The temporal core community was characterized by the organism being present in at least 90% of samples for all 13 weeks of the study. In all host species, the number of classes represented in the core community decreases in drought conditions, as compared to control conditions (Figure 5). RStudio was also utilized to construct boxplots showing the relative abundance of the overall core community when compared to all members of the phyllosphere community (Figure 6). The relative abundance of the core community decreases in drought, as compared to control in all three host species (Figure 6).

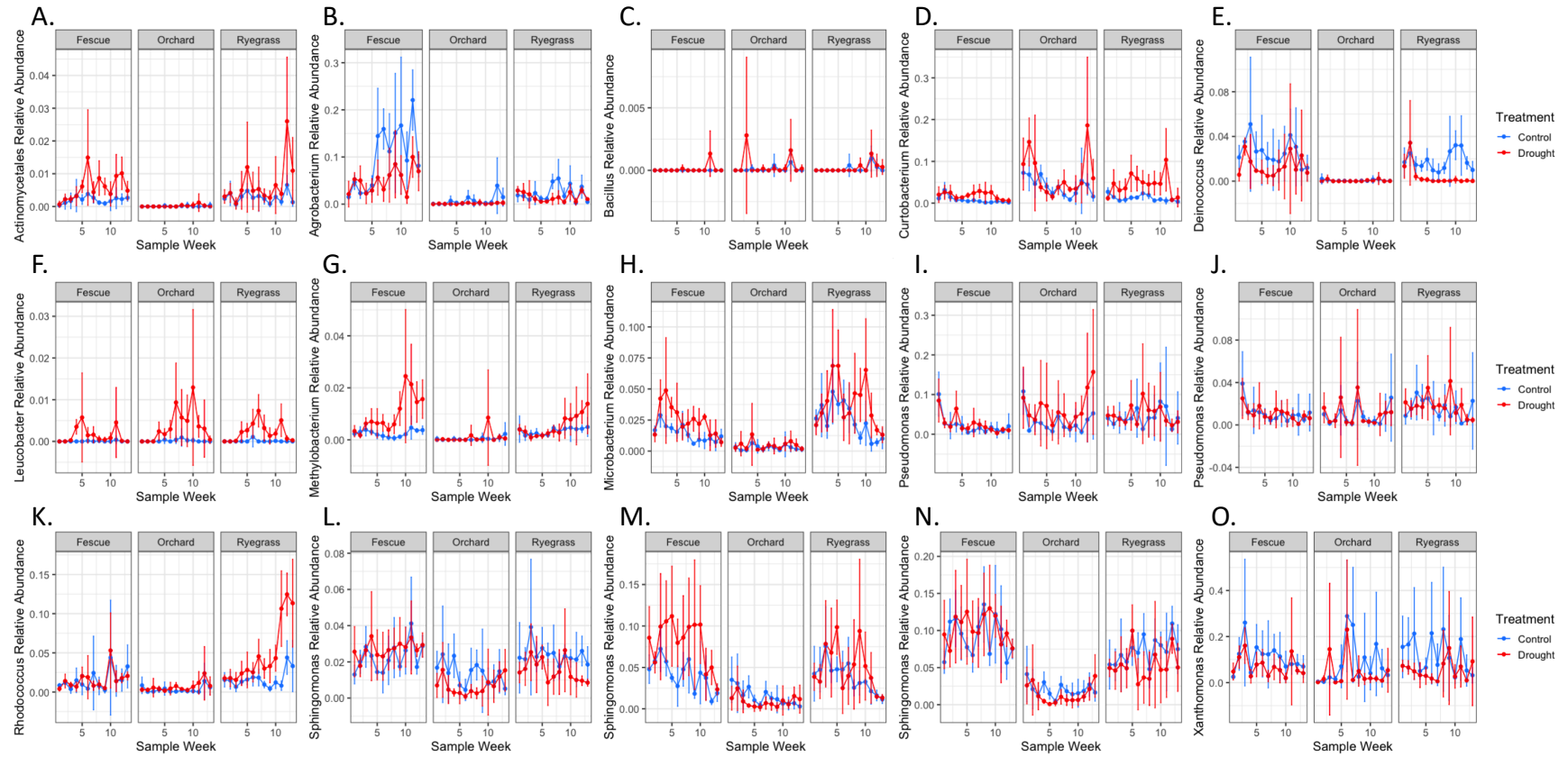


Figure 4. Graphs showing relative abundance of genera found in phyllosphere bacterial isolates in tall fescue, orchardgrass and ryegrass over a 13-week period in control and drought conditions. The drought recovery period begins at week 10.

