Fungal endophyte presence in relation to various nutrient regimes applied to *Gossypium hirsutum*

by

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Abstract

Proper nutrient conditions are necessary for optimal growth and yield of *Gossypium hirsutum*. This can be achieved through fertilizer application, but plants may also be assisted by fungal endophytes, potentially beneficial microorganisms in the tissue of plants. In the Southeastern United States, Cotton leafroll dwarf virus (CLRDV) is a concern for cotton producers; however, a potential management strategy is the use of fungal endophytes. Experiments in 2022 and 2023 evaluated culturable fungal endophytes from the foliage of *G. hirsutum* growing in various nutrient regimes at the Cullars Rotation (Auburn, AL), where the soil fertility treatments have been in place for over 100 years. The isolated genera included *Alternaria*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Phoma*, and *Stemphylium*. In 2022, the relative abundance of *Colletotrichum truncatum* was around 50% in the no potassium regime, *Diaporthe* sp. abundance was over 50%, in the no phosphorous regime, and *Alternaria* abundance was around 25% in all sampled regimes except the no potassium regime. The only endophytic species isolated from the no potassium regime in 2023 were *Stemphylium vesicarium* and *S. solani.* Relative to endophyte diversity, a significant difference ($P \leq 0.05$) was observed in 2023 between the no potassium regime and all other sampled regimes. Incidence of CLRDV was determined in the Fall of 2023 to observe whether infection differs among *G. hirsutum* treated with various nutrient regimes or if it relates to endophytic presence. The results were insufficient to further explore a relationship between CLRDV, endophytes, and nutrients. The fungal endophyte microbiome in *G. hirsutum* leaf tissue was analyzed in 2023. In relation to fungal endophyte diversity, the no potassium regime was significantly different ($P \leq 0.05$) from all other sampled regimes. The relative abundance of *Stemphylium* was over 50% of the population

in the no potassium regime. *Diaporthe* abundance was high, greater than 50% of the community composition in the no phosphorous regime. *Stemphylium* spp. and *Colletotrichum truncatum* were identified as indicator species, signifying differences in the community based on the applied nutrient regime. The findings of this research indicate that fungal endophyte presence may be impacted by nutrient regimes applied to *G. hirsutum*.

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I. Literature Review

Cotton, *Gossypium* spp., is an important agricultural crop in the Malvaceae family that grows in tropical and subtropical regions (Chaudhry and Guitchounts 2003). The highest producing countries are China, India, and the United States, respectively. The United States produces 3,553 metric tons annually; the top producing states are Arizona, California, Florida, Mississippi, and Texas (Aslam et al. 2020). Only four species of cotton are grown commercially: *Gossypium arboreum, G. herbaceum, G. barbadense, and G. hirsutum* (Chaudhry and Guitchounts 2003). *Gossypium arboreum* and *G. herbaceum* are diploid Old World cottons that originated from regions in Asia and Africa, respectively. *Gossypium barbadense* is native to Peru and *G. hirsutum* to Mexico. Both are tetraploid New World cottons (Chaudhry and Guitchounts 2003).

Out of the four species, *G. hirsutum*, commonly known as upland cotton, is the most widely planted, accounting for 90% of the world's cotton production (Aslam et al. 2020). As of 2023, 380,000 hectares of upland cotton were planted in Alabama with 374,000 hectares harvested. A total of 730,000 217 kilogram bales were produced, yielding 425 kg/hectare. They were sold at $0.683/kg for a total of $234,406,000 in production in Alabama (NASS 2023). *Gossypium* has a perennial growth habit (Rehman and Farooq 2005) but is grown as an annual shrub that reaches around 1.2 meters in height (Aslam et al. 2020). The growing season for *G. hirsutum* is between 150 to 180 days (Dugan 2009) and extends from April or May into September or October based on the region (Rehman and Farooq 2005). Development of *G. hirsutum* occurs in two general stages: vegetative and reproductive (Rehman and Farooq 2019). Vegetative development is the first stage and involves germination, root development, and leaf

and canopy development. Reproductive development occurs subsequently and represents the production of flowers, bolls, seeds, and fiber (Rehman and Farooq 2019).

