The effect of water stress on peanut soil microbiome and plant physiology

By

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Abstract

Arachis hypogea is an economically important crop in the Southeastern region of the United States. The kernel has a high nutritional value containing oil, proteins, and carbohydrates. However, 90% of farmers in Alabama do not irrigate their peanuts due to expensive irrigation and/or water availability. Peanuts in Alabama and nearby regions tend to grow in sandy soil under hot and dry periods, possibly leading to drought. Drought alone can take over $50 million out of the U.S. yearly and is one of the most severe abiotic stresses that affect plants. As the temperature rises and water becomes restricted, soil solute concentration increases, and moisture availability and soil nutrient content decrease. Peanuts under water stress symptoms include wilting, folding, and drooping of the shoot; closure of stomata leads to an increase in photorespiration and a reduction of photosynthesis, the plant weakens, facilitating the incidence of infection, and a decrease of yield can be observed. *Aspergillus flavus* is a fungus that infects peanuts under hot and dry conditions. This fungus produces secondary metabolites (aflatoxins) that are carcinogenic and hepatotoxic in animals and humans. Studies have found a higher concentration of aflatoxins in the kernel, possibly due to its high oil and protein concentration. Conversely, some microbes can help by improving growth by regulating and producing phytohormones, enhancing water and nutrient availability, and conferring biotic and abiotic tolerance to plants. The family Mortierellaceae and the species *Penicillium citrinum* have shown plant growth-promoting properties on various plant hosts.

Hence, detecting microbes that can antagonize *A. flavus* or confer drought tolerance to peanut plants is highly desired. Chapter one will provide an overview of peanuts and their importance, the effects of drought on the peanut plant and the soil microbiome, and soil fungal

species known for plant growth-promoting properties in drought-stress environments. Chapter two focused on testing fungi that can alleviate drought stress in peanuts using two different fungal collections. For this study, the fungal collections were screened for salt and high temperatures, and five fungal cultures were selected and used for inoculating peanut seedlings under drought and no drought conditions. Fungal treatments were *P. citrinum* CCH_F37_B, *Mortierella alpina* OEO-305, *M. calciphila* OEO-304, *Linnemannia elongata* OEO-198, *L. elongata* OEO-196. Under no drought conditions, *L. elongata* OEO-196 significantly increased shoot biomass, while *M. calciphila* OEO-304 trended lower root and shoot biomass but was not significant. However, interestingly, *M. calciphila* OEO-304 had a positive trend in the dry biomass and was no different from control without drought. The treatment with *P. citrinum* CCH_F37_B altered photosynthetic efficiency in both droughted and no-drought experiments, but the plant was smaller overall. More studies are needed to understand the mechanisms of alleviating water stress from these treatments in peanut plants, but some fungi show promising results.

In the third chapter, we designed experiments to understand how water regimes impact the microbial communities from two peanut soils. We hypothesized that applying different water regimes to the peanut soils would alter the microbial composition over time. Soils from two different fields at Wiregrass Research and Extension Center Headland, Alabama, were collected and transferred to a polyvinylchloride tube inside the growth chamber, where five different water treatments were applied each week for a total of nine weeks at 29ºC. Each week soil was collected, DNA extracted, and sequenced. Results show that the water regime applied created a gradient and had a significant impact on the microbial communities in both soils. Actinobacteriota was an indicator taxon for drier soils, while Proteobacteria and Planctomycetota were more associated with moist ones.

Drought is a major abiotic stress in peanut crops. Even though Alabama is one of the biggest peanut producers in the United States, a small percentage of farmers irrigate their crops. This study aims to show the necessity of alleviating peanut stress under water restriction that leads to yield loss. This detection of a microbe can not only antagonize *A. flavus* but alleviate water stress in plants by increasing water and nutrient availability, phytohormones synthesis, regulation of stomatal conductance, and solubilizing properties, among others. Understanding the microbiome under droughted conditions can also guide the detection of this microbe or set of microbes. The conclusions and impacts of this study are presented in chapter four.

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1. CHAPTER ONE

Introduction and Literature Review

Introduction to peanuts

The peanut crop, also known as groundnut (*Arachis hypogea*), is an herbaceous annual plant from the Fabaceae family. The peanut stem supports four leaflets that form a tetrafoliate leaf, and its structure is alternate and pinnate. The stem also bears papilionaceous flowers that emerge aerially, followed by geotropic movement of the gynophore to the ground. These structures, called pegs, are where pod formation happens below ground (Variath and Janila, 2017). The kernel is an important source of nutrition, containing 40 to 55% oil, 20 to 35% protein, and 10 to 20% carbohydrates, as well as several vitamins, minerals, antioxidants, biologically active polyphenols, flavonoids, and isoflavones (Janila et al. 2013; Variath and Janila 2017). Peanut is an economically important crop worldwide due to its nutritional value and is grown in over 100 countries (Janila et al. 2013). Alabama produced 637 million pounds out of 6.13 billion pounds of total peanut produced in the United States USDA-NASS, 2021. In this region, peanut grows in sandy soil, typically with low water holding capacity, often with little or no irrigation. Therefore, peanut production can be subjected to periodic droughts and higher temperatures between rain events, which strongly affects the formation of pegs and pod filling, leading to yield loss (Rosas-Anderson et al. 2014; Q. Zhang et al. 2022). In Alabama, only 10% of peanut farmers artificially irrigate. The peanut industry loses around $50 million yearly due to drought stress (Hamidou et al. 2013;

Hollis, 2020; Puppala et al. 2023). There are several mechanisms by which yield can be reduced by drought, including direct and indirect effects on the plant and associated microbiome.

Effect of drought on peanut

Drought is one of the most severe abiotic stresses and can be described as long-term exposure to water restriction (Seleiman et al. 2021; Shao et al. 2008; Zhen et al. 2022). Worldwide drought constrains the productivity and quality of peanut crops and is the main driver of yield reduction in the Southeastern United States (Chen et al. 2022; Zhen et al. 2022). Groundnut developmental stage, variety, and drought duration are crucial in the plant's biochemical, physiological, and morphological response (Seleiman et al. 2021; Zhen et al. 2022). The first symptom of water deficit in peanut plants is wilting, explained by the loss of turgor pressure. Above-ground biomass folds and drops due to the closure of the stomata, a known defense mechanism of plants under stress to evade the loss of water through transpiration, and by the reduction of nitrogen fixation activity in the root nodules (De Lima Pereira et al. 2016). It has been demonstrated that under water restriction, proline plays an important role in the performance of osmotic regulation (J. Zhang et al. 2021). An accumulation of proline leads to an increase in the solute concentration of the cytoplasm and has been shown to replace the water in some physiological processes during drought (Furlan et al. 2020; J. Zhang et al. 2021). However, if a drought period is prolonged, the closure of stomata will greatly affect photosynthesis and gas exchange and interrupt metabolism with direct actions on thylakoid electron transport, phosphorylation, and carboxylation (De Lima Pereira et al. 2016; Pilon et al. 2018; Shao et al. 2008). Groundnut flowering is receptive to light, temperature, and relative humidity (Variath and

Janila, 2017). For peanuts, flower, peg, and pod development are affected by a water deficit, and pod and seed production can decrease by approximately 30% (de Lima Pereira et al. 2016). Moderate water restriction followed by rehydration improved the Water Use Efficiency (WUE) of peanut leaves. However, under severe water restriction and rehydration, the plant may be unable to recuperate at normal photosynthesis rates (J. Zhang et al. 2021).

Effect of drought on the microbiome

The root system administers water acquisition and nutrient uptake in a plant. An important association for plant health is the interaction of plants and nearby microbial communities, such as those in the phyllosphere, rhizosphere, and endosphere, among others (Naylor and Coleman-Derr, 2018). Microorganisms are important in disease protection, nutrient cycling, increasing water and nutrient uptake, osmotic adjustment, nitrogen fixation, phosphate solubilization, and antimicrobial compound production. They can help resist biotic and abiotic stresses (Chen et al. 2022). Williams and de Vries (2020) have shown that root exudates influence the composition of the microbial communities and vice versa. Many studies suggest that root exudates play a role in alleviating stressful conditions (Oppenheimer-Shaanan et al. 2022). Root exudates from plants under stress have shown to be more selective to different microbial communities depending on their needs. The root community responds to drought stress by producing several plant hormones, including abscisic acid, cytokinin, indoleacetic acid, and other compounds like trehalose, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile organic compounds, and exopolysaccharides (Chen et al. 2022). Hence, even though a decrease in soil biodiversity in water-restrictive conditions is observed, some microbial species are more abundant in these environments (Chen et al. 2022). For example, as water restriction is imposed, an increase in Gram-positive

bacteria from phylum Actinobacteria, and genera *Streptomyces*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Acinetobacter*, *Burkholderia*, *Arthrobacter*, and *Paenibacillus* are observed in the rhizosphere, which can suggest a close interaction with the plant root under stress (Chen et al. 2022; Oppenheimer-Shaanan et al. 2022; Williams and de Vries, 2020). Furthermore, many of these mentioned are known plant growth-promoting rhizobacteria (PGPR) (Chen et al. 2022; Oppenheimer-Shaanan et al. 2022). In a study with peanuts, colonization by the genera *Bacillus* and *Pseudomonas* in the roots increased benzoic and salicylic acids known to facilitate root colonization by the bacteria and suppress fungal plant pathogens while promoting plant growth (Oppenheimer-Shaanan et al. 2022).

Like bacteria, fungi can interact with plants under stress regulating hormones, solubilization and mineralization of nutrients, evading pathogenic infections by producing volatile organic compounds and microbial enzymes, regulating plant defense responses, and alleviating in abiotic stresses (Hossain and Sultana, 2020). A study in rice shows that under drought, an increase in the relative abundance of actinobacteria and chloroflexi alongside root-associated fungal communities was potentially aiding the plant to tolerate drought (Chen et al. 2022). Plant roots have intimate associations with active microorganisms promoting plant nutrient uptake. For example, arbuscular mycorrhizal fungi (AMF) aiding in water assimilation thanks to fungal physical structure with the formation of the hyphal network that stretches out, obtaining a larger surface area possibly leading to an increase of water uptake and absorption of nutrients (Begum et al. 2019; Chen et al. 2022). Furthermore, fungi can help improve soil structure, texture, and, therefore, plant health (Begum et al. 2019). The AMF increases nutrient availability, such as nitrogen, phosphorus, and other elements found in the soil, facilitating water absorption and pathogen protection. At the same time, the plant provides carbon sources and protection to AMF

(Chen et al. 2022). It is estimated that more than 80% of plants can form interactions with AMF (Chen et al. 2022). In soybean, inoculation with AMF has improved biomass and proline content in drought conditions. Another example is fungi in the sub-phylum Mortierellomycotina which have increased the growth of many plant species such as *Arabidopsis sp.*, *Crocus sativus*, *Pinus taeda*, *Citrullus lanatus*, *Quercus* sp. (oak), *Zea mays*, *Paspalum notatum*, and *Solanum lycopersicum*, among many others (Yadav et al. 2010; K. Zhang et al. 2020). The interest in studying these fungi is that they have been able to plant growth-promoting fungi (PGPF) in many different species of plants, leguminous and non-leguminous, in different climates and soil structures. However, there is a lack of understanding of their mechanism of action, host specificity, and favorable conditions to promote growth or alleviate stress in crops, considering this group is ubiquitously found in rocks, caves, rivers, lakes, bulk soil, plant tissues, temperate and polar climates rhizosphere (Ozimek and Hanaka, 2021).

The basal fungi, *Mortierella* and *Linnemannia* species, from the sub-phylum Mortierellomycotina are widespread filamentous fungi that live in the soil and can be found as root endophyte or as soil saprotroph (Liao et al. 2019). Species from the family of Mortierellaceae are known to be growth promoters to several host plants and they have been found aiding in phosphorous mobilization, phosphatase activity, plant growth promotion under high salinity levels, nitrogen fixation, potassium solubilization, production of siderophores, phytohormones such as auxins (indole-3- acetic acid), cytokinins, gibberellic acid, and ethylene (precursor 1-aminocyclopropane-1-carboxylate deaminase) (Ozimek and Hanaka, 2021). These fungi fall in the same phylum (Mucoromycota) as the arbuscular mycorrhizal fungi (AMF). The phylum Mucoromycota comprises the subphyla Glomeromycotina, Mortierellomycotina, and Mucoromycotina (Liao, 2021). Mortierellomycotina is a sister group of Glomeromycotina. In

contrast to the AMF, can be cultured in the laboratory. Due to their history as plant-beneficial fungi and ease of use in the lab, they are of interest for further study. Furthermore, their interactions with peanuts under drought stress are largely unknown.

Just as there are fungi that may help alleviate drought stress, there are also fungi that thrive under low water availability. *Aspergillus flavus* is a fungus from the family Trichocomaceae, found as a saprotroph with omnipresent distribution. Well known for being soil-borne and prevalent in hot and dry environments (Amaike and Keller, 2011). Aflatoxins are naturally occurring carcinogenic substances produced by *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii*, with *A. flavus* being the more frequently encountered contaminating peanuts (Uppala et al. 2013). This toxin can be divided into aflatoxins B1, B2, G1, and G2, with B1 being the most potent carcinogen (Amaike and Keller, 2011). Peanut infection can start with roots, pegs, and even the seed. Many studies propose that the main source of infection is the soil and the flower (Diener, 1960). *A. flavus* can be found invading peanut plants before harvest as well as post-harvest. Infestation of plant parts by *A. flavus* is more likely under drought stress and insect damage (Tola and Kebede, 2016), and after harvest in storage conditions. It has been observed that the production of aflatoxins is enhanced with the lipids, proteins, and sugars found in the peanut kernel. Thus, high aflatoxin concentration in seeds are observed under improper storage conditions (moisture exceeding 8%, and temperature rises above 25ºC) (Tola and Kebede, 2016; Uppala et al. 2013). Norlia et al. (2020) studied the interaction of temperature and water in *A. flavus* growth and aflatoxin production. They demonstrated that infection of this fungus does not mean a direct production of aflatoxins. Some strains cannot produce the detrimental toxin. Cotty and Bayman (1993) further demonstrated that atoxigenic strains of *A. flavus* can effectively provide biocontrol against aflatoxin-producing strains in cotton crops. Following this discovery, the same idea was

commercialized and applied to different crops with positive results (Agbetiameh et al. 2019; Donner et al. 2010).

In the following chapters, we hypothesize that fungi tolerant to high heat and/or salt stress can alleviate plant water stress. Similarly, we hypothesize that applying different water regimes to peanut soils will alter the microbial composition over time and reveal target microbes that thrive under dry conditions. We tested fungi for their ability to alleviate drought stress in peanuts. To do this, we used two collections of fungi. One collection was sourced from plots of the Old Rotation, Auburn, AL. The other was a collection of *Mortierella* and *Linnemannia* isolated from cotton seedlings in Alabama. Second, we designed experiments to understand what happens to the diversity of microbial communities from peanut production fields (fungi and bacteria) when restricting water. This may help us pinpoint soil and microbial communities prone to *Aspergillus* infection and help identify microbial groups that increase in abundance under dry conditions. These two studies will help us better understand the effect of drought on peanuts and the microbiome so that we can help inform ecologically motivating strategies for drought management in peanuts.