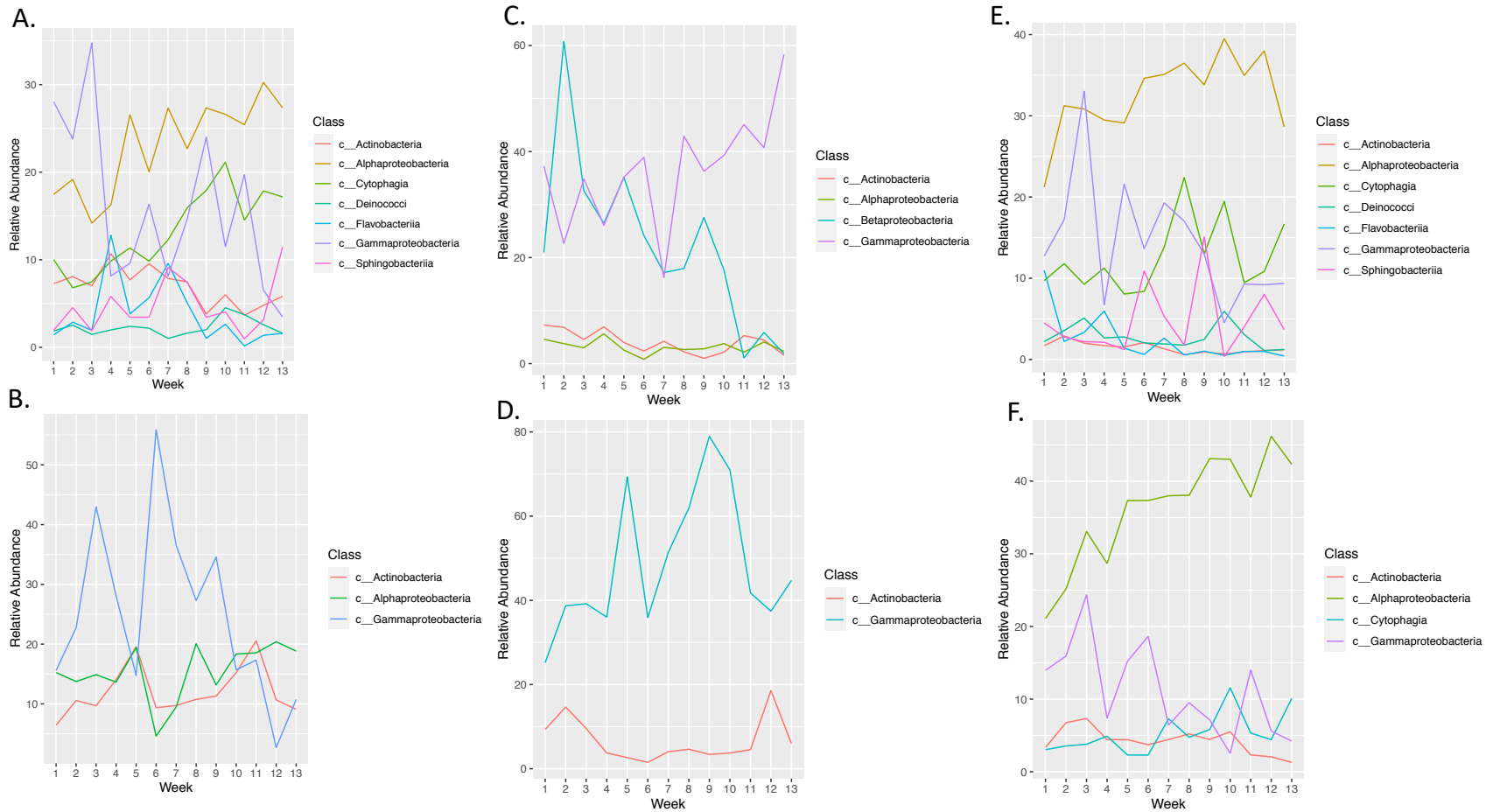


Figure 5. Graphs showing relative abundance of classes found in the temporal core community of tall fescue, orchardgrass and ryegrass compared to the overall phyllosphere community over a 13-week period. Graphs A and B represent ryegrass control and drought conditions, respectively. Graphs C and D represent orchardgrass control and drought conditions, respectively. Graphs E and F represent tall fescue control and drought conditions, respectively.