Nutrient deficiencies in *Gossypium* species can be a result of soil properties, environmental conditions, and grower application error. The most important nutrient for production in cotton and in all plants is nitrogen (Sawan 2013). This is due to its requirement in photosynthesis, plant growth, and the prevention of square and boll abscission (Sawan 2013). Nitrogen is most heavily required during boll filling (Malik 1998). Deficiency symptoms will present as stunted growth and detachment of bolls, but the main symptom is chlorosis because of the plant's need for nitrogen during photosynthesis (Khan et al. 2013).

Phosphorous contributes to increased root morphology as well as reproductive growth and yield (Iqbal et al. 2020). A phosphorous deficient plant will have purple leaf pigmentation, small, dark green leaves, decreased leaf development, and poor yield (Xiao and Yin 2020). Cotton requires phosphorus throughout the growing season (Malik 1998). Phosphorous mobility in soil is a limiting factor with regard to nutrient uptake, meaning *Gossypium* heavily relies on its roots for absorption (Malik 1998).

Second to nitrogen, potassium requirements are high for optimal growth and yield of *Gossypium* (Oosterhuis et al. 2013). Potassium is used for boll development as well as fiber quality (Fang et al. 2016). Due to the high uptake rate and poor ability of cotton to absorb potassium, a continuous supply is needed during the growing season (Makhdum et al. 2007). However, potassium uptake reaches its peak a few weeks after flowering begins (Oosterhuis 2002). Potassium deficiency in *Gossypium* occurs more often and is more extreme than in most agricultural crops (Oosterhuis 2002). Symptoms of deficiency have even been seen in *Gossypium* planted in soil not considered deficient (Oosterhuis et al. 2013). Potassium deficiency begins in

mature leaves and progresses to the younger leaves (Xiao and Yin 2020). The initial indication of deficiency is yellowing along the veins and margins of the leaves. This is followed by browning and curling of the leaf tips before leaf detachment from the plant (Xiao and Yin 2020).

While identification of nutrient deficiencies in *G. hirsutum* is possible through symptom recognition, certain plant diseases exhibit similar characteristics, leading to potential misidentification. Exemplifying this is Cotton leafroll dwarf virus (CLRDV), an aphid vectored virus within the genus *Polerovirus* affecting *Gossypium* throughout the Southeastern United States (Ramos-Sobrinho et al. 2021). The first documented instance of CLRDV infection in the United States occurred in Alabama in 2017 and has since been confirmed in neighboring states (Edula et al. 2023). CLRDV is a global issue, and was previously reported in Africa, Asia, and South America before being identified in the United States (Tabassum et al. 2021).

Cotton leafroll dwarf virus symptom presentation is highly variable and impacted by the growth stage of the plant. In the early growing season, stunting and leaf distortion are frequently observed (Edula et a. 2023). As the growing season progresses, symptoms are influenced by environmental conditions and plant infection age (Edula et al. 2023). Plants infected within 50 days post emergence will generally present symptoms such as maroon petioles and leaves, downward leaf drooping, and leaf puckering. In plants infected later in the season, wilting, leaf crinkling, and more vegetative growth in the upper areas of the plant are commonly observed (Edula et al. 2023). Management of CLRDV is feasible through integrated approaches such as weed and voluntary stalk removal (Edula et al. 2023). A potential strategy to mediate CLRDV infection in *Gossypium* species is through the use of fungal endophytes (Grabka et al. 2022). Fungal endophytes have the ability to synthesize secondary metabolites that may suppress the growth and development of plant pathogens (Gao et al. 2010). In particular, alkaloids have been

observed to reduce aphid-transmitted plant pathogen infection by inhibiting aphid reproduction and plant ingestion (Rua et al. 2013).