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2. CHAPTER TWO

Alleviation of water stress in peanuts with *Linnemannia elongata* and *Penicillium citrinum*

Abstract

Intensification and prolonged drought due to increases in temperature and changes in weather, such as altered rainfall periods and intensity or scarcity of water, are gravely affecting crops. Drought decreases the productivity and quality of peanuts (*Arachis hypogaea*). Further, peanut health suffers under drought, allowing opportunistic infection from aflatoxin-producing fungi such as *Aspergillus flavus*. The peanut is commonly grown in sandy soil making it highly susceptible to drought conditions. The detection of microbes that could help delay drought severity as well as aid in growth promotion is highly desired. Fungi have known to help plant cope with water restriction (drought) by expanding its mycelia for water and nutrient assimilation beyond the root zone, biofertilization, Phytohormones synthesis and regulation, and pathogen protection, among others. We screened 100 fungal isolates for heat and salt tolerance as some of them have been described to translate in plant drought tolerance. Then, due to screened phenotypes and literature suggesting beneficial plant associations, we focused on inoculation of *Penicillium citrinum*, *Mortierella*, and *Linnemannia* species, under no-drought and drought conditions. *L. elongata* OEO-196 significantly improved plant biomass accumulation in drought and non-drought conditions. On the other hand, *P. citrinum* was reported to improve the water status and photosynthetic efficiency of plants grown under drought stress. Overall results show a promising trend in peanut biomass potentially allowing peanuts to be more resilient to drought. However, more experimentation is needed to understand the mechanism for alleviating water stress in the peanut plant.

Introduction

High temperatures, decreases in precipitation, shifts in precipitation patterns, and increased salinity in soil conform to a major abiotic factor (Elliott et al. 2018). Drought is the main driver of yield reduction in the Southeastern United States (Chen et al. 2022; Zhen et al. 2022). The peanut crop, an economically important crop in the Southeastern U.S. is particularly vulnerable to drought. However, only 10% or less of peanut fields in Alabama are irrigated, leading to industry losses of approximately $50 million annually (Hamidou et al. 2013; Hollis, 2020; Puppala et al. 2023). In addition, drought can harm the plant's overall health, leaving it more prone to infections. Therefore, identifying microbes that can alleviate water stress in plants is a goal that will aid in understanding how microbes can help sustain plant growth under stress.

Several studies suggest that long exposure to changes in the soil, such as added irrigation, can influence soil microbial communities. With bacteria, irrigation has been shown to favor the abundance of Planctomycetes, Deltaproteobacteria, and Proteobacteria, with Betaproteobacteria and Gammaproteobacterial negatively affected by drought and enriched in roots (Hartmann et al. 2017; Naylor et al. 2023). The fungal Phyla that increased during irrigation were Mucoromycota and Zoopagomycota with *Mortierella*, *Umbelopsis*, and *Zygorhynchus* species (Hartmann et al. 2017). However, for many other species, such as Chloroflexi, Firmicutes, and fungi Ascomycota with Genus *Penicillium* sp. and *Aspergillus* sp. water availability does not seem to alter their presence in the soil (Hartmann et al. 2017; You et al. 2012).

Non-irrigated soil may also change the microbial communities by attracting microbes that can be more tolerant to the dryer, warmer soils, and soils with higher solute concentrations due to

the evaporation of water from the media. As such, these environments may favor microbes with thicker cell walls, increased osmolyte production, nutrient cycling, nitrogen fixation, antimicrobial compounds, phosphate solubilization, and microbial characteristics that help resist water limitation (Chen et al. 2022; Naylor et al. 2023). A prolonged lack of water, often combined with higher temperatures, can lead to drought that leads to solute concentration and salinity. Drought can change the soil microbiome and crop health. Bacterial groups found in non-irrigated soils are Actinobacteria, Acidimicrobiia, Betaproteobacteria, Blastocatellia, Actinophytocola, Cellulomonas, and Pedobacter (Naylor et al. 2023). Fungal groups found in non-irrigated sections can be Ascomycota, Basidiomycota, and Mucoromycota, among others (Hartmann et al. 2017).

Penicillium citrinum has been found in systems with high water availability and drought systems. This specie has been found near water sites (You et al. 2012), in soil, in the rhizosphere (Gu et al. 2023; Wang et al. 2022; Yadav et al. 2010), and as an endophyte of halophytes and non-halophytes (El-Neketi et al. 2013; Hakim and Yuwati, 2020; Kaur and Saxena, 2023; Khan et al. 2008; Sharma et al. 2021; Wang et al. 2022; You et al. 2012). *Penicillium citrinum* has also been isolated from stored peanut seeds, kernels, and in-shell peanuts (Horn, 2005; Xing et al. 2016). Furthermore, several reports about *P. citrinum* aiding plant growth promotion (Hakim and Yuwati, 2020; Kaur and Saxena, 2023) and providing protection from pathogens (Sharma et al. 2021; You et al. 2012). Gu et al. (2023) reported plant growth-promoting effects from the *P. citrinum* when inoculating a spore solution of $1–5 \times 10^5$ spores/mL into *Atriplex gmelinii*. A similar study was done in a different plant host (Khan et al. 2008), but instead of inoculating the plant with the fungal spores, the fungal filtrate was applied to the apical meristem of rice seedlings. Both studies show that the presence of gibberellin derivatives that promoted growth in different plant hosts. In addition, cytokinins (trans-zeatin and trans-zeatin ribosides), 1-Aminocyclopropane-1-carboxylic

343 acid (ACC), and phosphate solubilizing capabilities were observed in *P. citrinum* (Gu et al. 2023;
344 Jia et al. 1999; Yadav et al. 2010). *Penicillium citrinum* has been shown to interfere with the early
345 infection process and, to a certain capacity, limit the disease development of *A. rolfsii* in sunflower
346 plants (Sharma et al. 2021; Waqas et al. 2015) and possibly confer resistance against salt, heat,
347 and drought (Khan et al. 2008; Waqas et al. 2015; You et al. 2012).

348 One common and prevalent saprotroph under drought environments is *Aspergillus flavus*.
349 This soil-borne fungus can thrive with low water availability and in hot climates. *A. flavus* can
350 cause major losses not because it reduces yield with infection but because of its production of
351 aflatoxins. Aflatoxins are carcinogenic compounds that can cause liver cancer and failure. Losses
352 due to aflatoxins in peanuts have caused more than $25 million annually in the Southeastern United
353 States, Georgia (Ali et al. 2021).

354 Atoxigenic isolates of *A. flavus* have been shown to mitigate aflatoxin contamination.
355 Identifying additional microorganisms that can compete against *A. flavus* is imperative for
356 continued peanut production and human health. An antagonistic microbe to *A. flavus* (besides
357 itself) that also flourishes in hot, dry conditions or high salinity can be from the bacterial phylum
358 Actinobacteria, with Genera *Streptomyces, Bacillus, Pseudomonas, Enterobacter, Acinetobacter,*
359 *Burkholderia, Arthrobacter*, and *Paenibacillus* observed in the rhizosphere. Soils with water
360 restriction display an increase in these Genera (Chen et al. 2022; Naylor et al. 2023; Oppenheimer-
361 Shaanan et al. 2022; Williams and de Vries, 2020), as well as enrichment near the rhizosphere of
362 the plants, which suggests a close interaction with the plant root under stress (Chen et al. 2022;
363 Oppenheimer-Shaanan et al. 2022; Williams and de Vries, 2020). Another microbe found in hot,
364 dry conditions is *Penicillium citrinum*. A study of fungal diversity in in-shell and kernel peanuts
365 under storage demonstrated the presence of *A. flavus* and the genus *Penicillium* with *P. citrinum*

as a predominant specie (Xing et al. 2016). Interestingly, between 20 to 30 days of storage, the genera *Eurotium, Rhizopus*, and *Wallemia* had greater relative abundance than *Aspergillus*, which is explained by the idea that these fungi were xerophilic and are known to grow well under low water availability. As these fungi colonized and grew in the peanut shell and kernel, they released metabolic water allowing *Aspergillus* to grow (they tend to be less xerophilic than *Eurotium, Rhizopus*, and *Wallemia*). Later, after 30 to 90 days, a rise in the relative abundance of *Aspergillus* and decreased *Eurotium, Rhizopus*, and *Wallemia* was observed (Xing et al. 2016).

Another phylum of importance found in peanut pods is from the Phylum Mucoromycota. In the peanut soil, Mortierellomycotina with *Mortierella* and *Linnemannia* are highly abundant species (H. Li et al. 2022). In other studies, *Mortierella* and *Linnemannia* were the most abundant at the genus level (Frene et al. 2022). Mortierellomycotina is a sister group of Glomeromycotina. The basal fungi *Mortierella* and *Linnemannia* species from the sub-phylum Mortierellomycotina are widespread fungi which are found in rocks, caves, rivers, lakes, bulk soil, plant tissues, and rhizosphere (Ozimek and Hanaka, 2021). Ozimek and Hanaka (2021) mention that *Mortierella* species are the most common filamentous fungi in soils. Filamentous fungi use mycelia to expand through the soil surface area, improving soil structure, texture, and increasing water availability, nutrient availability, and translocation in plants (Begum et al. 2019; Ozimek and Hanaka, 2021). Plant growth-promoting effects have been observed in many *Mortierella* and *Linnemannia* species. These fungi have been found to aid in mobilizing phosphorous, phosphatase activity, plant growth promotion under high salinity levels, nitrogen fixation, potassium solubilization, production of siderophores, phytohormones such as auxins (indole-3- acetic acid), cytokinins, gibberellic acid, and ethylene (precursor 1-aminocyclopropane-1-carboxylate deaminase) (Ozimek and Hanaka, 2021). Several *Mortierella* species are endophytic strains obtained from roots, stems, leaves, fruits,

bulbs, and seeds, among other plant parts (Ozimek and Hanaka, 2021). *Mortierella candelabrum* was obtained from the seeds of *Crataegus azarolus*; *M. hyalina, M. isabellina,* and *M. ramanniana* were isolated from *Holcus lanatus* grassroots; *M. gamsii*, *M. verticillate,* and *Mortierella zonata* were obtained from *Fragaria vesca* roots; *M. hyalina* and *M. indohii* were the most abundant of the *Mortierella* genus found in the tomato roots, and *M. elongata* recovered from *Populus trichocarpa* (Ozimek and Hanaka, 2021). Many of these endophytes were also reported to aid the plant, producing phytohormones.

In this study, we aimed to describe fungi's *Mortierella*, *Linnemannia,* and *P. citrinum* ability to alleviate water stress in peanuts. The isolates chosen scored best in growing on high NaCl concentrations and were not plant or human pathogens, and the continuation of isolates from the family Mortierellaceae was more based on previous reports of fungi from the sub-phylum Mortierellomycotina aiding in growth promotion (Cesari et al. 2019; Liao, 2021; Vandepol et al. 2021) and mitigating drought stress (Vandepol et al. 2021). Salt (NaCl) was used to screen for drought since salinity has very similar response to drought. We hypothesize that screening fungi from the culture collection from the Old Rotation and cotton seedlings for high salt and heat will lead to the detection of drought resistant fungi which may help peanuts survive a water deficiency.

Methods

Fungal collections and isolation. Fungi were isolated from two sources representing two hypotheses. First, *Mortierella* and *Linnemannia* fungi were isolated from cotton seedlings grown on corn meal agar with pentachloronitrobenzene (50 mg/liter), ampicillin (250 mg/L), rifampicin (10 mg/L), pimaricin (5 mg/L) and benomyl (10 mg/L) (PARPB) in a 100 mm Petri dish (Olofintila

and Noel, 2023). Second, fungi were isolated from soil collected from irrigated and non-irrigated sections of the “The Old Rotation” at Auburn, Alabama in January 2021. Irrigated sections have received supplemental irrigation for the past 20 years, while the non-irrigated section receives only rainfall. Three soil samples were collected from irrigated and non-irrigated sections of plots of a cotton-corn-wheat-soybean rotation with legume cover crops. Soybean seeds were used as bait to enrich fungi from the soil collected. After 72 hours, the soybean seeds were taken out of the soil and immersed in 10 ml 1X PBS. This solution was vortexed to separate the seed from the soil, and the diluted soil was plated on 2% Malt Extract Agar (MEA) with antibiotics rifampicin and chloramphenicol. Pure cultures of all fungi were obtained by hyphal tipping, which consisted of extracting a small piece of hyphae from the tip of a fresh growing culture and subculturing it in a new clean plate. Once in pure culture, fungi were maintained on 2% MEA agar slants or as colonized agar chunks stored in sterile water until use. Unless otherwise stated, fungi were grown on 2% MEA.

DNA extraction and identification of Fungi. DNA extraction of fungi was done by lysing the cell with an extraction solution and applying a 95°C for 10 minutes and finishing with 10°C on the thermal cycler (Thermal cycler Eppendorf USA) following DNA extraction (Noel et al. 2021), followed by the addition of bovine serum albumin (BSA) (Sigma-Aldrich, USA) 3%. The DNA region to be amplified was the internal transcribed spacer region (ITS) primer ITS1f and ITS 4. The cycle program used on the thermal cycler was 94°C for 1 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 8 minutes, following DNA extraction (Noel et al. 2021). PCR products were visualized in 1% agarose gel. Clean-up of the sequences was based on a mix of 5 µl PCR product with 5 µl mix of Exonuclease 1 at 3.57U,

Antarctic Phosphatase at 0.29U, buffers, and ddH20 per µl reaction (Olofintila and Noel, 2023). Sequences were sequenced by Sanger sequencing with ITS1f primer. Received sequences were placed on Geneious Prime (Geneious Prime, New Zealand), and sequences were trimmed to eliminate regions exceeding a one percent chance of error per base. The trimmed sequences were blasted against the nucleotide database (Genbank) BLASTn from the National Institute of Health. Most sequences were identified to species, and all were identified to Genera.

Screening for temperature and salt tolerance. Fungi were screened for salt and heat tolerance since these two stressors are often associated with drought and are easier to screen than desiccation stress in the lab. Fungi were grown on MEA for three to five days then 50 mm plugs were transferred to three replicate 100 cm Petri dishes containing MEA with and without 10% NaCl and incubated at 24ºC and 31ºC (Cole et al. 1985). Measurements of the diameter of the mycelia growth were taken daily for seven days. Some cultures of interest growing in the salt treatment presented scattered growth making mycelial growth measurements difficult; hence, we performed a liquid culture fungal biomass assay as a verification method. For the liquid culture biomass assay, spores were harvested and adjusted to 1 x 10^5 spores per milliliter (Gu et al. 2023). One-hundred microliters of spore suspension were inoculated into 100 ml of Malt Extract Broth (MEB) with and without 10% NaCl with three replicates at the temperatures mentioned previously. Fungal cultures were incubated in the shaker at 150 RPM for one week. The biomass produced by each fungus was dried at 105°C for 48 hours and weighed. The most tolerant fungi that were not pathogenic, according to literature searches, were selected for the greenhouse and growth chamber (Led Plant Growth Chamber, Caron, Marrieta, OH) project, described below.