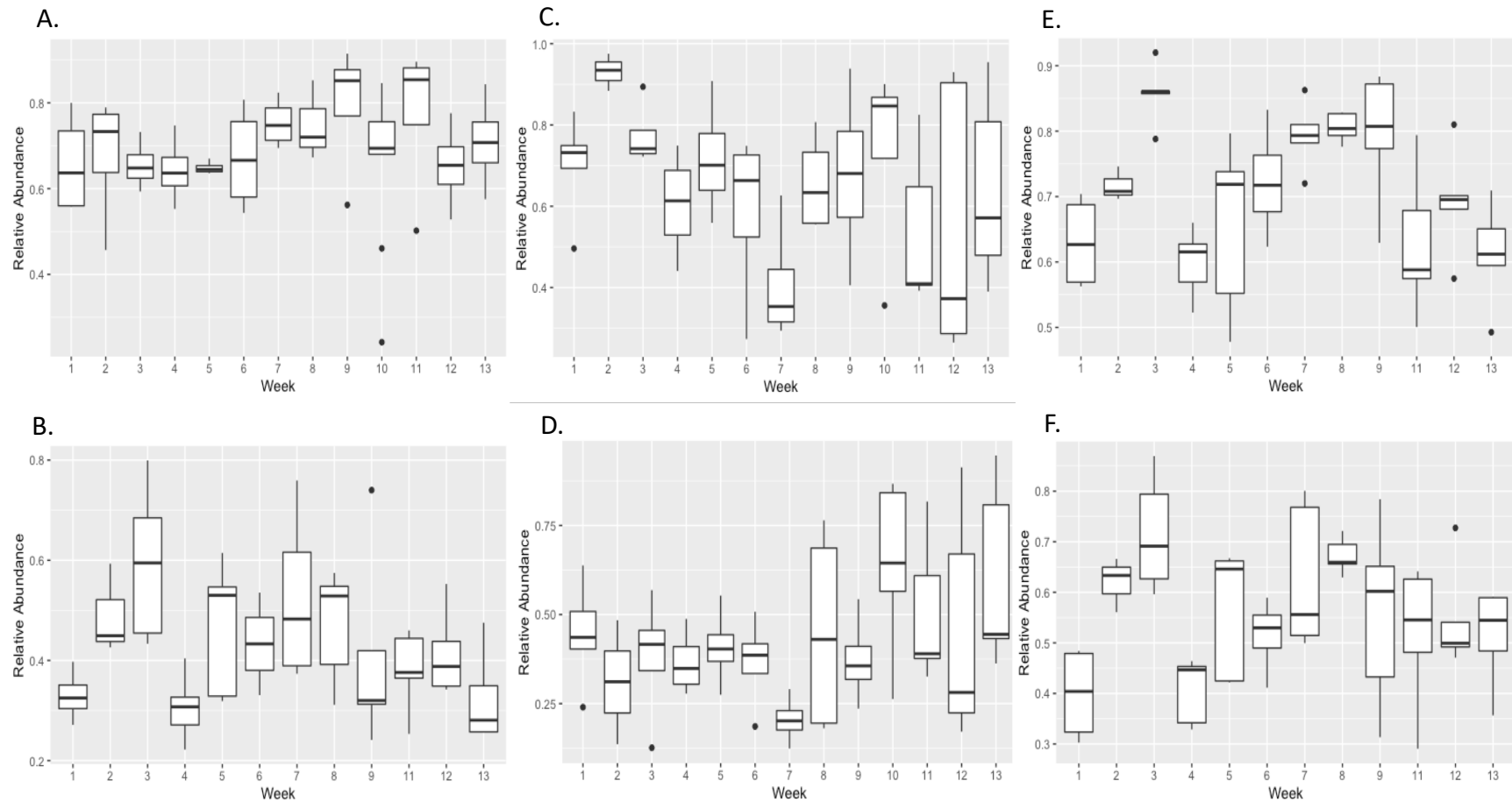


Figure 6. Boxplots showing relative abundance of the temporal core community represented in Figure 5 compared to the overall phyllosphere community over a 13-week period. Boxplots A and B represent ryegrass control and drought conditions, respectively. Boxplots C and D represent orchardgrass control and drought conditions, respectively. Boxplots E and F represent tall fescue control and drought conditions, respectively.

To check for the presence of the *nifH* gene, all samples underwent a PCR assay using primers that amplified this gene. After gel electrophoresis of the PCR products, it was found that none of the samples showed amplification of the *nifH* gene.

DISCUSSION

The goal of this study was to analyze the communities that are present in the phyllosphere of forage grasses, and how they are affected by drought over time. Additionally, the relationship between host species and their respective phyllosphere community was investigated. To first understand how the host plant is affected by drought, the relative water content (RWC) of hosts throughout the field study was calculated. In general, the RWC of the drought-exposed plants was lower than the RWC of control plants, which is expected (Figure 1). The RWC peaked at week 8 for all three host species in both control and drought conditions, which is unexpected in drought since drought conditions would likely lead to lower levels of moisture in these plants, especially towards the end of the drought period (Figure 1). This may be a result of not keeping moisture levels at an adequately low level for drought conditions, which may be attributed to the soil being from an area that was previously a riverbed. Having drought growing conditions that do not accurately represent drought may have skewed results; phyllosphere communities may not have been affected by true drought conditions, so this must be accounted for when drawing conclusions from obtained data. Additionally, neither tall fescue nor ryegrass experienced a great increase in RWC during the recovery period, which likely also affected results, as a true recovery in water content did not occur for these host species (Figure 1). In the future, ensuring adequate drought and recovery conditions is essential, and may be accomplished by using a different type of soil.