Rua et al. (2013) conducted a greenhouse experiment to study the use of the fungal endophyte species *Neotyphodium coenophialum* against Barley yellow dwarf virus – PAV (BYDV) in two cultivars of tall fescue grass, Kentucky 31 and Texoma. The two cultivars were either infected with the chosen endophytic species or remained uninfected and used as control plants. Plants were then inoculated with BYDV through aphid feeding and monitored before being harvested for analysis. Researchers found that endophyte infection reduced total aphid numbers as well as aphid reproduction in both Kentucky 31 and Texoma. These results support the idea that fungal endophytes have the potential to protect their host plant against aphid vectored viruses.

Endophytes are symbiotic microorganisms colonizing the interior tissue of plants that have the ability to protect against biotic and abiotic stressors such as drought, temperature fluctuations, salinity, and plant pathogens (Baron and Rigobelo 2021). They also demonstrate the potential to enhance plant growth and nutrient uptake (Baron and Rigobelo 2021). To distinguish between epiphytes, microorganisms that live on the surface of a plant, it is thought that endophytes cannot be washed off the surface of a plant or inactivated with surface sterilization (Porras-Alfaro and Bayman 2011). The term ‘endophyte’ originates from work by Anton De Bary in 1886, describing them as fungal species that live inside host tissue (Kumari et al. 2022). Ascomycota is the dominant phylum of fungal endophytes, and a few of the most common genera include *Alternaria, Cladosporium, Epicoccum, Fusarium, Phoma,* and *Pleospora* (Aghdam and Brown 2021).

Fungal endophytes colonize their host plant by way of horizontal transmission and vertical transmission (Bard et al. 2024). The process of horizontal transmission involves the spread of endophytes by way of the environment, such as the soil, rain, or air (Bard et al. 2024). One of the main factors that effects endophyte frequency is weather (Grabka et al. 2022). An increase in precipitation has been observed to increase the occurrence of horizontally transmitted endophytes (Grabka et al. 2022). This is because moisture can be important for host colonization and spore dispersal (Grabka et al. 2022). Endophytes in the phyllosphere access the plant through epidermal tissue such as the stomata (Bashir et al. 2022). Rhizosphere inhabiting endophytes enter the plant through the roots (Chaturvedi 2022). Endophytic colonization is also sensitive to plant age and can vary in plants of the same species depending on their health (Baron and Rigobelo 2021). Vertical transmission is from the seed to the plant and occurs during seed germination (Samreen et al. 2021). This method of transmission is most often seen in grass species (Grabka et al. 2022). A study on endophyte colonization in above and below ground tissue of *Aristolochia chilensis* found that *Cladosporium, Fusarium, Meyerozyma, Penicillium, Preussia, Talaromyces,* and *Trichoderma* were isolated from both the leaves and roots (Guevara-Araya et al. 2020).

Isolation of fungal endophytes can be done through surface sterilization of plant material followed by cultivation on growth medium (Porras-Alfaro et al. 2011). Polymerase Chain Reaction (PCR) is a commonly used molecular analysis method to study fungal endophytes (Reis et al. 2022) The technique involves the amplification of the 5.8 gene, ITS1, and ITS2 rDNA regions of pure cultured isolates using primers such as ITS 1F and ITS4 (Ek-Ramos et al. 2013). Sample sequencing can be done using Sanger sequencing, and online databases, such as the NCBI (National Center for Biotechnology Information) BLASTn tool (Basic Local Alignment

Search Tool) may be used for fungal identification (Reis et al. 2022). Ek-Ramos et al. (2013) studied fungal endophyte variation in *G. hirsutum* using cultured isolates and found that *Alternaria*, *Fusarium*, and *Phoma* were the most commonly isolated species. Other fungal endophytes species observed were *Colletotrichum* and *Epicoccum*.