Growth promotion of peanuts with fungi in growth chamber and greenhouse: Fungal growth. The fungal species used for inoculation were Penicillium citrinum CCH_F37_B, L. elongata (OEO-196, OEO-198), M. alpina OEO-305, and M. calciphila OEO-304. Penicillium citrinum was selected because it showed salt and heat tolerance (Gu et al. 2023) and is plant beneficial with the production of gibberellin (Khan et al. 2008), cytokinins (Gu et al. 2023), ACC deaminase (Jia et al. 1999), and phosphate solubilizing (Liao, 2021). L. elongata and M. alpina are well-documented plant growth-promoting fungi, but little is known about M. calciphila (G. J. Li et al. 2016). Peanut seedlings were inoculated with the fungus of interest by each of two methods. For the dip root method, peanut seedling roots were dipped into a spore suspension. This method was used for Penicillium citrinum since spores are easily harvested and quantified. The spore suspension was prepared by adding 10 ml of water to five-to-seven-day-old colonies in Petri dishes and scraping the spores with a glass rod. The harvested spores were quantified using a hemacytometer and adjusted to 1 x 105 per milliliter. The second method was mycelial root inoculation, used for all Mortierella and Linnemannia isolates. To inoculate peanut germlings with Mortierella or Linnemannia fungal mycelium, the fungi were grown on MEA for three to five days, then 4 plugs of 50 mm each were transferred to three replicate Erlenmeyer flasks containing 100 ml Malt Extract Broth (MEB). The flasks were placed in the shaker at 150 RPM for one week. After this, the mycelial mat was blended for five to six seconds, filtered, and washed twice with 0.5X Phosphate-buffered Saline solution (PBS), rinsed three times with sterile water. The mycelial bits were moved to a sterile 50 ml tube with 50 ml of sterile water. Five and a half milliliters of mycelia suspension were used to inoculate peanut seedlings; L. elongata OEO-196 quantity ranged from 35 mg to 100 mg, M. calciphila OEO-304 ranged from 12 mg to 100 mg, L. elongata OEO-198 with 50 mg to 200 mg, and M. alpina OEO-305 with 25 mg to 100 mg of mycelium.

Growth promotion of peanut with fungi under well water conditions in the growth chamber. The peanut variety used was runner AU-NPL 17, selected due to its commercial use in Alabama (Q. Zhang et al. 2022). Peanut seeds were surface sterilized by soaking in a 6% sodium hypochlorite solution for 10 minutes, and, subsequently, washed three times with sterile water (Sauer and Burroughs, 1986). Potting soil Pro-Mix BX General Purpose (Premier tech, Canada) was added to a 50.8 mm x 25.4 mm plastic tray with the surface sterilized seeds and placed in the germination chamber at 25°C to 30°C with 70% humidity and no light. Germination was observed after five to seven days, and the seeds with visible radicles were chosen and moved to 11.43 x 10.67 cm^2 pots in a growth chamber (Led Plant Growth Chamber, Caron, Marrieta, OH) with 25°C to 30°C with 70% humidity, and a range of fluorescence of 600 μmole m^{-2} s^{-1} with a 14-hour day/night cycle. All the seedlings were placed at 5 cm consistent depth in the pots with soil. Tip root peanut inoculation was used for *P. citrinum*, and 5.5 ml of mycelia solution was inoculated for *L. elongata* OEO-196, *L. elongata* OEO-198, *M. calciphila* OEO-304, and *M. alpina* OEO-305 treatments. After inoculation, the pots were randomized within each tray, and six trays served as the blocking factor for the experiment. Pots had the following treatments: no fungi control, *L. elongata* OEO-196, *L. elongata* OEO-198, *M. calciphila* OEO-304, *L. elongata* OEO-198, and *M. alpina* OEO-305 fungal isolate inoculations. Peanut seedlings were grown for two weeks or until they reached three to four full leaves. At this point, Phi2 was measured using the MultispeQ device (PhotosynQ, Lansing MI). Phi2 measures the amount of incoming light that the plant uses for photosynthesis (Scharnagl and TerAvest, 2017). Peanut roots were un-potted cleaned and a sample of the tip of the root was transferred to corn meal agar with pentachloronitrobenzene (50 mg/liter), ampicillin (250 mg/L), rifampicin (10 mg/L), pimaricin (5 mg/L) and benomyl (10 mg/L)

(PARPB) in a 100 cm petri dish (Olofintila and Noel, 2023) with the objective of re-isolating the inoculated fungus. Isolation from root tissue was done for epiphytic and endophytic fungi. The remaining plant material was separated into shoot and root, moved into envelopes, and dried in the oven for 48 to 72 hours at 60°C to obtain dry shoot and root biomass data. Data analysis was done using R programming version 4.1.1 (2021-08-10). Package ggplot2 was used for visualization (Wickham, 2016). A linear model was done to analyze the effect of the treatment through time or growth compared to the control without fungi with One-way ANOVA and Tukey to test and find out which specific treatment means are different from each other.

Greenhouse terminal drought stress experiment. In this experiment the peanut seeds were surface sterilized by soaking in a 6% sodium hypochlorite solution for 10 minutes, and, subsequently, washed three times with sterile water (Sauer and Burroughs, 1986). Potting soil Pro-Mix BX General Purpose (Premier tech, Canada) was added to a 50.8 mm x 25.4 mm plastic tray with the surface sterilized seeds and placed in the germination chamber at 25°C to 30°C with 70% humidity and no light. Germination was observed after five to seven days, and the seeds with visible radicles were chosen and moved to 7.82 cm x 7.62 cm pots in the greenhouse with temperatures of 24°C to 29°C and no light supplementation was used. All the seedlings were placed at 5 cm consistent depth in the pots with soil. Tip root peanut inoculation was used for inoculation of *P. citrinum*, and 5.5 ml of mycelia solution were inoculated for *L. elongata* OEO-196, *L. elongata* OEO-198, *M. calciphila* OEO-304, and *M. alpina* OEO-305 treatments. After inoculation, the pots were randomized within each tray, for a total of six trays. The entire experiment was repeated three times during the months of July 2022, September 2022, and October 2022. Pots had the following treatments: well-water control with no fungal inoculation, drought

with no fungal inoculation, *L. elongata* OEO-196, *L. elongata* OEO-198, *M. calciphila* OEO-304, *L. elongata* OEO-198, and *M. alpina* OEO-305. The seedlings continued to be watered to field capacity for two weeks or until three to four full leaves were expanded in each plant. At this point, only drought treated seedlings and no-fungi drought control were subjected to drought treatments for two weeks by not watering the pots under drought. Well-water controls were watered to field capacity every three days and the drought treatments were watered to field capacity every seven days. To observe how peanut plants physically respond to water restriction over time, a rating scale was taken every two days (Fig. 1) with the scoring modified from (Sarkar et al. 2021). The scale runs from "0" representing healthy plants with no wilting or leaf drooping, "1" leaves folded but still upright, "2" leaves are folded up and no longer upright with minimal drooping of petioles and stem, "3" folded leaves and drooping of leaves and wilting of the petiole stem, "4" all leaves are wilted, leaves, petiole, and stem are wilted, some leaves start to become opaque, chlorotic, or necrotic, "5" more than 50% of leaves are crisp and dry presenting an opaque green color to the plant, leaves can show light green to yellow color change, the plant is almost physiologically dead. Phi2 was measured using the MultispeQ device (PhotosynQ, Lansing MI). At the end of the drought, root and shoot biomass was collected separated into shoot and root, moved into envelopes, and dried in the oven for 48 to 72 hours at 60°C to obtain dry biomass data. Analysis were done using R programming version 4.1.1 (2021-08-10). Package ggplot2 was used for visualization (Wickham, 2016). Linear model was used to analyze the effect of the treatment through time compared to the control without fungi with One-way ANOVA and Tukey to test and find out which specific treatment means are different from each other.

Growth chamber-controlled drought experiment (20% of soil water content). To measure the amount of water to be added to maintain a 20% soil water content (SWC) relative to field capacity an additional ten pots were filled with Potting soil Pro-Mix BX General Purpose (Premier tech Canada) and weighed to determine soil dry weight and soil field capacity. Soil field capacity was obtained by watering the five pots twice until saturated and draining out the bottom. Pots were allowed to drain overnight, and the five pots were weighed and averaged, providing an estimate of the water needed to reach field capacity. This was compared to dry soil (i.e., oven dried for five days at 60°C). Controls without drought were watered to field capacity every two days. For the drought treatments soil moisture was kept at 20% field capacity by weighing each pot and adding back water to maintain a drought-like condition. Wilting severity ratings and Phi2 were taken every two days after the start of the drought which was started when the plants reached 4 true leaves. After three weeks, roots were washed with water and a sample of the tip of the root was transferred to CMAPARP-B media with the objective of re-isolating the inoculated fungus. The remaining plant material was separated into shoot and root, moved to envelops, and dried in the oven for 48 to 72 hours at 60°C to obtain shoot and root biomass. Data analysis was done using R programming version 4.1.1 (2021-08-10). Package ggplot2 was used for visualization (Wickham, 2016). Linear model was used to analyze the effect of the treatment through time compared to the control without fungi with One-way ANOVA and Tukey to test and find out which specific treatment means are different from each other.

Results

Selecting fungal cultures from salt and temperature screening. Out of the 100 fungal isolates screened for salt and heat tolerance, only three cultures had nucleotide sequences similar to *Penicillium citrinum* specie by BLAST with a 99% query cover. These isolates showed growth at 31ºC and on high salinity media (MEA 10% NaCl); only *P. citrinum* CCH_F37_B was used for the remainder of the experiments, (Fig. 2) *Mortierella* sp. and *Linnemannia* sp. isolates showed no visible sign of growth on 10% salt but did not differ in growth rate at either temperature (24ºC or 31ºC).

Fungal isolate effects on peanut biomass under non-drought conditions. All the variables under study (dry biomass, Phi2, and rating) had an impact in the peanut plant (Table 1). *Linnemannia elongata* OEO-196 significantly increased shoot, but not root biomass compared to the non-inoculated control ($P = 0.04$). However, treatment with *L. elongata* OEO-196 resulted in numerically greatest root biomass. *Linnemannia elongata* OEO-198 did not follow this same trend, indicating a unique feature for *L. elongata* OEO-196 (Fig. 3). In contrast, *M. calciphila* OEO-304 trended to decrease root and shoot biomass. Phi2 results show a significant higher Phi2 value in *P. citrinum* CCH_F37_B ($p = 0.0032$) in comparison with the no inoculated control on the twelfth day of measurement (Fig. 3). Re-isolation of *L. elongata*, and *M. calciphila* was successful from epiphytic root samples, indicating that these fungi successfully colonized roots.

Fungal isolates effect on acute water stress of peanut seedlings in the greenhouse. Analysis of the dry biomass, Phi2 and rating had an impact on the peanut plant (Table 2). *Mortierella calciphila* OEO-304, *P. citrinum* CCH_F37_B, and *M. alpina* OEO-305 inoculated seedlings had significantly higher shoot biomass than the drought control but were still significantly lower than

the non-droughted peanuts, which were watered to maintain soil moisture at field capacity (Fig. 4a). Only *L. elongata* OEO-198 allowed plant shoot development that was not significantly different from the non-droughted control. All peanut plants inoculated with fungi had significantly greater root dry biomass than the drought control except inoculation with isolate *P. citrinum* CCH_F37_B (Fig. 4b). Additionally, *M. calciphila* OEO-304 and *L. elongata* OEO-196 root biomass was not significantly lower than the non-droughted control. For the rating analysis, physical differences were observed as early at four days post-drought. Still, after ten days with water restriction, all plants except for the non-droughted control had folded, drooping leaves and wilting of the petiole stem. Impressively, *L. elongata* OEO-196 and *P. citrinum* CCH_F37_B sustained similar morphological ratings to non-droughted control up to day six, indicating they may help the peanuts sustain a drought for an additional two days. Further, *P. citrinum* CCH_F37_B recorded no differences between the control without drought at days post-drought eight. Equivalently, at six days post drought changes in photosynthesis (Phi2) were detected. At timepoint eight, *P. citrinum* CCH_F37_B, followed by *L. elongata* OEO-196 and *M. calciphila* OEO-304, were the treatments closest to the control with no drought. In ten days post-drought *P. citrinum* CCH_F37_B is significantly better differing from control drought ($p = 0.0264$).

Peanut seedling fungal inoculation under drought conditions (**20% of soil water content in Growth Chamber).** We subjected peanut seedlings to 20% of soil water content with and without fungal inoculation. All peanut plants with the fungal treatments had significantly lower biomass compared to the control without drought (Fig 5). Neither root nor shoot biomass showed any significant difference compared to controls with no water restriction. However, the same numeric trend was observed as in the greenhouse, i.e., dry shoot biomass from peanuts inoculated with *M.*

calciphila OEO-304 and *P. citrinum* CCH_F37_B had numerically higher biomass compared to the droughted control (Fig. 5a) and dry root biomass from peanuts inoculated with *M. calciphila* OEO-304 and *L. elongata* OEO-196 increased relative to the droughted control (Fig. 5b). Ratings results slightly changed starting at day six and at twelve days post-drought, all the treatments are different from the control without drought (Fig. 5c). All treatments maintained the same response in Phi2 results (Fig. 5d), where at twenty-five days post-drought, treated plants start diverging from the control no-drought, and two days later, all fall significantly different from the no-droughted control. The analysis of variance for the growth chamber under water restriction is observed in (Table 3).

Discussion

A single day can become a decisive factor in life and death, high productivity, and decreased plant yield under drought. For peanuts, drought can also mean an increased risk of *A. flavus* infection. Certain fungi may help plants survive stressful situations like drought through various mechanisms. We found that some fungi, especially in the Mortierellaceae family, could increase plant biomass. *Linnemannia elongata* OEO-196 in particular had a positive effect on dry shoot biomass. *Mortierella calciphila* OEO-304 treatment performed better during the drought experiments than under no drought conditions, and *P. citrinum* had higher photosynthetic efficiency and rating results overall treatments.