PCR amplification of the 16S rRNA gene and subsequent gel electrophoresis was performed on all bacterial isolates. Bands were present at approximately 1465 bp, which is equivalent to the length of the 16S rRNA gene (Clarridge 2004). These results indicate that DNA

from the bacterial isolates was properly extracted. Also, the gel results confirmed that the PCR functioned properly and the PCR products had the proper length of approximately 1465 bp.

The colony forming units per gram (CFUs/g) of leaf were calculated across all host species and growing conditions. Due to lower levels of water and nutrition from host species in drought growing conditions, it was expected that there would be fewer CFUs/g in drought compared to control conditions, but this was not the case. In all three host species, there was no significant difference between the number of CFUs/g of leaf between control and drought growing conditions (Figure 2). A lack of variation between the conditions may be a result of inadequate experimental drought conditions. It may also be possible that the number of viable bacteria present in the phyllosphere is not greatly affected by drought. When drought conditions occur, it is possible that when certain bacterial species lose viability, they are replaced by other bacteria that can survive in harsh conditions. The CFU/g results also show that the ryegrass control plants had significantly more CFUs/g than the orchardgrass control plants, which may show that the ryegrass phyllosphere contains more organisms per unit of leaf surface area.

After Sanger sequencing was performed on the isolate DNA, BLAST was used to find matches between the DNA sequences and amplicon sequence variants (ASVs) in the NCBI database. Graphs showing the relative abundance of genera over the course of the study were created (Figure 4). The relative abundance of these organisms varied greatly, based on host species. The graphs clearly demonstrate that different host species house different bacterial genera in the phyllosphere. It should be noted that these are general trends, and the relative abundance of genera varies greatly by week. The genera with the highest relative abundance in tall fescue are *Agrobacterium*, *Sphingomonas*, and *Xanthomonas*. *Curtobacterium*, *Pseudomonas* and *Xanthomonas* were the most abundant in orchardgrass. In general, *Rhodococcus*,

Sphingomonas and *Xanthomonas* are the most abundant genera in ryegrass. The species that are found in the phyllosphere of tall fescue and ryegrass are often distinct from those found in that of orchardgrass. For example, there are *Actinomycetales*, *Agrobacterium* and *Deinococcus* found in both tall fescue and ryegrass but are generally not found in orchardgrass (Figure 4a, 4b, 4e). In drought conditions, some genera had increases in relative abundance during the recovery period, such as *Actinomycetales*, *Methylobacterium* and *Rhodococcus* in ryegrass (Figure 4a, 4g, 4k). This may reveal that these bacteria are prevalent in optimal growing conditions, but not widespread in drought.

To learn what microorganisms made up the core community of the phyllosphere over the course of the study, a temporal core community was determined. One notable result from this data is that there are 7 classes represented in the core community of ryegrass grown in control conditions, while there are only 3 core community classes present in drought conditions (Figure 5a-b). Moreover, the number of classes goes from 4 to 2 in orchardgrass and from 7 to 4 in tall fescue (Figure 5c-f). The diversity of the core community was decimated by drought, which may be a result of plants closing stomata in drought to prevent moisture loss (Agurla *et al.*, 2018). This takes water away from microorganism in the phyllosphere. Additionally, the forage grasses used in this study use C₃ carbon fixation, and these types of plants cannot carry out photosynthesis when stomata are closed, so fewer metabolic processes are occurring (Sivaram *et al.* 2018). This could result in fewer nutrients being available for microbial growth.

The relative abundance of the classes found in the core community varies by host species. In drought, *Gammaproteobacteria* is the most abundant core community class in ryegrass and orchardgrass, whereas *Alphaproteobacteria* is the most abundant class in tall fescue. This difference may be an indication of the importance of host species in determining core community

assembly. In addition, *Actinobacteria* and *Gammaproteobacteria* are present in all three host species core communities in drought and control conditions, so these classes are likely important for the core community. Some research suggests that *Actinobacteria* have been found to disease soils, so they be opportunistic pathogen core community members (Jia *et al.*, 2020). Indeed, their relative abundance decreases during the recovery period (Figure 5b,d,f). This may be a result of pathogens being eliminated in favorable growing conditions, as commensal bacteria take their place. Moreover, we were able to identify to the genus level for many bacteria, and some of the *Gammaproteobacteria* present were found to be *Pseudomonas*. *Pseudomonas* is a genus that contains a wide variety of species, some of them being plant pathogens (Xin *et al.*, 2018). The relative abundance of this class also decreases during the recovery period, which may be due to the same reason as *Actinobacteria* prevalence decreasing.

The organisms that make up the core community also became less abundant in drought conditions as compared to control conditions (Figure 6). This phenomenon can be seen in all three host species for nearly all weeks of the study. The cause of this drop in core community abundance could be the result of several factors. For example, core community members may simply not fare as well as other phyllosphere symbionts in severe weather conditions. More evidence would need to be obtained to substantiate this postulation.