The endophytic microbiome is comprised of fungal communities inhabiting the interior tissue of a plant. Its diversity may be influenced by factors such as environmental conditions, host plant species, and nutrient availability (Porras-Alfaro and Bayman 2011). These factors also play a role in an endophyte's lack of pathogenicity. Their nonpathogenic, or asymptomatic, nature can be attributed to loss of virulence or a prolonged latency period (Ek-Ramos et al. 2013). Latent pathogens may become active during a state of stress, conducive environmental conditions, or due to host age (Bamisile et al. 2018). However, endophytes tend to stay in an asymptomatic state (Faeth and Fagan 2002). Some species of *Fusarium* have the potential to exhibit both endophytic and pathogenic phases during their lifecycle (Padhi et al. 2015).

Assessing a plant's endophytic fungal microbiome can be done without culturing, instead using direct plant samples (Reis et al. 2022). Surface sterilization is required to remove any unwanted epiphytic microbiota. This is followed by DNA extraction, PCR (Reis et al. 2022), and a next generation sequencing (NGS) platform such as Illumina (Satam et al. 2023). Unlike Sanger sequencing, which sequences individual DNA fragments for analysis, NGS sequences millions of DNA fragments (Satam et al. 2023). This method also provides information on culture-dependent and independent fungal endophytes, while Sanger sequencing is culture dependent only (Reis et al. 2022).

There are four classes of endophytes, categorized as clavicipitaceous or non-clavicipitaceous, location within a plant, and transmission method (Sravani et al. 2022).

Clavicipitaceous endophytes are classified within the Phylum Ascomycota and are found only in grasses (Kuldau and Bacon 2008), while non-clavicipitaceous endophytes are within the Phylum Ascomycota, Basidiomycota, or Mucoromycota and inhabit vascular and non-vascular plants (Rashmi et al. 2019).

Class one endophytes are clavicipitaceous fungi with a narrow host range consisting of cool and warm season grasses (Rodriguez et al. 2009). They are present in the shoots of their host plant and are transmitted horizontally and vertically (Sravani et al. 2020). Benefits from infection include an increase in drought tolerance and plant growth (Rodriguez et al. 2009).

Class two endophytes are non-clavicipitaceous fungi, predominantly from Phylum Ascomycota, that have a broad host range (Sravani et al. 2020). They inhabit both the above and below ground plant tissue and are transmitted vertically as well as horizontally (Rodriguez et al. 2009). Class two endophytes are found to be at an elevated level of colonization in plants exposed to stress (Rodriguez et al. 2009). Several genera include *Phoma* (Rodriguez et al. 2009), *Penicillium, Aspergillus, Fusarium, Colletotrichum,* and *Beauveria* (Baron and Rigobelo 2021).

Class three endophytes are non-clavicipitaceous fungi found in the shoots of their host plant. They are primarily from the Phylum Ascomycota and their method of transmission is horizontal (Rodriguez et al. 2009). While they have a broad host range, they form localized communities in their host plant (Sravani et al. 2020). Class two and class three endophytes contain some of same endophytic genera, but this is dependent on environmental conditions, host, and mode of transmission (Baron and Rigobelo 2021). Class four endophytes, non-clavicipitaceous fungi found in the roots of their host plant, are also known as ‘dark septate endophytes’ (Rodriguez et al. 2009). This is due to their brown/black coloration (Rodriguez et al.

2009). They have a broad host range and are only transmitted horizontally. They provide benefits such as increased plant growth and production of secondary metabolites (Sravani et al. 2020).

Fungal endophytes can positively impact plant growth by increasing nutrient accessibility (Sarkar et al. 2021). In turn, this affects the host's root growth, plant size, and biomass production (Rana et al. 2020). The incorporation of fungal endophytes as biofertilizers can help supply plants with nutrients necessary for enhanced growth and yield (Fasusi et al. 2021). Biofertilizers are products containing microorganisms that improve nutrient uptake and development of plants. They can be applied in a liquid or granular form (Vassilev et al. 2022). Quality control of biofertilizers is necessary to prevent microorganisms from becoming pathogenic (Agarwal et al. 2021). This can be implemented by understanding the microorganism's bio-properties, such as mode of action, natural occurrence, and environmental effect (Vassilev et al. 2022). Microscopy, culturing, molecular analysis, and proper regulation are also used for quality control (Vassilev et al. 2022). A better understanding of the roles of endophytic fungi in plants in relation to nutrient regimes will better assist the effectiveness of biofertilizers for grower application.