Under no water stress, *L. elongata* OEO-196 improved shoot biomass accumulation but did not under drought stress conditions. Interestingly, the other treatment with *L. elongata* OEO-198 did not improve biomass accumulation, indicating within-species variation. A study used three different plant growth-promoting *L. elongata* isolates from different geographic locations, and

same as observed in this study, different isolates will respond differently; hence, the intensity of plant growth-promoting activities can vary between isolates (K. Zhang et al. 2020). For example, inoculation of different *L. elongata* isolates have led to an increase in plant dry biomass and leaf expansion in cottonwood, pine, corn (Liao, 2021), watermelon, tomato, squash, and bahiagrass, among other plants (K. Zhang et al. 2020). For example, the three different *L. elongata* isolates (PMI77, PMI624, PMI93) led to variation in plant height, leaf area, and plant dry biomass on watermelon, corn, tomato, squash, bahiagrass, okra, and soybean. Isolate PMI624 increased plant height in watermelon, corn, tomato, and bahiagrass, leaf area increase was observed in watermelon, corn, and squash, and an increase in dry biomass was detected in watermelon, tomato, squash, and bahiagrass compared to control. However, the same isolate PMI624 had no effect on the plant height of okra, and a decrease in leaf area and dry biomass was observed. Interestingly, *L. elongata* isolate PMI93 decreased soybean by more than 30% in its height and reduced plant dry weight by 7% compared to control plants while the same isolate increased plant height of corn by 12%, and leaf area by 9.1% (K. Zhang et al. 2020). Another study worked with the *L. elongata* isolate PMI93 on *Populus trichocarpa* for growth promotion and discovered that this isolate can form biofilm on plant roots, showing direct interaction with roots (Liao et al. 2019). In our results, a similar response is observed among the *L. elongata* OEO-198 and *L. elongata* OEO-196 treatment possibly expressing a similar within-species variation.

Penicillium citrinum CCH_F37_B and *L. elongata* OEO-198 produced no significant differences in dry biomass but a positive trend was observed. Likewise, the implementation of acute water stress of peanut seedlings in the greenhouse showed a growing trend in dry biomass in all treatments. The shoot biomass with *L. elongata* OEO-198 is the closest to control No-drought, while there is no evidence to suggest that root biomass treatments with *Mortierella*

663 *calciphila* OEO-304, followed by *L. elongata* OEO-196, are different from control No-drought. A
664 35-year-old experiment with the application of organic fertilizers reported *L. elongata* to increase
665 plant growth under normal conditions. The dominant species that responded to the organic
666 fertilizers was *L. elongata* which increased the dry weight of *Zea mays* and leaf area. Additionally,
667 higher concentrations of auxins (IAA) and ABA were detected in roots and increased
668 concentrations of bioavailable phosphorous were found in the soil (F. Li et al. 2018, p. 20; Liao,
669 2021; Ozimek and Hanaka, 2021). This is a possible mechanism that Linnemannia and Mortierella
670 are using in our studies, maybe quantifying the hormone ABA since is considered a stress hormone
671 in charge of regulating stomatal conductance, gene up regulation, compatible solute (such as
672 proline) under drought stress environments (Hirayama & Mochida, 2022; Iqbal et al. 2022).

673 A scale was developed in this study to evaluate the effect of water restriction (drought)
674 imposed on peanut seedlings with possible plant growth promoting fungi. The rating scale results
675 in the greenhouse under acute drought showed *P. citrinum* CCH_F37_B physiology sustained a
676 similar rating score compared to the non-droughted control. Moreover, Phi2 changes started to be
677 observed as early as six days post drought, and *P. citrinum* CCH_F37_B maintained a close rating
678 to the control peanut seedlings without drought up to eight days post drought, indicating that *P.*
679 *citrinum* helps peanuts be more resilient to drought (Khan et al. 2008; Waqas et al. 2015) You et
680 al. (2012) reported that *P. citrinum* growing in salt environments help alleviate salt and
681 temperature stress in plants. *Penicillium citrinum* has been found as an endophyte in halophytic
682 plants raising questions about tolerance to harsh environments in plants (Leitão and Enguita, 2016;
683 You et al. 2012). Jia et al. (1999) studied the production of ACC in *P. citrinum*, Kanhayuwa et al.
684 2023 detected *P. citrinum* antibiosis effects inhibiting the growth of *Micrococcus luteus,*
685 *Staphylococcus aureus, S. epidermidis, MRSA, Salmonella Typhi, Candida albicans, Aspergillus*

686 *fumigatus*, and *Microsporum canis*, and Khan et al. (2008) studied a new *P. citrinum* strain with
687 higher gibberellins production capacity than the *G. fujikuroi* wild type. The discovery of
688 gibberellin production by *P. citrinum* has prompted the understanding of the production of
689 gibberellins from the endophytic *P. citrinum* as a crucial piece in allowing plants to overcome
690 salinity, temperature, and drought stress (Leitão and Enguita, 2016). Further, endophytic *P.*
691 *citrinum* isolated from wheat (*Triticum aestivum L*.) improved seedling growth under drought
692 using polyethylene glycol 6000 (PEG) as the drought imposer and under normal conditions (Kaur
693 and Saxena, 2023; H. Zhang et al. 2011).

694 The Mortierellaceae isolates used in this study sustained similar growth to controls (24ºC)
695 in higher temperatures (31ºC) experiments but did not survive at high salt concentrations. As water
696 restriction is imposed there is not enough water to buffer against the heat affecting soil and plants.
697 Mortierellaceae family have been found to contain P solubilizing properties that have been studied
698 in conjunction with arbuscular mycorrhizal fungi to boost nutrient acquisition, promoting higher
699 shoot plant biomass in *Leucaena leucocephala* plant and stress alleviation of saline environments
700 on *Ricinus communis L.* plant (H. S. Zhang et al. 2014). *Mortierella calciphila* OEO-304 growth
701 trended higher at 31ºC than in the control temperatures. This aligns with the increased growth trend
702 of shoot and root biomass in the drought-imposed experiments, while in the growth chamber
703 experiment under well-watered conditions, *M. calciphila* OEO-304 decreased plant shoot and root
704 biomass. Although not significant, this flip in the response under stress potentially demonstrates a
705 stress-dependent alteration in the interaction of *M. calciphila* and peanuts. The role of interaction
706 between *Mortierella* and plants is not entirely understood, leaving questions about which factors
707 such as soil structure, nutrients availability, phytoregulators interacting on the rhizosphere, or even

biotic factors are critical to have a favorable interaction among legume and non-leguminous plant growth.

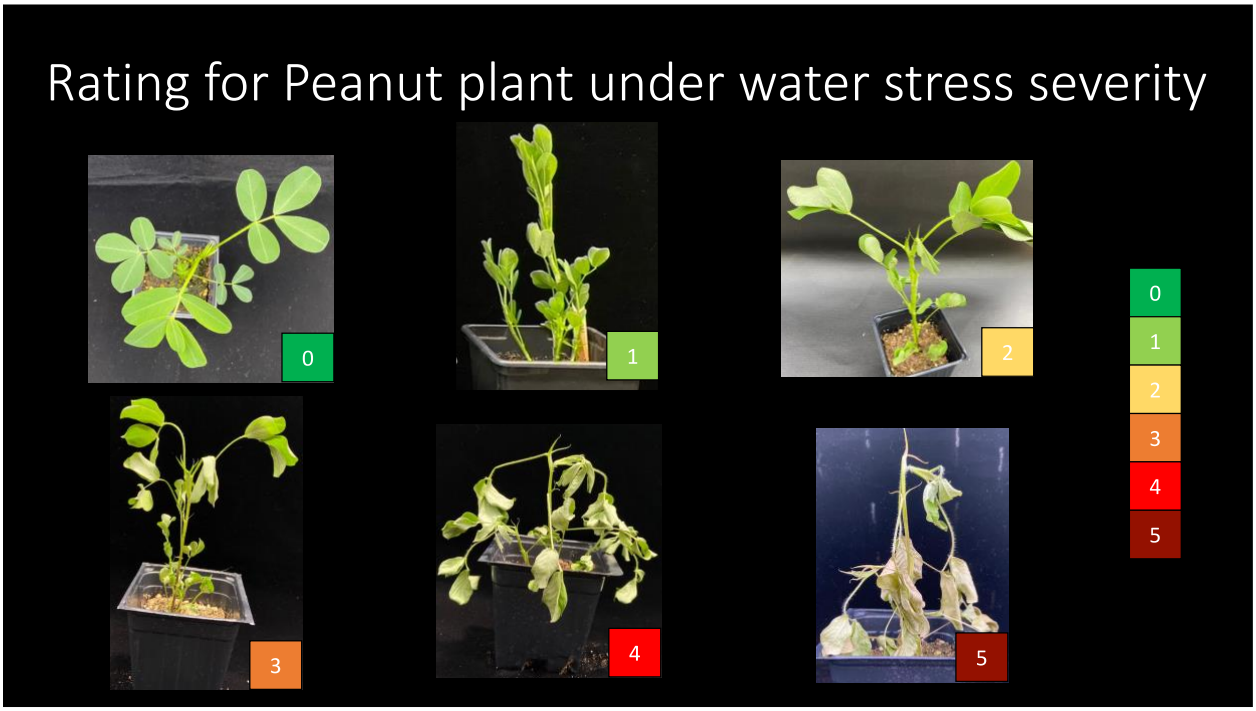

Figure 1. Rating scale constructed for peanut physiological response to water restriction, modified from (Sarkar et al. 2021). The scale starts at 0) representing healthy plants with no wilting or leaf drooping, 1) leaves folded but still upright, 2) leaves are folded up and no longer upright with minimal drooping of petioles and stem, 3) folded leaves and drooping of leaves and wilting of the petiole stem, 4) all leaves are wilted, leaves, petiole, and stem are wilted, some leaves start to become opaque, chlorotic, or necrotic, 5) more than 50% of leaves are crisp and dry presenting an opaque green color to the plant, leaves can show light green to yellow color change, the plant is almost physiologically dead.

Table 1. Analysis of variance table for the growth chamber under well water conditions experiment.

Variable	Factors	[a]Df	Fvalue	Pvalue
Shoot dry biomass	Treatment	5	6.0	0.001
Root dry biomass	Treatment	5	4.7	0.003
Phi2	Treatment	5	4.5	0.001
	Days post drought	5	2.4	0.041
	Treatment: days post drought	25	0.7	0.803

[a]Degree of freedom

752 Table 2. Analysis of variance table for the greenhouse experiment.

Variable	Factors	[a]Df	Fvalue	Pvalue
Shoot dry biomass	Treatment	6	13.3	< 0.001
Root dry biomass	Treatment	6	11.6	< 0.001
Phi2	Treatment	6	12.2	< 0.001
	Days post drought	6	36.2	< 0.001
	Treatment: days post drought	36	1.3	0.106
Rating	Treatment	6	27.8	< 0.001
	Days post drought	6	70.9	< 0.001
	Treatment: days post drought	36	2.2	< 0.001

753 [a]Degree of freedom
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767 Table 3. Analysis of variance table for the growth chamber under water restriction experiment.

Variable	Factors	[a]Df	Fvalue	Pvalue
Shoot dry biomass	Treatment	6	28.1	< 0.001
Root dry biomass	Treatment	6	15.4	< 0.001
Phi2	Treatment	6	8.3	< 0.001
	Days post drought	15	49.9	< 0.001
	Treatment: days post drought	90	2.1	< 0.001
Rating	Treatment	6	86.2	< 0.001
	Days post drought	14	69.1	< 0.001
	Treatment: days post drought	84	2.4	< 0.001

768 [a]Degree of freedom
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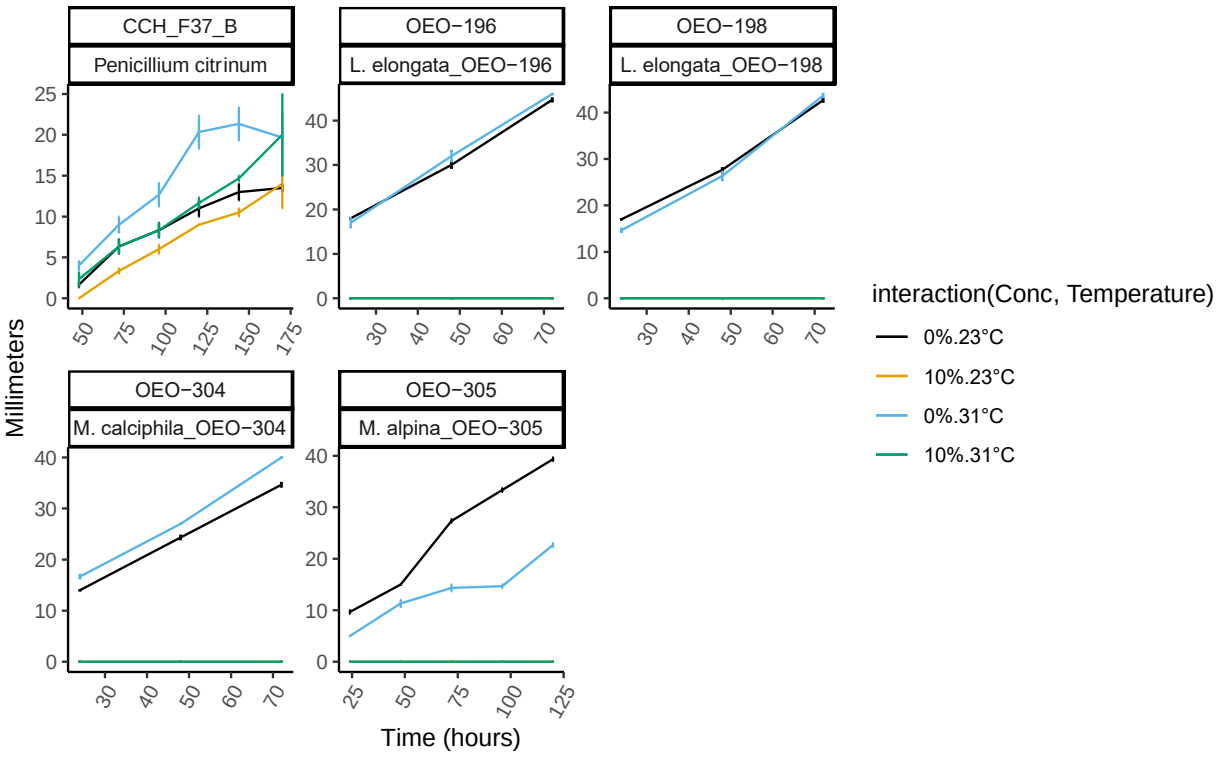

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779 Figure 2. Salt and temperature screenings from *P. citrinum*, *M. calciphila*, *L. elongata*, and *M.*

780 *alpina*.