A PCR assay was used on the bacterial isolate DNA samples to look for the presence of *nifH*, a gene known to be involved in bacterial nitrogen fixation. The PCR and subsequent gel electrophoresis showed no amplification of the *nifH* gene in any of the bacterial isolates. It was expected that the *nifH* gene might be found in the phyllosphere community, as nitrogen fixation is a trait believed to be possessed by some phyllosphere bacteria (Agurla *et al.* 2018). A lack of the *nifH* gene may indicate that the microorganisms present in the phyllosphere are not involved

in nitrogen fixation, or there may be another gene that these bacteria use for fixing nitrogen. At the same time, only a portion of the entire community was represented in the isolates, so the *nifH* gene may not be present in these samples. It is also possible that no amplification occurred due to errors in the PCR assay. For example, an excess of inhibitors in the PCR reactants may have led to issues with amplification.

CONCLUSIONS & FUTURE RESEARCH

The results indicate that the number of viable bacteria in the phyllosphere is not related to the occurrence of drought. Additionally, the classes and genera of microorganisms present are highly influenced by the species of the host. Ryegrass and tall fescue each have phyllosphere compositions that are much more similar to each other than the phyllosphere composition of orchardgrass. Furthermore, the temporal core community composition is highly affected by drought; the diversity of bacteria that make up the temporal core is greatly reduced, and the relative abundance of core community members decreases in drought. Also, some *Actinobacteria* and *Gammaproteobacteria* found in the phyllosphere of forage grasses are likely opportunistic pathogens that dominate the phyllosphere in times of drought.

As a result of the lack of amplification of the *nifH* gene during PCR, it is possible that the gene is not present in the phyllosphere of forage grasses. Another possible explanation could be that the *nifH* gene is not present in the bacterial isolates but is found in the overall phyllosphere community that was not isolated. There is also a high possibility that the lack of amplification was merely due to errors involving the PCR process. In future studies, PCR should be completed with a different buffer and sterile technique must be ensured to minimize the chance of contamination by PCR inhibitors.

It is important to keep in mind that interactions between microorganisms have an enormous impact on phyllosphere community assembly. The relative abundance of genera is likely significantly impacted by factors such as competition for resources (Bringel and Couée 2015). Additionally, due to the large impact of the environment on the phyllosphere, the organisms that are present may be swayed by environmental events, such as wind, rainfall and levels of sunlight (Bringel and Couée 2015). The particular weather patterns and environmental

conditions that occurred during the study may have created a unique set of data that would be different from data taken at another time or location. Moreover, the drought conditions in this study do not necessarily represent accurate drought conditions, as the soil retained water well. Despite the lack of true drought conditions, there were still differences in the phyllosphere community. Therefore, future work should investigate if microorganisms are responding to drought stress before the host plant. As such, it is critical to repeat similar experiments in the future so that patterns between studies can be observed and stronger conclusions on phyllosphere composition can be made.

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APPENDIX

Appendix A. RStudio Workflow Examples

Workflow for Figure 1

```
``{r}
library(tidyverse)
library(ggplot2)
``
```

```
``{r}
rwc <- read_csv("rwc.csv")
``
```

```
``{r}
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                 varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}

df3 <- data_summary(rwc, varname="RWC",
                  groupnames=c("Species", "Treatment", "week"))
```

```
# Default line plot
water<- ggplot(df3, aes(x=week, y=RWC, group=Species, color=Treatment)) +
  geom_line(aes(group=Treatment)) +
  geom_point()+
  facet_wrap(~Species)+
  geom_errorbar(aes(ymin=RWC-sd, ymax=RWC+sd), width=.2,
               position=position_dodge(0.05)) +
  ylab("Relative Water Content")+
  xlab("Sample Week") +
  theme_bw(base_size=30) +
```

```

  scale_color_manual(values=c('dodgerblue4','red4'), name = "Treatment", labels =
c("Control", "Water Reduction"))+
  geom_vline(xintercept = 10.5, color = "black", linetype = 2)
  #scale_color_manual(values=c('darkseagreen4','goldenrod2'))
water

```

Workflow for Figure 2

```

library(ggplot2)
library(tidyverse)

## — Attaching packages ————— tidyverse 1.
3.1 —

## ✓ tibble 3.1.4    ✓ dplyr 1.0.7
## ✓ tidyr 1.1.3    ✓ stringr 1.4.0
## ✓ readr 2.0.1    ✓ forcats 0.5.1
## ✓ purrr 0.3.4

## — Conflicts ————— tidyverse_conflict
s() —
## x dplyr::filter() masks stats::filter()
## x dplyr::lag()     masks stats::lag()

colonies <- read_csv("Colony_Counts.csv")

## Rows: 30 Columns: 6

## — Column specification —————
## Delimiter: ","
## chr (3): Plot, Species, Treatment
## dbl (3): Colonies at 10^-4 Dilution, Leaf mass (g), Counts
##
## ⓘ Use `spec()` to retrieve the full column specification for this data.
## ⓘ Specify the column types or set `show_col_types = FALSE` to quiet this
message.

ANOVA <- aov(Counts ~ Treatment*Species, data =colonies)
summary(ANOVA)

##           Df    Sum Sq  Mean Sq F value Pr(>F)
## Treatment    1 9.978e+13 9.978e+13  0.003 0.9567
## Species      2 2.276e+17 1.138e+17  3.430 0.0489 *
## Treatment:Species  2 1.016e+16 5.080e+15  0.153 0.8588
## Residuals   24 7.961e+17 3.317e+16
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