One endophytic mechanism to increase nutrient uptake is the production of phytohormones, such as auxin, gibberellin and cytokinin (Baron and Rigobelo 2021). Phytohormones promote plant growth; plants with a larger surface area, especially of roots, are able to take up a larger quantity of nutrients (Sabagh et al. 2022). Auxin contributes to shoot and root development, gibberellin aids in fruit formation, senescence, stem elongation, and seed development (Baron and Rigobelo 2021), and cytokinin promotes cell division and impacts seed dormancy and apical dominance (Rana et al. 2020). Khan et al. (2015) studied the effects of *Fusarium tricinctum* and *Alternaria alternaria* isolates inoculated in Dongjin rice plants. The

study found that inoculated plants exhibited an increase biomass, root length, and shoot length. This may be attributed to the endophyte's ability to produce indole-3-acetic acid, which was observed in an in vitro experiment.

Phosphorus is one of the three macronutrients necessary for plant growth. While it is found in large quantities in the soil, most forms are insoluble (Fadiji and Babalola 2020). Research has shown an increase in phosphorous and even nitrogen content in the shoots and roots of endophyte inoculated plants (Sarkar et al. 2021). Endophytes use enzymes, such as phosphatase, to solubilize organic phosphorus into inorganic phosphorus (Rana et al. 2020). *Aspergillus* and *Penicillium* have been identified as some of the most prevalent phosphorous solubilizing endophytes, but others include *Curvularia* and *Piriformospora* (Mehta et al. 2019). The application of *Penicillium pinophilum, Aspergillus niger*, and *A. fumigatus* suspensions to faba bean plants grown in phosphorous treated soil was found to increase plant yield as well as phosphorous uptake. Specifically, *P. pinophilum* had the greatest solubilization ability of the three endophytes during an in vitro analysis (Wahid and Mehana 2000). Another endophyte with the ability to solubilize phosphorous is *Epichloe* (Soto-Barajas et al. 2015). This endophyte is found only in the above ground tissue of plants (Soto-Barajas et al. 2015). In a study by Malinowski et al. (1998), a release of compounds with phosphorous solubilizing capability was observed from roots of tall fescue plants infected with *Epichloe coenophiala.*

Nutrient transfer is an example of symbiosis between an endophyte and its host plant (Garcia-Latorre et al. 2021). Plant nutrient uptake is primarily through the roots, so dark septate fungi, class four endophytes, are typically the main facilitators (Sarkar et al. 2021). The plant provides carbohydrates to the endophytes, and in return the microbes aid in nutrient absorption (Garcia-Latorre et al. 2021). However, endophytes inhabiting both root and foliar tissue and even

foliar tissue only can also assist with nutrient transfer; these include *Epichloe*, *Colletotrichum*, and *Phomopsis* (Sarkar et al. 2021).

The effect of *Phomopsis liquidambari* inoculated in *Oryza sativa* undergoing various levels of nitrogen application was studied by Li et al. (2017). Plants infected with *P. liquidambari* demonstrated a significant increase in shoot, root, and biomass under conditions of low nitrogen. No significant differences were seen between middle and high levels of nitrogen in endophyte infected plants. In comparison to uninfected plants, endophyte infected plants showed an increase of auxin and cytokinin content in their roots and shoots under low nitrogen conditions. Ethylene content was also seen to increase in endophyte infected plants. Auxin has the ability to regulate nitrogen signals from the shoots to the roots of plants. Based on the experimental results, it was hypothesized that *P. liquisambari* may adjust the phytohormone concentration as a method of improving *O. sativa* nitrogen uptake.