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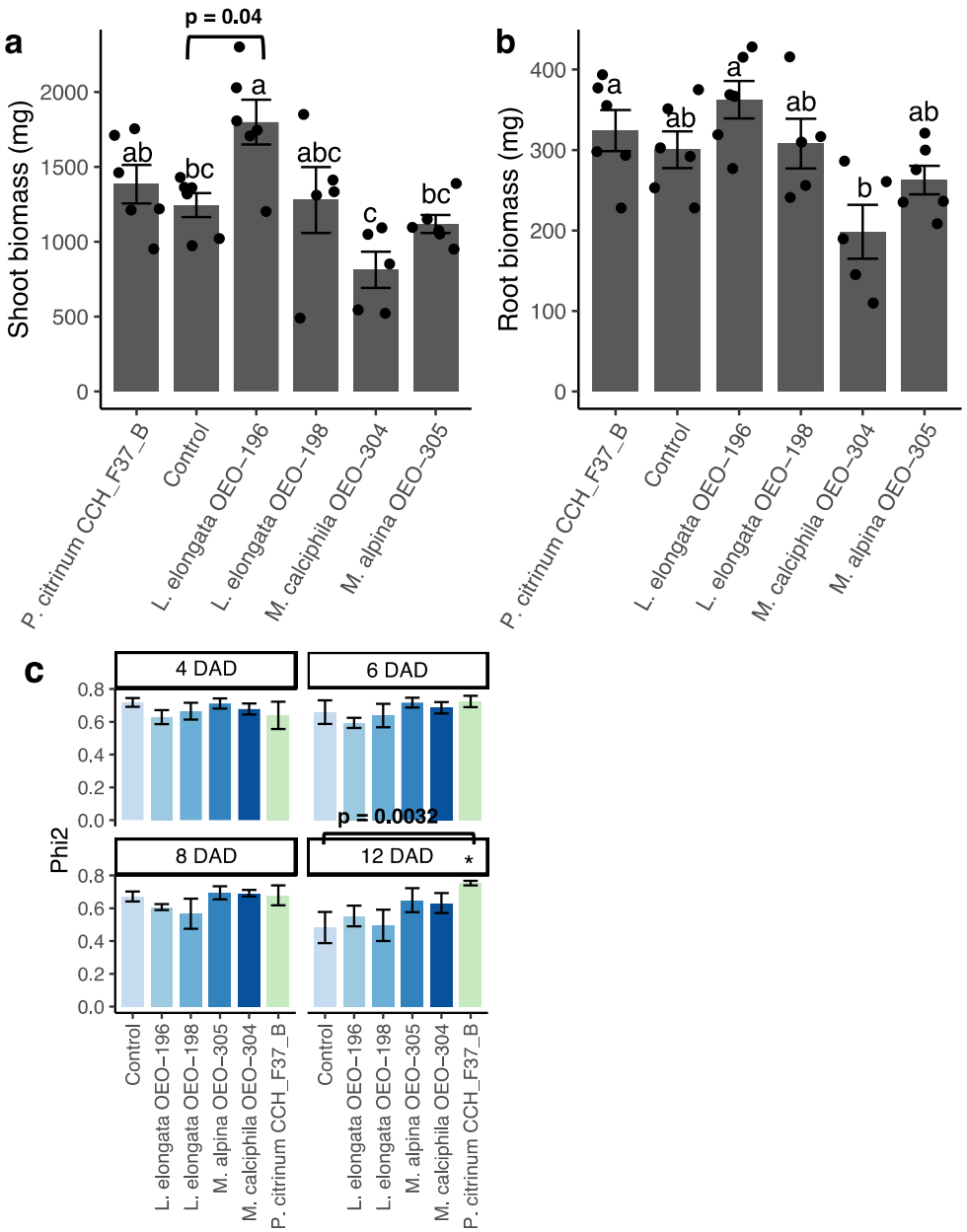

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784 Figure 3. Shoot and root biomass of peanut seedlings under fungal inoculation growing in growth

785 chamber under no-drought conditions. (a) *L. elongata* OEO-196 accumulated significantly greater

786 shoot biomass than the control. (b) No significant difference but an increased trend was observed

787 on *L. elongata* OEO-196, followed by *P. citrinum* CCH_F37_B, and *L. elongata* OEO-198. (c)

788 Phi2.

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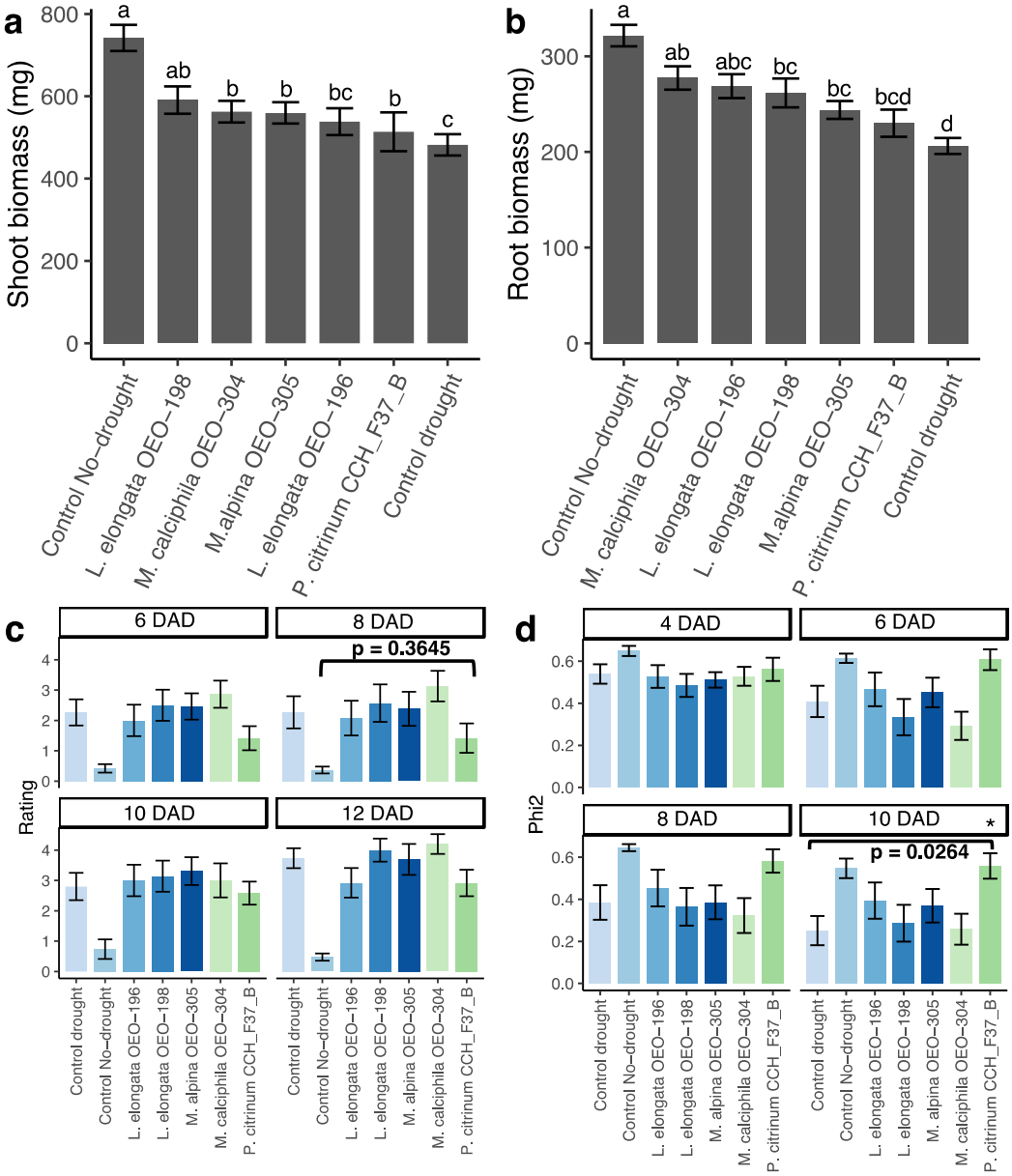

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791 Figure 4. Greenhouse under acute water stress, (a) shoot biomass and (b) root biomass of

792 inoculated peanut seedlings with *L. elongata* OEO-196, *P. citrinum* CCH_F37_B, *L. elongata*

793 OEO-198, *M. calciphila* OEO-304, *M. alpina* OEO-305, and controls. (c) Rating, and (d) Phi2.

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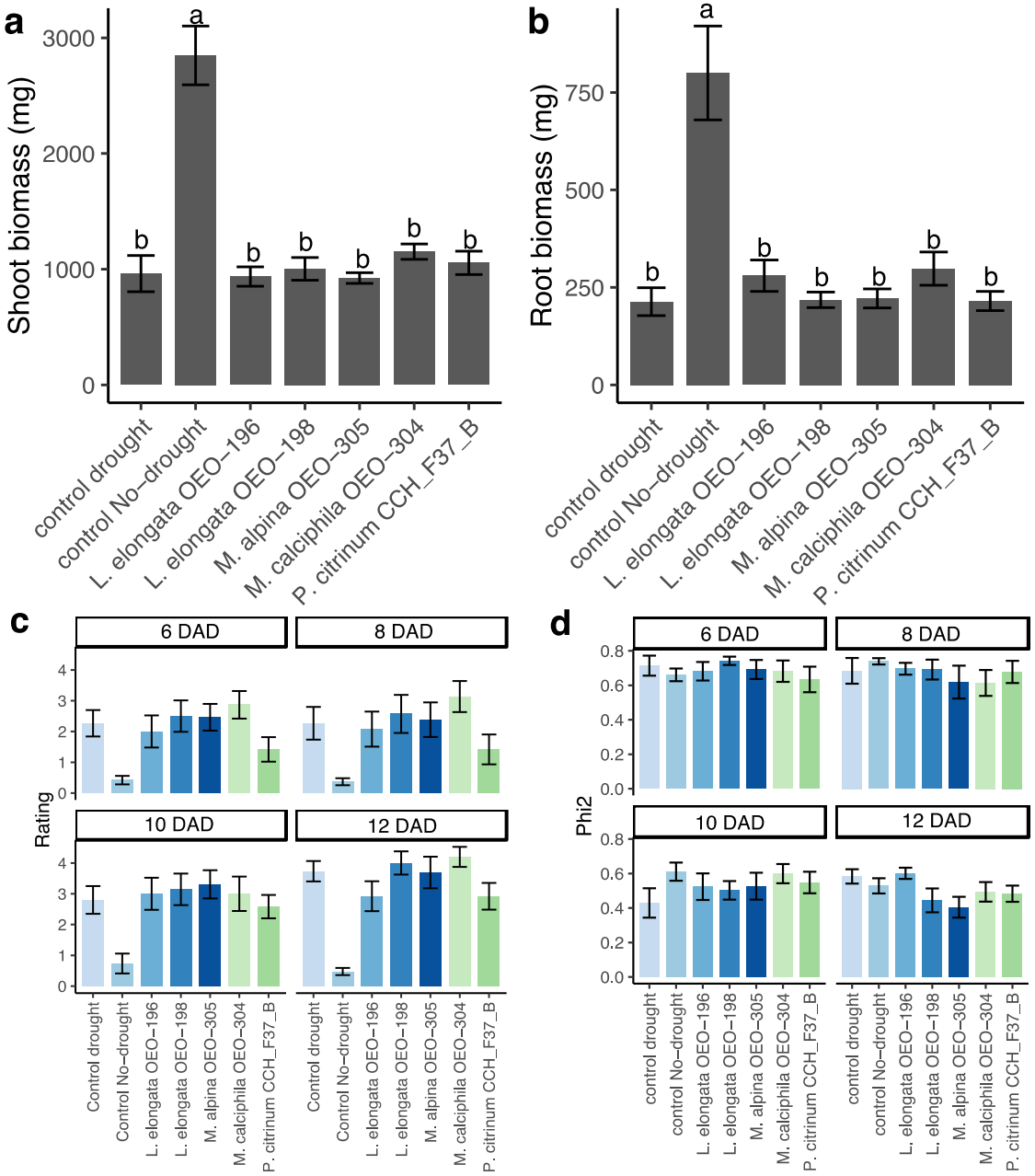

Figure 5. Growth chamber ratings result under water restriction. (a) dry shoot biomass and (b) dry root biomass of peanut seedlings. (c) rating of peanuts plants through the drought implementation. The scale is structured where "0" is the plant in well-water conditions, slowly increasing to "5" where the plant is almost physiologically dead. (d) Phi2.

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3. CHAPTER 3

Changes in fungal and bacterial diversity over a peanut soil moisture gradient

Abstract

The soil microbiome plays an important role in the overall health and development of a plant. Stresses such as drought can affect the abundance of soil microbial communities as well as plant productivity and plant health. Peanut (*Arachis hypogaea*) is an economically important crop worldwide that can be at risk of infection with *Aspergillus flavus* during drought. This fungus is known to produce carcinogenic aflatoxins. We hypothesize that applying different water regimes to peanut soils will alter the microbial composition over time and reveal microbes that may also thrive under dry conditions to compete with *A. flavus*. Two soils from different fields with a history of peanut production were collected at Wiregrass Research and Extension Center Headland, Alabama. The soils were placed in polyvinylchloride tubes inside a growth chamber (29°C). Five water regimes represented a gradient from dry to wet conditions. They were sampled through a nine-week experiment and sequenced to determine the effect of the moisture treatments on the fungal and bacterial communities. Time, soil, and treatments had a significant impact on both bacterial and fungal communities. Bacterial Phylum Actinobacteriota thrived in drier treatments, and Proteobacteria and Planctomycetota on moist ones. This helps us understand that Actinobacteria may be a good target against *A. flavus* because it can survive under dry soil conditions.

Introduction

Drought is a serious environmental stress that affects plant productivity and plant health (Seleiman et al. 2021). Drought can be explained as long-term exposure to water deficit. Over time, drought directly interferes with the normal function of the plant (Shao et al. 2008). Plants restricted to water will have a decline in photosynthesis, morphological changes, and a decrease in physiological functions (Sun et al. 2020). An economically important legume crop affected by drought is peanuts (*Arachis hypogea*). In the United States, 80% of the peanuts grown are in the southeast, including Georgia, Alabama, Florida, North Carolina, and Texas (Zhang et al. 2022). Production of peanuts in these states tends to be in sandy or sandy loam soil under rainfed conditions (Zhen et al. 2022). Periodic drought can affect peanut size, quality, and yield (Rosas-Anderson et al. 2014; Zhang et al. 2022), and annual losses from drought can reach up to $50 million (Dai et al. 2019; Zhang et al. 2022).

The first morphological indications of drought stress in peanut plants are the closure of the leaflets followed by leaf and stem epinasty and dulling of foliage. The intensification and extension of water stress decreases stomatal conductance leading to a decline in photosynthesis and other metabolic processes that can decrease leaf area and even reduce yield (Shao et al. 2008; Shrief et al. 2020). As drought is imposed on peanut plants, they become more susceptible to infections by fungi like *Aspergillus flavus* (Uppala et al. 2013).