```

tukey <-TukeyHSD(ANOVA)
tukey

## Tukey multiple comparisons of means
## 95% family-wise confidence level
##
## Fit: aov(formula = Counts ~ Treatment * Species, data = colonies)
##
## $Treatment
##          diff          lwr          upr          p adj
## Drought-Control 3647420 -133606308 140901147 0.9567147
##
## $Species
##          diff          lwr          upr          p adj
## Ryegrass-Orchardgrass 200137826 -3261702 403537354 0.0543824
## Tall Fescue-Orchardgrass 36080547 -167318981 239480075 0.8979335
## Tall Fescue-Ryegrass -164057279 -367456807 39342249 0.1304077
##
## `$Treatment:Species`
##          diff          lwr          upr
## Drought:Orchardgrass-Control:Orchardgrass 53533105 -302611412 409677623
## Control:Ryegrass-Control:Orchardgrass 231116267 -125028250 587260785
## Drought:Ryegrass-Control:Orchardgrass 222692489 -133452028 578837007
## Control:Tall Fescue-Control:Orchardgrass 79930633 -276213884 436075151
## Drought:Tall Fescue-Control:Orchardgrass 45763565 -310380952 401908083
## Control:Ryegrass-Drought:Orchardgrass 177583162 -178561355 533727680
## Drought:Ryegrass-Drought:Orchardgrass 169159384 -186985134 525303901
## Control:Tall Fescue-Drought:Orchardgrass 26397528 -329746990 382542045
## Drought:Tall Fescue-Drought:Orchardgrass -7769540 -363914057 348374978
## Drought:Ryegrass-Control:Ryegrass -8423778 -364568296 347720739
## Control:Tall Fescue-Control:Ryegrass -151185634 -507330152 204958883
## Drought:Tall Fescue-Control:Ryegrass -185352702 -541497220 170791815
## Control:Tall Fescue-Drought:Ryegrass -142761856 -498906373 213382662
## Drought:Tall Fescue-Drought:Ryegrass -176928924 -533073441 179215594
## Drought:Tall Fescue-Control:Tall Fescue -34167068 -390311585 321977450
##          p adj
## Drought:Orchardgrass-Control:Orchardgrass 0.9969477
## Control:Ryegrass-Control:Orchardgrass 0.3678533
## Drought:Ryegrass-Control:Orchardgrass 0.4074244
## Control:Tall Fescue-Control:Orchardgrass 0.9808928
## Drought:Tall Fescue-Control:Orchardgrass 0.9985524
## Control:Ryegrass-Drought:Orchardgrass 0.6421493
## Drought:Ryegrass-Drought:Orchardgrass 0.6863765
## Control:Tall Fescue-Drought:Orchardgrass 0.9999009
## Drought:Tall Fescue-Drought:Orchardgrass 0.9999998
## Drought:Ryegrass-Control:Ryegrass 0.9999997
## Control:Tall Fescue-Control:Ryegrass 0.7754311
## Drought:Tall Fescue-Control:Ryegrass 0.6007102
## Control:Tall Fescue-Drought:Ryegrass 0.8133633

```

```
## Drought:Tall Fescue-Drought:Ryegrass      0.6456180
## Drought:Tall Fescue-Control:Tall Fescue   0.9996483

ggplot(colonies, aes(x=Species, y=Counts, fill=Treatment)) +
  geom_boxplot() +
  ylab("CFU/g") +
  theme_classic()
```

Workflow for Figure 4

```
library(tidyverse)

## — Attaching packages ————— tidyverse 1.
3.1 —

## ✓ ggplot2 3.3.5      ✓ purrr  0.3.4
## ✓ tibble  3.1.4      ✓ dplyr  1.0.7
## ✓ tidyr   1.1.3      ✓ stringr 1.4.0
## ✓ readr   2.0.1      ✓ forcats 0.5.1

## — Conflicts ————— tidyverse_conflict
s() —
## x dplyr::filter() masks stats::filter()
## x dplyr::lag()     masks stats::lag()
```

Read in the files

```
FC_2R <- read_csv("FC_3R_2.csv")

## Rows: 21 Columns: 3

## — Column specification —————
## Delimiter: ","
## chr (2): ID, ASV
## dbl (1): percent

##
## ⓘ Use `spec()` to retrieve the full column specification for this data.
## ⓘ Specify the column types or set `show_col_types = FALSE` to quiet this
message.