Oono et al. (2020) studied the correlation between fungal endophyte diversity and nutrient availability in a natural environment. Nutrient content of leaves from two species, *Pinus muricata*, the Bishop Pine, and *Vaccinium ovatum*, the California Huckleberry, were analyzed. Fungal endophyte colonization was determined through culturing and sequencing. For both plant species, the results depicted an increase in fungal endophyte diversity in plant leaves containing a smaller nitrogen-to-phosphorus ratio and a decrease in endophytic presence in plant leaves with a higher ratio. The optimal nitrogen to phosphorous ratio for most plants is 15:1 (Luo et al. 2016). The results of this experiment indicate that nutrient stress may impact endophyte colonization in a plant.

Further research is necessary to determine the relationship between fungal endophytes and *G. hirsutum* in relation to nutrients. The goals of this study are to assess the impact of fungal

endophytes in the foliage of *G. hirsutum* growing in various nutrient regimes as well whether CLRDV infection varies among *G. hirsutum* plots with varying nutrient regimes or relates to endophytic presence. Methods of evaluation include lab and field trials as well as molecular analysis to determine endophytes present in cotton treated with varying nutrient regimes.

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II. Presence of fungal endophytes and Cotton Leafroll Dwarf Virus Incidence in the foliage of *Gossypium hirsutum* grown under various nutrient regimes

Introduction

Fungal endophytes are potentially beneficial microorganisms that colonize the interior tissue of plants (Baron and Rigobelo 2021). Endophytes enter their host through horizontal or vertical transmission (Bard et al. 2024). During horizontal transmission, spores are spread from the environment by factors such as air, rain, and soil (Bard et al. 2024). Vertical transmission is from the seed to the plant (Samreen et al. 2021). Endophytes have the ability to protect their host plant against biotic and abiotic stressors, such as drought, temperature fluctuations, salinity, and plant pathogens (Baron and Rigobelo 2021). They can also potentially enhance plant growth and nutrient uptake (Baron and Rigobelo 2021). Endophytes are able to produce phytohormones, secondary metabolites that promote plant growth (Sabagh et al. 2022). These include, but are not limited to, auxin, gibberellin, and cytokinin (Baron and Rigobelo 2021). Auxin contributes to shoot and root development, gibberellin assists with elongation and seed development (Baron and Rigobelo 2021), and cytokinin promotes cell division (Rana et al. 2020). An example of fungal endophyte's role in improving nutrient uptake can be seen with phosphorous solubilization (Rana et al. 2020). While phosphorous is found in large quantities in the soil, most forms are insoluble (Fadiji and Babalola 2020). To mediate this, endophytes use enzymes, such as phosphatase, to mineralize organic phosphorus into inorganic phosphorus (Rana et al. 2020).

Nutrient stress can reduce the growth and production of *Gossypium* spp., such that it is necessary to maintain the plant's specific nutrient requirements. Nitrogen is the most important nutrient for cotton production due to its role in photosynthesis, plant growth, and the prevention

of square and boll abscission (Sawan 2013). The main symptom of nitrogen deficiency is chlorosis. Other symptoms include stunted growth and boll detachment (Khan et al. 2013). Phosphorous is needed by the plant for reproductive growth and yield (Iqbal et al. 2020). A phosphorous deficiency presents as purple leaf pigmentation, small, dark green leaves, decreased leaf development, and poor yield (Xiao and Yin 2020). Potassium is needed for boll development and optimal fiber quality (Fang et al. 2016). Potassium deficiency in *Gossypium* occurs more often and is more extreme than in most agricultural crops (Oosterhuis 2002). The first symptom of potassium deficiency is yellowing along the leaf veins and margins followed by browning and curling of the leaf tips (Xiao and Yin 2020). According to the National Agricultural Statistics Service, an average of 29 kilograms of nitrogen per acre, 23 kilograms of phosphorous per acre, and 34 kilograms of potassium per acre were applied to cotton fields across Alabama 2021 (NASS 2021).

Cotton leafroll dwarf virus (CLRDV) is an aphid vectored virus in the genus *Polerovirus* affecting *Gossypium* spp. throughout the Southeastern United States (Ramos-Sobrinho et al. 2021). This disease was first observed in Alabama in 2017 and has since been found in other cotton producing areas in the United States (Edula et al. 2023). Symptom manifestation of CLRDV is related to environmental conditions and age at plant infection (Edula et al. 2023). These include downward leaf drooping, maroon petioles and leaves, and wilting (Edula et al. 2023).