Aspergillus flavus is an opportunistic fungal soil saprotroph known to be associated with several economically important crops such as maize, cottonseed, rice, wheat, sorghum, and peanuts (Amaike and Keller, 2011; Kebede et al. 2012). This fungus is known as a saprobe and human pathogen that is found in agricultural soils, storage areas, and processing facilities (Tola and

Kebede, 2016). Additionally, some *A. flavus* strains and other species in sections Flavi and Ochraceorosei can produce aflatoxins, which are highly carcinogenic and mutagenic (Amaike and Keller, 2011). These fungal species produce toxins B1, B2, G1, and G2. The most life-threatening and potent carcinogen is aflatoxin B1. Infection of peanuts with this fungus does not necessarily reduce yield but its production of aflatoxin contaminates the grain, nuts, and seeds, preventing commodity sales, and causing major economic losses (Uppala et al. 2013). The southeast United States peanut industry can lose over $25 million annually due to aflatoxin contamination (Amaike and Keller, 2011; Robens and Cardwell, 2003). Consequently, the Food and Drug Administration (FDA) has highly regulated guidelines for total aflatoxins in food or feed with a max of 20 parts per billion (ppb) in food or feed, and 0.5 ppb in milk (Alam et al. 2020). Aflatoxin contamination from *A. flavus* is a major problem exacerbated by drought.

As plants direct their energy to osmotic adjustments and try to stay alive during water deficit, the roots expand and penetrate the soil looking for water and nutrients. Root microbial communities have been shown to interact with stressed plants and aid plants by improving nutrient circulation and water acquisition, producing compounds such as secondary metabolites and hormones that can protect the crop from biotic and abiotic stresses (Chen et al. 2022; Oppenheimer-Shaanan et al. 2022). However, the microbial community and abundance can be affected by drought. Some bacteria are known to ameliorate crop productivity by the production of 1-aminocyclopropane-1carboxylate (ACC) deaminase, formation of exopolysaccharides (EPS), osmotic adjustment by the production of hormones, and antioxidant defenses, among others (Saberi Riseh et al. 2021). In the case of fungi, evidence suggests they can aid the plant through the regulation of hormones, solubilization and mineralization of nutrients, evading pathogenic infections by producing volatile organic compounds and antimicrobial compounds, regulating

plant defense responses, and aiding in abiotic stresses (Hossain and Sultana, 2020). Plant roots that have active microorganisms promoting plant nutrient uptake may make changes to that microbial community. Several studies propose that plants grown in arid regions and drought-affected environments are more selective in their microbial counterparts (Naylor and Coleman-Derr, 2018; Saberi Riseh et al. 2021). In this study, we focus on understanding and detecting changes in microbial communities of peanut soils under five different water regimes over a nine-week trial. We hypothesize that applying different water regimes to peanut soils will alter the microbial composition over time and reveal target microbes that thrive under dry conditions.

Materials and Methods

Study site and sample collection. The soil was collected from the Wiregrass Research and Extension Center (WGREC) in Headland, Alabama, USA. The soil was collected on 29 June 2021 in two separate fields (A and B), each with peanuts growing in them. For each location, the soil was collected in a "w" transect 12.7 cm deep and then sieved through a 1 mm screen for uniform particle size. A subset of each soil was sent to the Auburn University Soil Testing Laboratory for percent sand, percent silt, percent clay, soil organic matter, nutrient levels (phosphorus, potassium, magnesium, and calcium), and pH, textural class, and water availability recorded in Table 7.

Growth chamber soil moisture regimes. Approximately 574.32 cm^3 of sieved soil was added to a polyvinylchloride (PVC) cylinder 7.5 cm diameter with 15.24 cm length and a mesh screen with 1 mm openings affixed to the bottom to prevent soil disturbance while allowing drainage. The PVC tubes were arranged in a randomized complete block design with six replicates and five soil

moisture treatments. Five water regimes were established to represent a gradient of dry to wet conditions and were as follows: 1) never watered, 2) 20 ml of water once per week, 3) 20 ml of water twice a week, 4) 50 ml of water once per week, and 5) 50 ml of water twice per week. The experiment ran for nine weeks at a temperature of 29°C inside a growth chamber with no light. Sterile water for the treatments was added using a micropipette Repeater M4 (Eppendorf, USA). Every week from week zero to week nine, before adding the water, 2 to 3 grams of soil were collected in a sterile coin envelope, and 250 mg of soil was added to 2ml disruptor tubes from OMEGA E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). After the nine-week sampling period, a subsample of soil was taken from each PVC tube to determine the fluctuation in soil moisture content for each treatment experienced over one week. With five treatments, six replicates, and two soils sampled over five time points, the total number of samples processed was equal to 300 soil samples. To determine the soil moisture, one gram of soil was weighed before and after drying at 105°C for 24 - 48 hours (Robertson and VanderWulp, 2019).

Genomic DNA extraction and library preparation. DNA was extracted from soils at weeks 0, 2, 5, 7, and 9 using OMEGA E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and quantified using a Nanodrop (Thermo Scientific, USA) or a Qubit 3.0 fluorometer (Thermo Fisher, USA). Amplicon libraries were then constructed targeting the internal transcribed spacer (ITS) region of the fungal rRNA gene and the V4 region of the 16S rRNA gene. The fungal and bacterial amplicon libraries were prepared according to modified protocols from Lundberg et al. (2013). Followed by library preparation composed of three rounds of polymerase chain reaction (PCR), followed by normalization, concentration, and finalizing with a clean-up and agarose gel before sending for sequencing. The PCR steps, the reagents, primers, and cycling conditions are

disclosed in Tables 4, 5, and 6, respectively, adapted from (Noel et al. 2022). At least one negative extraction control, a PCR negative control, and a positive control were included in each 96-well plate and sequenced. The Invitrogen SequealPrep kit (Thermo Fisher, USA was used to normalize sample concentrations. Individual libraries were then pooled then concentrated 20:1 using the Amicon® Ultra 0.5 mL filters (EMDmillipore, Germany). AMPure beads (Beckman Coulter, USA) at 0.7X were used to purify the pooled library. Finally, the Qubit 3.0 fluorometer was used to quantify the pooled library. The pooled amplicon libraries were sent to MiGs (Pittsburgh, PA) for sequencing Illumina MiSeq 2x300 bp V3 chemistry.

Bioinformatics and statistical analysis. Prokaryote raw sequences were merged using vsearch 2.14.1 (Rognes et al. 2016). Fungal and prokaryote primers were removed using cutadapt 4.0 (Martin, 2011). Sequences were filtered using an expected error threshold of 1.0 for fungi and 0.5 for bacteria and trimming to 250 bp length. For fungi, the conserved 18S region was trimmed from the front of the reads. Qualified reads were then dereplicated, chimeras removed, and clustered into operational taxonomic units (OTUs) at traditional 97% identity vsearch v2.14.1 (Rognes et al. 2016). OTU sequences were then assigned taxonomy with the SINTAX algorithm using vsearch v2.14.1 (Rognes et al. 2016). Fungal ITS sequences were compared to the UNITE and prokaryotes with SILVA 138.1 database.

Data analysis. Metadata, taxonomy, and OTU table were uploaded into R. The R packages phyloseq v134.0 (McMurdie and Holmes, 2013) and vegan 2.5-7 (Oksanen et al. 2020) were primarily used for the analysis. Unclassified taxonomy at the kingdom level and OTUs matching positive control mock sequences were filtered. OTUs that were also found in the sample negative

controls were removed by using R package decontam (Davis et al. 2018). Samples with lower than 5,000 reads per sample were discarded due to low sequence coverage.

We assessed rarefaction analysis by undergoing the dataset to the lowest sequencing depth. Counts were then normalized based on cumulative sum scaling and subjected to a principal coordinate analysis with subsequent Bray-Curtis distance matrix. To determine the differences in community structure between soils, moisture treatment, and time, we ran a permutational analysis of variance (PERMANOVA) with the function *adonis2*. To know which microorganisms were significantly associated with the moisture treatments, we ran an indicator species analysis 1.7.12 to determine OTUs significantly associated with the five different moisture treatments (De Cáceres et al. 2010). Operational taxonomic units were regarded as significant if the p-value was below 0.01.

Results

Soil Analysis. The analyzed soils from two field sites (soil A, soil B) collected from the Wiregrass Research and Extension Center (WGREC) in Headland were analyzed for percent sand, percent silt, percent clay, soil organic matter, nutrient levels (phosphorus, potassium, magnesium, and calcium), pH, textural class, and water availability (Fig. 4). The two soils had similar sand content, pH, water availability, and organic matter but differ in silt, calcium, phosphorous, potassium, and magnesium. Soil A had a higher silt percentage (1.88%), phosphorous (41 pounds per acre), and potassium (236 pounds per acre) while soil B has higher results for clay percentage (20%), calcium (575 pounds per acre), and magnesium (63 pounds per acre).

Overall sequencing output. To understand the microbial shifts from the peanut soils under different water treatments, fungi, and bacteria were sequenced for both soils (A and B). Three fungal OTUs were filtered that were found to be positive contaminants. After decontamination and filtering, we obtained 22,957,826 high-quality clean fungal reads across 298 samples. Two samples were removed due to low sequencing coverage. For bacterial sequences, twenty-five OTUs were filtered due to possible contamination leaving a total of 9,073,713 high-quality bacterial reads across 291 samples. Seven samples were removed due to low sequencing coverage. Rarefaction analysis showed the number of unique sequences in our dataset and indicated that the median read depth per sample captured the majority of the OTUs present in each soil (Fig. 6).

Beta-diversity analysis. To determine the factors that contribute to dissimilarities in community structure, a principal coordinate analysis (PCoA) was conducted, followed by a PERMANOVA. In Fig. 7, each point represents a single community; hence, the closer they are together, the more similar the community composition, and the further apart the points, the more dissimilar communities. Two clusters detached from each other representing soils A and B were observed on the first principal coordinate for both fungal (Table 8; Fig. 7a; $p < 0.0001$, $R^2 = 0.164$) and bacterial (Table 8; Fig. 7b; $p < 0.0001$, $R^2 = 0.286$). Significant changes were also observed for time (Table 8; fungal: $p < 0.0001$, $R^2 = 0.096$; bacterial: $p < 0.0001$, $R^2 = 0.077$), which was observed separated on the second principal coordinate, and moisture treatment (Table 8; fungal: $p = 0.0057$, $R^2 = 0.018$; bacterial: $p < 0.0001$, $R^2 = 0.032$).

Fungi associated with specific moisture treatments. Fungal indicator species analysis highlights the specific fungal OTUs significantly associated with the five water treatments (Fig. 8).

Ascomycota had one OTU associated with the driest treatment (treatment 1) in soil A, while in soil B Ascomycota had one OTU associated with treatment 4 (50 ml of water) and three OTUs in moist treatment (treatment 5). In soil B, Basidiomycota had one OTU in treatment 3. One OTU from Blastocladiomycota was observed in soil A in treatment 3.

Relative abundance of *Aspergillus flavus* in different moisture regimes. We analyzed the relative abundance of *A. flavus* for soil A and B (Fig. 9). Soil A had a low relative abundance relative to soil B. In soil B, there was a spike in week seven for drier treatment (treatment 1), treatment 3 (20 ml of water twice a week), and treatment 4 (50 ml of water a week). Treatment 3 maintained a pattern of increasing relative abundance in weeks two and seven and decreasing abundance in weeks five and nine. Soil B treatment 1 and treatment 2 had the lowest relative abundance in week cero and two and an increase in week five, seven, and nine. Treatment 3 and treatment 5 a gradient is detected while for treatment 4 increase in relative abundance is observed in week two with a decrease in week five and slowly increasing in week seven and nine.

Bacteria associated with specific water treatments. The bacterial community in the soils changed over the treatments applied and analyzed using the indicator species analysis (Fig. 10). Overall, more bacterial OTUs were associated with treatment 5 (50 ml of water twice weekly) than with drier treatment (treatment 1). Many bacterial Phyla were strongly associated with specific water treatments. Proteobacteria had more OTUs in treatment 5 (soil A = 15 OTUs, soil B = 34 OTUs) than in the drier treatment (treatment 1) with (soil A = 4 OTUs, soil B = 4 OTUs). They increased in relative abundance with progressively wetter soils (Fig. 11). Similarly, in the moist treatment (treatment 5), Acidobacteriota had more indicator OTUs in soil B (n = 15) compared to

soil A with five. Likewise, Planctomycetota was most associated with treatment 5 for both soils, with 24 indicators in soil A and 11 in soil B. On the other hand, Actinobacteriota was mostly associated with drier treatment (treatment 1) and treatment 2 in both soils. Eighteen indicators were observed in treatment 1 (dry) and nineteen counts in treatment 2 of soil A, while treatment 5 had one OTU. In soil B, eight counts in treatment 1 (dry) and 26 counts in treatment 2, while two OTUs were indicators in treatment 5. The imposed water treatment induced a gradient in the relative abundance of Actinobacteriota (Fig. 11). A higher relative abundance for Actinobacteria was detected in the dry treatment and slowly decreased with decreasing water for both soils.

Discussion

In this study, we aimed to understand the changes in peanut soil microbial communities varying a moisture gradient. Despite the similar geographical location of the peanut soils, both fungal and bacterial communities had different community compositions in the two soils. The moisture treatment implemented throughout the nine-week study had an impact on the microbial composition of fungi and especially on bacteria. Some taxa groups were strongly associated with moisture changes. For example, we observed that Actinobacteriota increased its relative abundance in the drier treatment and decreased in the moist ones. Not alone, they were found to be indicators for drier treatments in both soils and, therefore, may be a target for alleviating drought stress and/or antagonism against *Aspergillus flavus*.

Changes in soil moisture can impact the microbial composition of soils (Xie et al. 2021). Bacterial taxa were more associated with moist treatments than drier ones. Proteobacteria were found at a higher count in the moist treatments and a greater relative abundance in treatments containing 50 ml of water weekly (treatment 4) and 50 ml of water twice weekly (treatment 5).

Proteobacteria have previously been associated with irrigated and moist soils and respond to rewetting events (Hartmann et al. 2017; Meisner et al. 2018; Naylor and Coleman-Derr, 2018). Proteobacteria play a large role in carbon, nitrogen, and sulfur cycling (Abdul Rahman et al. 2021; Hartmann et al. 2017). In contrast to our results, Proteobacteria have also been found as core members of root microbiomes under drought and the most abundant Phylum in soil, bringing into question the selection of these microbes by the plant in drought conditions (Xie et al. 2021). For example, the gram-negative nitrogen-fixing bacteria *Rhizobium* infects legumes through root hairs and forms nodules (where nitrogen fixation happens). This source of nitrogen aids in plant fitness and stress tolerance (Abdul Rahman et al. 2021). Bacteroidota and Planctomycetota are other Phyla that increased their abundance under irrigation (Dai et al. 2019; Hartmann et al. 2017). Planctomycetota are gram-negative bacteria Phyla highly associated with droughted conditions. However, depending on the subgroup detected, they have also been found in irrigated environments (Hartmann et al. 2017) like our results. In our results, Acidobacteriota was found in soil B with a greater number of indicators OTUs in high moisture treatments. Following other studies, Acidobateriota under irrigation is associated with lower bacterial aggregation, yet an increase in carbon sequestration (Frene et al. 2022; Naylor and Coleman-Derr, 2018). However, the Phylum Acidobacteriota have shown a very heterogeneous response to irrigation, possibly due to its phylogenetic, and metabolic divergence at lower taxonomic ranks(Hartmann et al. 2017). Hartmann et al. (2017) observed that in periods of short drought treatments, the Acidobacteria tended to decrease in abundance, and that this Phylum has been found in irrigated and in non-irrigated soils (Hartmann et al. 2017; Meisner et al. 2018; Naylor and Coleman-Derr, 2018). Not alone, Acidobacteriota has been observed occupying different ecological niches (Hartmann et al. 2017). Many times, this Phylum has been identified as a widespread, drought-tolerant, and

oligotrophic bacteria thriving in nutrient-limited conditions (Hartmann et al. 2017) and is known for carbon cycling (Hartmann et al. 2017), degrading cellulose and lignin (Abdul Rahman et al. 2021), and producing exopolysaccharides (Meisner et al. 2018). The production of EPS facilitates the survival of nearby microbes by creating moist micro-niches under droughted conditions (Meisner et al. 2018). Another factor that stands out about Acidobacteriota is that they are pH sensitive, increasing their relative abundance under low pH and decreasing with an increase in alkalinity (Abdul Rahman et al. 2021); becoming a possible drought-tolerant microbe due to its microbial facilitation mechanism and its low pH growth conditions. Due to the non-consistent results at the phylum level, it may be prudent to look for associations at lower taxonomic ranks for Acidobacteria.