align <- read_csv("rarefied-feature-table.csv")

## Rows: 5399 Columns: 374

## — Column specification —————
## Delimiter: ","
## chr (1): ASV
```

```
## dbl (373): Ryegrass_Control_2D_10, Fescue_Control_2C_11, Fescue_Control_2C_1...
##
## i Use `spec()` to retrieve the full column specification for this data.
## i Specify the column types or set `show_col_types = FALSE` to quiet this message.
```

Join isolate ASVs w/ community sequence data

```
joined <- FC_2R %>%
  left_join(aligned)
## Joining, by = "ASV"
```

Remove all NAs from data

```
joined_na <- na.omit(joined)
abundance <- joined_na %>%
  pivot_longer(4:376) %>% #changes shape of table so columns become rows
  group_by(name) %>% #group
  summarize(total=sum(value)) %>%
  mutate(abun=total/1500) %>%
  separate(name, into = c("species", "Treatment", "replicate", "week"), sep="
_")
abundance$week <- as.integer(abundance$week)
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                 varname)
  data_sum <-rename(data_sum, c("mean" = varname))
  return(data_sum)
}
df3 <- data_summary(abundance, varname = "abun",
                   groupnames=c("species", "Treatment", "week"))
## Loading required package: plyr
## -----
##
## You have loaded plyr after dplyr - this is likely to cause problems.
## If you need functions from both plyr and dplyr, please load plyr first, th
```

```

en dplyr:
## library(plyr); library(dplyr)

## -----
----

##
## Attaching package: 'plyr'

## The following objects are masked from 'package:dplyr':
##
##   arrange, count, desc, failwith, id, mutate, rename, summarise,
##   summarize

## The following object is masked from 'package:purrr':
##
##   compact

p3 <- ggplot(df3, aes(x=week, y=abun, group=species, color=Treatment)) +
  geom_line(aes(group=Treatment)) +
  geom_point()+
  facet_wrap(~species)+
  geom_errorbar(aes(ymin=abun-sd, ymax=abun+sd), width=.2,
               position=position_dodge(0.05)) +
  ylab("Deinococcus Relative Abundance") +
  xlab("Sample Week") +
  theme_bw(base_size = 14) +
  scale_color_manual(values=c('dodgerblue1', 'red2' ))

p3

```

Workflows for Figures 5 and 6

```

library(tidyverse)

## — Attaching packages ————— tidyverse 1.
3.1 —

## ✓ ggplot2 3.3.5      ✓ purrr  0.3.4
## ✓ tibble  3.1.4      ✓ dplyr  1.0.7
## ✓ tidyr   1.1.3      ✓ stringr 1.4.0
## ✓ readr   2.0.1      ✓ forcats 0.5.1

## — Conflicts ————— tidyverse_conflict
s() —
## x dplyr::filter() masks stats::filter()
## x dplyr::lag()    masks stats::lag()

```



```

library(ggplot2)
library(ggvenn)

## Loading required package: grid

data <- read_csv("rarefied_species_adjusted.csv")

## Rows: 668 Columns: 374

## — Column specification —————
## Delimiter: ","
## chr (1): index
## dbl (373): Ryegrass_Control_2D_10, Fescue_Control_2C_11, Fescue_Control_2C_1...
##
## ⓘ Use `spec()` to retrieve the full column specification for this data.
## ⓘ Specify the column types or set `show_col_types = FALSE` to quiet this message.

d1 <- data %>%
  pivot_longer(2:374, names_to = "sample", values_to = "count") %>%
  separate(sample, into = c("Species", "Treatment", "Replicate", "Week"), sep = "_") %>%
  filter(Species=="Ryegrass" & Treatment=="Drought") %>%
  filter(count>0.5) %>% # this removes 0s so that we don't have NAs later on
  mutate(value=count/count) %>%
  group_by(index, Species, Treatment) %>%
  summarise( sum = sum(value)) %>%
  arrange(desc(sum)) %>%
  ungroup %>%
  filter(sum>58)

## `summarise()` has grouped output by 'index', 'Species'. You can override using the `.groups` argument.

#group_by(ID, Species, Treatment, Day) %>%
#summarise( sum = sum(count)) %>%
#arrange(desc(sum)) %>%
#ungroup

write_csv(d1, "Ryegrass_Drought_90_v3.csv")

library(tidyverse)

## — Attaching packages ————— tidyverse 1.3.1 —
## ✓ ggplot2 3.3.5      ✓ purrr 0.3.4
## ✓ tibble 3.1.4      ✓ dplyr 1.0.7

```

```

## ✓ tidyr 1.1.3 ✓ stringr 1.4.0
## ✓ readr 2.0.1 ✓ forcats 0.5.1

## — Conflicts ————— tidyverse_conflicts() —
## x dplyr::filter() masks stats::filter()
## x dplyr::lag() masks stats::lag()

library(ggplot2)
library(ggvenn)

## Loading required package: grid

data <- read_csv("rarefied_species_adjusted.csv")

## Rows: 668 Columns: 374

## — Column specification —————
## Delimiter: ","
## chr (1): index
## dbl (373): Ryegrass_Control_2D_10, Fescue_Control_2C_11, Fescue_Control_2C_1...