Application of integrated approaches is a possible management strategy for CLRDV. These practices include weed and voluntary stalk removal (Edula et al. 2023). Another potential management strategy is through the use of fungal endophytes that incite plant defense mechanisms (Grabka et al. 2022). Fungal endophytes also synthesize secondary metabolites that

may suppress the growth and developmental process of plant pathogens (Gao et al. 2010). For example, secondary metabolites, such as alkaloids, have been observed to reduce aphid-transmitted plant pathogen infection by inhibiting aphid reproduction and plant ingestion (Rua et al. 2013).

Exploring the relationship between fungal endophytic genera and *G. hirsutum* in relation to nutrient regimes is important to further improve the growth and yield of the crop. Research can also aid in improving efficient use of nutrients in the plant that work alongside common practices of nutrient application. The experimental hypothesis is that the number of fungal endophyte isolates will be higher in plants experiencing nutrient deficiencies and fungal diversity will vary based on nutrient regimes. The first objective of this work was to observe the diversity and number of endophytic isolates in foliage of *G. hirsutum* growing in varying nutrient regimes. The second objective was to establish whether CLRDV infection differs among *G. hirsutum* plots with varying nutrient regimes or relates to endophytic presence.

Materials and Methods

Gossypium hirsutum Sampling 2022

Sample collection of *G. hirsutum* foliage took place at the Cullars Rotation (circa 1911) in Auburn, Alabama. The experimental design of the rotation allows researchers to observe the long-term effects of specific nutrient deficiencies in corn, cotton, wheat, and soybean (Mitchell et al. 2012). The plots include 14 nutrient regimes with crops on a three-year rotation (Mitchell et al. 2012). The selected experimental plots for this study were Plot A: no nitrogen + winter legumes, Plot B: no nitrogen and no winter legumes, Plot 1: nitrogen and no winter legumes, Plot

2: no phosphorous, Plot 3: NPK, Plot 6: no potassium, and Plot 10: NPK + micronutrients (Table 1).

Sample collection occurred in September of 2022 during the cotton's cut-out growth stage. Samples were selected arbitrarily and between four and six leaf and petiole cuttings of cotton plants were gathered from each plot. Samples were placed on ice and transported to the laboratory for sterilization and endophyte isolation. Leaves were surface sterilized by soaking in a 10% bleach solution (0.75% sodium hypochlorite) for fifteen minutes. Leaves were then cut into pieces and washed in a three-part sterile water bath for thirty seconds per beaker. Samples were divided into smaller pieces and placed onto acidified potato dextrose agar. Fungal growth from plant tissue was subcultured onto new petri dishes with media after 6 to 8 days of growth to obtain pure isolates.

Gossypium hirsutum Sampling 2023

The sampling process was repeated in September of 2023 with minor changes to the protocol. The selected plots were Plot B: no nitrogen and no winter legumes, Plot C: no soil amendments, Plot 2: no phosphorous, Plot 3: NPK, and Plot 6: no potassium (Table 1). The addition of Plot C and removal of Plot A, Plot 1, and Plot 10 allowed a focus on the three major macronutrients without additional factors such as winter legumes and micronutrients. Ten leaves were collected from each plot. Samples were placed on ice and transported to the laboratory for fungal culture. Subsamples were taken to the Agricultural Services and Research Building for Cotton leafroll dwarf virus testing. The sterilization and culturing procedures followed the same protocol as with the 2022 samples. Petri dishes were left at 24°C in the dark for 7 days, and any fungal growth that occurred during that time was recultured onto fresh media plates.