The Actinobacteria results show an increase in OTUs count and relative abundance on the drier treatment (dry and moderate. Actinobacteriota are Gram-positive bacteria positively associated with drought environments due to its fast stress response. The adapted tolerance to moisture-limited soils from Actinobacteriotas might come as life strategies from certain bacterial groups, drought response mechanisms due to host specificity or fast responses to stressors (Evans et al. 2020; Frene et al. 2022; Hartmann et al. 2017; Naylor and Coleman-Derr, 2018; Xie et al. 2021), leading to adaptation to dry environments and conferring tolerance to other hosts such as plants. This Phylum is known to have a dense cell wall (Gram-positive) and tends to have an oligotrophic lifestyle (Frene et al. 2022; Naylor and Coleman-Derr, 2018). They are a widespread Phylum known for producing stress proteins and osmoprotectants conferring stress resistance for themselves and for other hosts, such as plants (Xie et al. 2021). Some groups have filamentous growth (Actinobacteriota), biofilm formation (EPS), production of stress-resistant spore-forming ability (exospores and endospores), and aid in aggregate formation in soils (Abdul Rahman et al.

2021; Frene et al. 2022; Hartmann et al. 2017; Xie et al. 2021). Naylor and Coleman-Derr, (2018) and Hartmann et al. (2017) mention Actinobacteria as having genes for complex carbon degradation, hence, facilitating degradation and utilizing recalcitrant carbon (important for the soil carbon cycle) in dry environments. In drought environments, plant selection has also been observed in many rhizospheric microbial studies (Xie et al. 2021). Actinobacteria is one of the most abundant and enriched Phyla in the rhizosphere, and studies have detected plant growth promotion and alleviation of different stresses such as desiccation, higher temperatures, and salinity (Abdul Rahman et al. 2021; Finkel et al. 2020; Hartmann et al. 2017). For example, they have been associated with impeding dissemination and development of plant pathogens such as *Erwinia amylovora* (fire blight) and *Agrobacterium tumefaciens* (crown gall disease) (Abdul Rahman et al. 2021), providing ammonium fixation (Abdul Rahman et al. 2021; Naylor and Coleman-Derr, 2018), phosphorus mobilization (Naylor and Coleman-Derr, 2018; Vargas Hoyos et al. 2021), degradation of cellulose and lignin, production and regulation of phytohormones (leading to changes in cell division, elongation, and differentiation), antibiotics, vitamins, and amino acids (Abdul Rahman et al. 2021). Streptomycetes have shown plant growth-promoting properties and drought tolerance. They have been shown to reduce reactive oxygen species (ROS), phosphate solubilization, siderophore production, increase dry biomass at different developmental stages in tomato (Hu et al. 2020), produce antimicrobial properties against *Phytophthora palmivora* (Nonthakaew et al. 2022), increase *Z. mays* biomass under normal and saline conditions (Nozari et al. 2021), and confer drought stress tolerance in *Sorghum* plants (Abdul Rahman et al. 2021). Maybe using this Phylum to antagonize *Aspergillus flavus* in peanut settings can be a direction in future studies.

The fungal community had a significant response to the water addition treatments. However, it was difficult to parse strong trends at the phylum level, like the bacteria. Evans et al. (2020) reported that bacterial communities responded more to drought treatments, while fungi responded less. Ascomycota and Basidiomycota are large phyla with physiologically and ecologically diverse species, considering this, it is no surprise to find little divergence across moisture treatments. However, Ascomycota is known to dominate over Basidiomycota in droughted conditions (Hartmann et al. 2017). In other studies, the Eurotiomycetes had a higher abundance under drought (Frene et al. 2022; Meisner et al. 2018). In our study, Eurotiomycetes (Ascomycota) were found in both soils in dry and moderately dry treatments. Eurotiomycetes are known for their diverse capabilities for growing in different environments, at low water activity, high salt concentration, resistant to desiccation and osmotic stress, as a psychrotroph or as thermophilic degrading capabilities, and many are known to be human and plant pathogens (Hartmann et al. 2017). *Penicillium citrinum* is a fungus from this Class that has shown PGPF properties in a broad host of plants. The application of spore solution or fungal filtrate have lead to increased growth in *Atriplex gmelinii* (Gu et al. 2023), and *Oryza sativa* (Khan et al. 2008), tolerate drought and salt stress (Kaur and Saxena, 2023), and has been shown to detain early infection of *A. rolfsii* (Sharma et al., 2021; Waqas et al., 2015).

Aspergillus flavus falls within Eurotiales, has a wide distribution, and is found as a saprotroph in dry and hot environments. Under drought conditions, aflatoxins produced by *A. flavus* tend to contaminate peanut shells and kernels, causing major losses in several economically important crops, including peanuts (Ali et al. 2021; Horn, 2005; Pitt and Hocking, 2009; Tola and Kebede, 2016; Uppala et al. 2013; Xing et al. 2016). We analyzed the relative abundance of this fungus in our peanut soils, and the relative abundance was low, especially for soil A, while soil B

seemed to increase with time. The lack of plants in our experiment, hence, the lack of a rhizosphere, may help explain the above results.

The Dothideomycetes from Phylum Ascomycota in soil A were distributed throughout the treatments, and in soil B the counts are observed in dry treatments. This class was reported to increase in water limitation as observed by Hartmann et al. (2017) and Meisner et al. (2018). Leotiomycetes were found in moderate and moist environments, like Hartmann et al. (2017) where they were found in irrigated soil. Many of them are degraders of organic matter, and maybe vegetation plays an important role in its abundance. Hartmann et al. (2017) found that Agaricomycetes from Basidiomycota were abundant in soil under water limitation. However, in our study, they were distributed across dry, moderate, and moist treatments. At the order level, there is an inclination of several groups to water-limited treatments. For example, Agaricales were only detected at dry treatments, alongside Polyporales and Hymenochaetales shifts between the soils. Agaricomycetes is a big class containing macroscopic mushrooms, decomposers, pathogens, and symbiotic relationships such as ectomycorrhizas (Nehls, 2008) Ectomycorrhizal fungi aid in nutrient and water absorption and help tolerate water limited conditions, thus the detection of Agaricomycetes group can vary on presence or absence of vegetation (Hartmann et al. 2017; Policelli et al. 2020).

Overall, drought poses a significant threat to agriculture and to peanut production. We identified changes in the microbial community associated with changes in soil moisture, which may help pinpoint potential antagonists against problematic fungi like *Aspergillus flavus*, and better understand how microbiomes change under stressful conditions. For example, we identified Actinobacteria as a bacterial phylum that significantly associated with dry conditions. Studying the microbiome at a lower taxon can incite more precise results of the community present, in our

case, in the soils under a moisture regime. Physiological and ecological diversity among groups at the phylum level, especially for fungi, might be missing significant shifts at a lower taxonomic rank.

Table 4. Three-step amplicon library preparation for bacteria and fungi adapted from Noel et al. (2022).

Bacteria		Fungi	
Reagent	**Volume per reaction (uL)**	**Reagent**	**Volume per reaction (uL**
Step 1		**Step 1**	
2X Platinum Green Taq Master Mix(Thermo Fisher,USA)	6.25	2X Dream Taq Green PCR Master Mix (Thermo Fisher,USA)	6.25
10 uM 515F Primer (IDT, USA)	0.375	10 uM ITS 1F Primer (IDT, USA)	0.375
10 uM 806R Primer (IDT, USA)	0.375	10 uM ITS 4 Primer (IDT, USA)	0.375
Bovine Serum Albumin (BSA, 3%)	1	Bovine Serum Albumin (BSA, 3%)	3
GC Enhancer (Thermo Fisher,USA)	2	H2O	1
H2O	1	Extracted DNA	1
Extracted DNA	1		
Step 2		**Step 2**	
2X Platinum Green Taq Master Mix(Thermo Fisher,USA)	6.25	2X Dream Taq Green PCR Master Mix (Thermo Fisher,USA)	6
Primer Frameshift mix 2 uM (IDT, USA)	0.6	Primer2 Mix* 2 uM (IDT, USA)	0.6
Bovine Serum Albumin (BSA, 3%)	0.65	Bovine Serum Albumin (BSA, 3%)	3
GC Enhancer (Thermo Fisher,USA)	2	H2O	0.4
H2O	0.5	Step 1 Product	2
Step 1 Product	2		
Step 3		**Step 3**	
2X Platinum Green Taq Master Mix (Thermo Fisher,USA)	8	2X Dream Taq Green PCR Master Mix (Thermo Fisher,USA)	8
Barcode Forward Primer	0.5	10 uM Forward Primer F	0.5
H2O	1	H2O	1.5
GC Enhancer (Thermo Fisher,USA)	0.5	10 uM Barcode Primers R	1
Unique 10 Nucleotide Barcode	1	Step 2 Product	4
Step 2 Product	4		

*Frameshift Primers ITS 1F and ITS 4 added together

Table 5. Primers used for both bacteria and fungi library preparation adapted from Noel et al. (2022)

	Prokaryote Primer Sequences		Fungal Primer Sequences	
Step 1	Sequence	Primer name	Sequence	Primer name
	GTGCCAGCMGCCGCGGTAA	515F	CTTGGTCATTTAGAGGAAGTAA	ITS 1F
	GGACTACHVGGGTWTCTAAT	806R	AGCCTCCGCTTATTGATATGCTTAART	ITS 4
		Frameshifts (combination of 6)		Frameshifts (combination of 6)
Step 2[a]				
	NNNNNNNN GA GTGCCAGCMGCCGCGGTAA	515F F1	NNNNNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F1
	NNNNTNNNN GA GTGCCAGCMGCCGCGGTAA	515F F2	NNNNTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F2
	NNNNCTNNNN GA GTGCCAGCMGCCGCGGTAA	515F F3	NNNNCTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F3
	NNNNACTNNNN GA GTGCCAGCMGCCGCGGTAA	515F F4	NNNNACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F4
	NNNNGACTNNNN GA GTGCCAGCMGCCGCGGTAA	515F F5	NNNNGACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F5
	NNNNTGACTNNNN GA GTGCCAGCMGCCGCGGTAA	515F F6	NNNNTGACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS 1F F6
	NNNNN AC GGACTACHVGGGTWTCTAAT	806R F1	NNNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F1
	NNTNNN AC GGACTACHVGGGTWTCTAAT	806R F2	NNTNNN AG AGCCTCCGCTTATTGATATGCTTAART	IT4 F2
	NNCTNNN AC GGACTACHVGGGTWTCTAAT	806R F3	NNCTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F3
	NNACTNNN AC GGACTACHVGGGTWTCTAAT	806R F4	NNACTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F4
	NNGACTNNN AC GGACTACHVGGGTWTCTAAT	806R F5	NNGACTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F5
	NNTGACTNNN AC GGACTACHVGGGTWTCTAAT	806R F6	NNTGACTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F6
Step 3				
	AATGATACGGCGACCACCGAGATCTACACGCCTCCCT CGCGCCATCAGAGATGTG	PCR F	AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGC GCCATCAGAGATGTG	PCR F

[a]Framshift primers are used in PCR reactions at an equal molar ratio of forward and reverse primers

Table 6. Library preparation cycling conditions used on the PCR processes for bacteria and for fungi adapted from Noel et al. (2022)

Bacteria								
Step 1			Step 2			Step 3		
Time	Temperature (c)	Cycles	Time	Temperature (c)	Cycles	Time	Temperature (c)	Cycles
5:00	95		5:00	95		5:00	95	
0:30	95		0:30	95		0:30	95	
0:30	50	15X	0:35	50	10X	0:35	63	10X
0:45	72		0:50	72		0:55	72	
7:00	72		7:00	72		7:00	72	
Infinite	10		Infinite	10		Infinite	10	

Fungi								
Step 1			Step 2			Step 3		
Time	Temperature (c)	Cycles	Time	Temperature (c)	Cycles	Time	Temperature (c)	Cycles
5:00	95		5:00	95		5:00	95	
0:30	95		0:30	95		0:30	95	
0:30	50	15X	0:35	50	10X	0:35	63	10X
1:00	72		1:05	72		1:10	72	
7:00	72		7:00	72		7:00	72	
Infinite	10		Infinite	10		Infinite	10	

Table 7.Field collection and soil properties description for soil A and soil B.

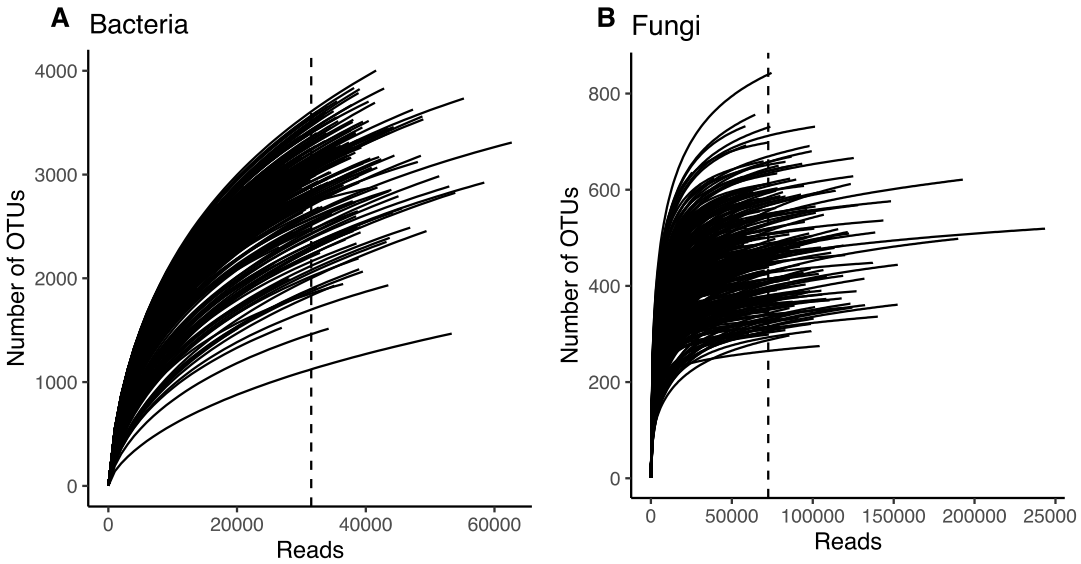
Soils	Year	Coordinates	Sampled	Soil Classification	%Sand	%Silt	%Clay	pH	Organic Matter	Calcium[a]	Phosphorus pounds/acre	Potassium pounds/acre	Magnesium pounds/acre
WGREC (A)	2021	31.373138, -85.317434	29-Jun	Sandy loam	79.38	1.88	18.75	6	1.4	139	41	236	18
WGREC (B)	2021	31.353885, -85.323304	29-Jun	Sandy clay loam	79.38	0.63	20	6	1.9	575	27	112	63

[a]The addition of calcium to the soil is in the form of gypsum.