##
## ⓘ Use `spec()` to retrieve the full column specification for this data.
## ⓘ Specify the column types or set `show_col_types = FALSE` to quiet this message.

rye <- read_csv("Fescue_Drought_90.csv")

## Rows: 668 Columns: 2

## — Column specification —————
## Delimiter: ","
## chr (1): index
## dbl (1): sum

##
## ⓘ Use `spec()` to retrieve the full column specification for this data.
## ⓘ Specify the column types or set `show_col_types = FALSE` to quiet this message.

d1 <- rye %>%
  filter(sum>.9)

rel <- data %>%
  mutate_if(is.numeric, funs(z = ./sum(.))) %>%
  select(1, ends_with("z"))

```

```

## Warning: `funs()` was deprecated in dplyr 0.8.0.
## Please use a list of either functions or lambdas:
##
## # Simple named list:
## list(mean = mean, median = median)
##
## # Auto named with `tibble::lst()` :
## tibble::lst(mean, median)
##
## # Using lambdas
## list(~ mean(., trim = .2), ~ median(., na.rm = TRUE))

rye_core_abundance <- d1 %>%
  left_join(rel) %>%
  select(1, starts_with("Fescue")) %>%
  pivot_longer(2:57, names_to = "Species" , values_to = "n") %>%
  separate(Species, into = c("species", "treatment", "replicate", "day"), sep
= "_") %>%
  group_by(replicate, day) %>%
  summarize(sum=sum(n))

## Joining, by = "index"

## Warning: Expected 4 pieces. Additional pieces discarded in 784 rows [1, 2,
3, 4,
## 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, ...].

## `summarise()` has grouped output by 'replicate'. You can override using th
e `.groups` argument.

rye_core_abundance$day <- as.factor(rye_core_abundance$day)

level_order <- c('1', '2', '3', '4', '5', '6', '7', '8', '9', '10', '11', '12
', '13')
plot <- ggplot(rye_core_abundance, aes(x=factor(day, level=level_order), y=su
m)) +
  geom_boxplot() +
  ylab("Relative Abundance") +
  xlab("Week")

plot

rye_genus <- d1 %>%
  left_join(rel) %>%
  select(1, starts_with("Fescue")) %>%
  pivot_longer(2:59, names_to = "Species" , values_to = "n") %>%
  separate(Species, into = c("species", "treatment", "replicate", "day"), sep
= "_") %>%
  group_by(index, day) %>%
  summarize(ave=mean(n)*100) %>%
  ungroup() %>%

```

```

  separate(index, into = c("Kingdom", "Phylum", "Class", "Order", "Family", "Genus", "Species"), sep = ";") %>%
  group_by(Class, day) %>%
  summarize(sum=(sum(ave))) %>%
  ungroup()

## Joining, by = "index"

## Warning: Expected 4 pieces. Additional pieces discarded in 812 rows [1, 2, 3, 4,
## 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, ...].

## `summarise()` has grouped output by 'index'. You can override using the `groups` argument.

## `summarise()` has grouped output by 'Class'. You can override using the `groups` argument.

level_order <- c('1', '2', '3', '4', '5', '6', '7', '8', '9', '10', '11', '12', '13')
plot <- ggplot(rye_genus, aes(x=factor(day, level=level_order), y=sum, group = Class)) +
  geom_line(aes(color = Class)) +
  ylab("Relative Abundance") +
  xlab("Week")

plot

```

Appendix B. Isolate Identities

Identity	ASV Percent Similarity
<i>Xanthomonas oryzae</i>	99.64
<i>Deinococcus gobiensis</i>	100
<i>Pseudomonas oleovorans</i>	98.26
<i>Agrobacterium rubi</i>	96.53
<i>Pseudokineococcus marinus</i>	99.65
<i>Microbacterium oxydans</i>	99.58
<i>Bacillus safensis</i>	99
<i>Sphingomonas melonis</i>	99.31
<i>Sphingomonas sanguinis</i>	100
<i>Sphingomonas paucimobilis</i>	98.96
<i>Rhodococcus fascians</i>	98.93
<i>Curtobacterium flaccumfaciens</i>	99.29
<i>Methylobacterium bullatum</i>	99.65
<i>Leucobacter tardus</i>	98.48
<i>Pseudomonas putida</i>	98.96

Table B1. Organism names of bacterial isolates based on amplicon sequence variants (ASVs) from BLAST on the NCBI database. Sanger sequencing was performed using the products of PCR amplification of the 16S rRNA gene, and poor quality reads were removed using Four Peaks.