Table 8. PERMANOVA of bacterial and fungal communities influenced by soil, week, and treatments.

	Factors	[a]Df	[b]R^2	Fvalue	Pvalue
Bacteria	Soil	1	29.00%	132.2	<0.0001
	Week	4	7.70%	8.9	<0.0001
	Treatment	4	3.15%	3.6	<0.0001
Fungi	Soil	1	16.40%	65.1	<0.0001
	Week	4	9.50%	9.5	<0.0001
	Treatment	4	1.80%	1.8	0.0057

[a]Degree of freedom; [b]Coefficient of determination



1415 Figure 6. Rarefaction curve of A) bacterial samples, and B) fungal samples. The dash line
1416 represents the median read depth (fungal: 72,526 and bacteria 31,517 reads).

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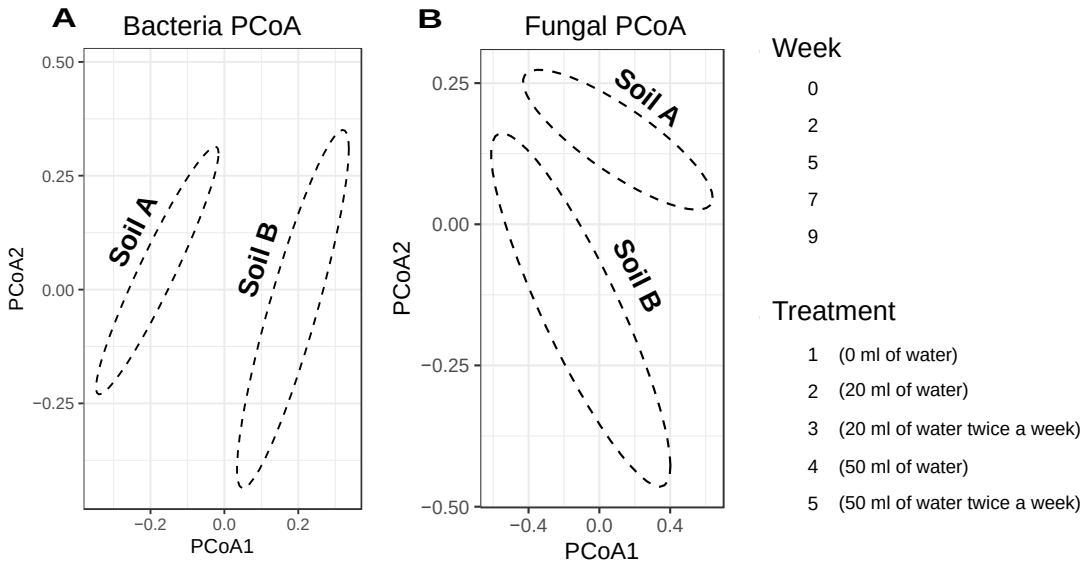

Figure 7. Principal Coordinates Analysis for a) bacteria and b) fungal soils. Two big clusters in both fungal ($p < 0.0001$) and bacterial ($p < 0.0001$). The color is represented by the week and the shapes as the moisture treatments. 95% confidence ellipses shown for soil type (soil A and B).

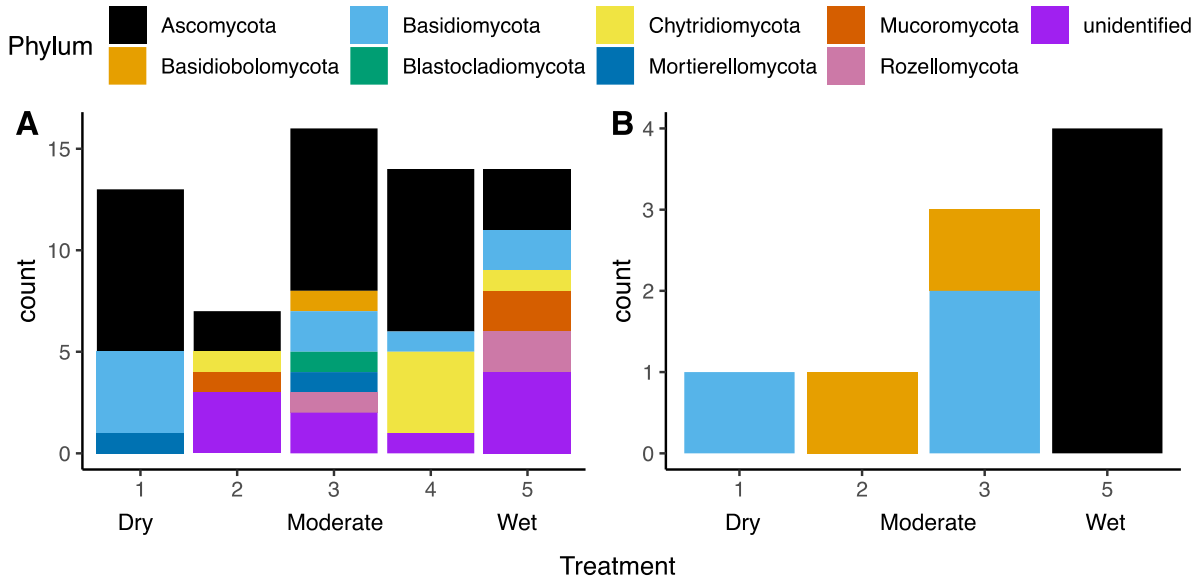

Figure 8. Fungal indicator species analysis at OTUs level ($p < 0.01$) on peanut (**A**) soils A and (**B**) soil B through the treatments from dry to moist conditions.

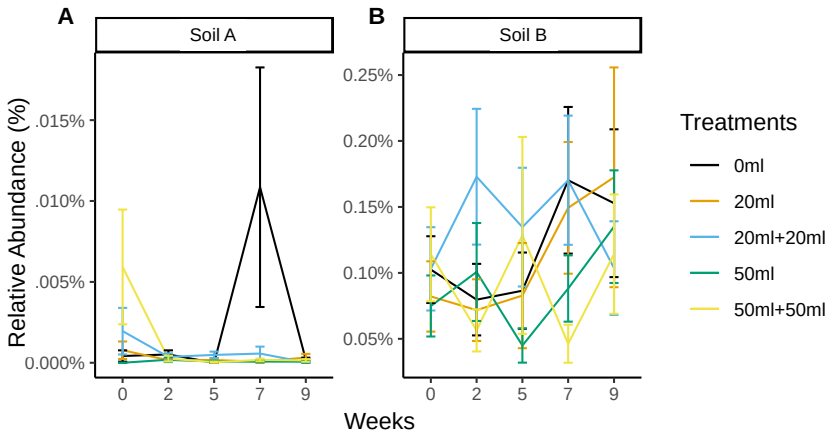

Figure 9. Relative abundance of *Aspergillus flavus* in peanut soil A (A) and soil B (B). Treatments gradient from dry to moist condition represented by color.

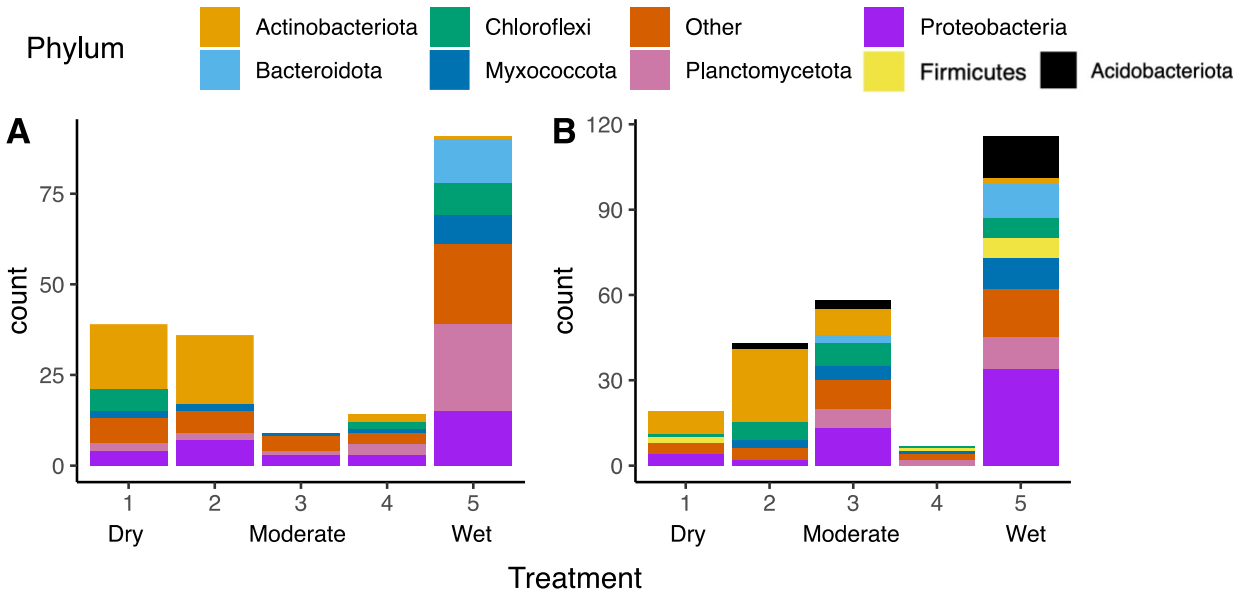

Figure 10. Bacterial indicator species analysis at OTUs level ($p < 0.01$) on peanut **A**) soils A and **B**) soil B.

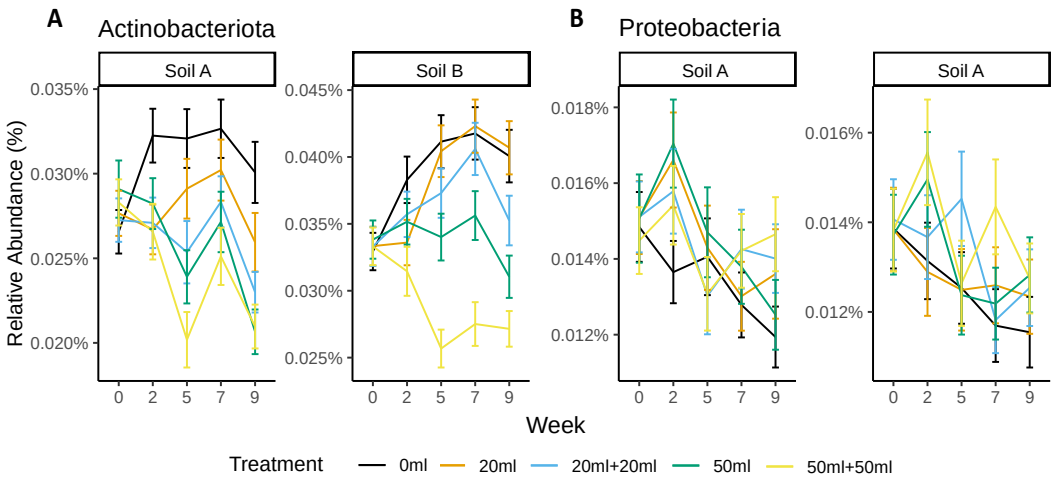

Figure 11. Relative abundance over time of A) Actinobacteria and B) Proteobacteria over time in soil A and soil B.

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4. CHAPTER 4

Conclusions and Impacts

Conclusion

Given the economically influential crops such as peanuts and the risk of mycotoxin accumulation in peanuts under drought, the main objective of this thesis was to 1) determine if fungi could alleviate water stress in peanut plants (Chapter 2), and 2) determine changes in the microbial communities of peanut soils under a moisture regime (Chapter 3). Several fungal species used were promising in increasing or maintaining plant biomass under drought conditions and in non-drought (*M. calciphila* OEO-304, *L. elongata* OEO-196, *L. elongata* OEO-198). Especially *L. elongata* OEO-196, which increased shoot biomass in peanut seedlings under non-droughted conditions, and the ability to improve root biomass under water-stressed conditions compared to droughted peanuts without fungal inoculation. Additionally, *M. calciphila* OEO-304 grew faster at higher temperatures and improved biomass when peanuts were under stress but trended to decrease plant biomass under non-water-stressed conditions. However, more study is needed to understand the mode of interaction of *Linnemannia* and *Mortierella* species with plants. With these results, future studies can focus on the fungi from the sub-phylum Mortierellomycotina and potentially expose more mature peanuts to chronic drought stress.

In this second study, we wanted to observe how restriction and implementation of water alter the fungal and bacterial communities on two peanut soils collected at Headland, AL. We hypothesized that applying different water regimes to peanut soils will alter the bacterial and fungal communities over time and reveal target microbes that thrive under dry conditions. The soil used for this study was geographically similar, but their microbial communities were significantly different. The moisture gradient and time also impacted the microbial community structure.

Especially for bacteria, where we detected some groups that clearly thrive in certain environments. We did observe higher bacteria taxa associated with moist treatments than drier ones. However, the fungal communities resulted in fewer changes throughout the study and less clear results over the water treatments. Maybe extending the experiment duration can give us a better idea of how the fugal microbiome is changing with the treatments. Regarding *Aspergillus flavus*, measuring its DNA via qPCR through the nine-week experiment may be more precise in tracking its abundance and relate to our sequence reads.

Impacts

This study has presented at the Alabama-Florida Peanut Trade Show 2023, Auburn University College of Agriculture research symposium on year 2021 and 2022, and at the American Phytopathological Society Southern Division 2021 and 2022. The objective of exposing this study to the scientific community and the general audience is to increase knowledge and spread awareness of present problems that Alabama is facing in agricultural settings, and possible solutions. More research is needed but this study's results are showing a promising trend in the use of microbes, in this case fungi, that can help maintain plant fitness under water limitation stress. Additionally, studying the soil microbiome under drought can give a better understanding of how microbial communities transition, which can lead to detecting species that thrives in dry and hot conditions and has growth-promoting properties to antagonize plant pathogen and alleviate stress symptoms.