Fungal Endophyte Identification

Fungal hyphae were scraped from petri dishes and transferred to VWR® PCR 8-strip tubes for DNA extraction. The protocol was adapted from methods in Noel et al. (2021). Each tube contained 20 µl of Extraction Solution (ES). The tubes were incubated at room temperature for 10 minutes and then incubated in a thermocycler for 10 minutes at 95 °C (Noel et al. 2021). This process created a DNA template that would be used for PCR amplification of the internal transcribed spacer region (ITS). Once incubation was completed, 60 µl bovine serum albumin (BSA 3%) was pipetted into each tube. Each DNA template was then pipetted into new 8-strip tubes that contained ITS1 (forward primer) (Table 2.2), ITS4 (reverse primer) (Table 2.2), water, and DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Waltham, Massachusetts). The tubes were placed in the thermocycler for 1 minute at 94°C, 30 seconds at 94°C, 30 seconds at 50°C, one minute at 72°C, 8 minutes at 72°C, and 10°C for holding.

The PCR products were analyzed using gel electrophoresis and cleaned by combining 5 µl of PCR product and 5 µl Exo-AP Master Mix to sterile PCR 8-strip tubes. The Exo-AP Master Mix working concentration contained 270 µl double distilled water and four components from New England Biolabs (Ipswich, MA): 40 µl Antarctic phosphatase, 50 µl Exonuclease I, 70 µl Antarctic Phosphatase Buffer, and 170 µl Exonuclease I reaction buffer. The cleaned products and an ITS1F primer were sent to Eurofins (Louisville, KY) for Sanger Sequencing. The sequenced DNA were trimmed using Geneious Prime. The National Center for Biotechnology Information's BLASTn program was used to compare the nucleotide sequences against similar sequences in the database to identify the fungal genera. In 2022, a total of forty-one fungal endophytes were identified, and in 2023 there was a total of ninety-eight fungal endophytes.

Data were analyzed using R(Version 4.3.2). Least square means (LSMeans) were determined, and the significance between nutrient regimes relative to endophyte diversity was observed. Pairwise comparisons of the LSMeans were conducted using the 'emmeans' package v.1.10.0.90001. The 'Kruskal-Wallis test' package v.0.6.0 was used to determine significance between number of isolates in relation to nutrient regime. The relative abundance of genera among various nutrient regimes was also determined.

Results

Fungal Endophyte Results 2022

The pairwise comparisons showed no significant difference between nutrient regimes relative to endophyte diversity. The 'Kruskal-Wallis test' showed no significant difference between nutrient regimes relative to number of isolates. Data were analyzed to determine relative abundance of genera in relation to various nutrient regimes (Figure 2.1). Isolated genera belong to either class two or class three endophyte groups based on location in the plant. *Diaporthe* and *Alternaria* were isolated from all sampled regimes except the no potassium regime. *Diaporthe*'s abundance was significant, over 50% of the population in the no phosphorous regime, and *Alternaria*'s relative abundance was observed to be around 25% in all regimes. *Colletotrichum truncatum* was only isolated from the no potassium regime, and the relative abundance was observed to be over 50% of the community. *Stemphylium solani* was isolated from the NPK regime, with a relative abundance around 15%, and the NPK + micronutrients regime, with a relative abundance of over 50%.

Fungal Endophyte Results 2023

Several fungal genera isolated in 2023 were previously isolated in 2022. A portion of these include *Alternaria*, *Colletotrichum*, *Diaporthe*, *Epicoccum*, and *Fusarium*. Pairwise comparisons showed that the no potassium regime was significantly different ($P \leq 0.05$) from the NPK regime, the unamended regime, the no nitrogen regime, and the no phosphorous regime in relation to endophyte diversity. The 'Kushal-Wallis test' showed no significant difference between nutrient regimes relative to number of isolates. The relative abundance of fungal endophytic genera in 2023 was determined to show the distribution of genera among various nutrient regimes (Figure 2.2). *Colletotrichum* sp. was only isolated from the unamended regime, with a relative abundance of less than 10%. *Alternaria* relative abundance was not significant, and the endophyte was isolated from only the no nitrogen and no phosphorous regimes. *Diaporthe* was isolated from all sampled regimes except the no potassium regime. In the no potassium regime, the abundance of *Stemphylium* spp. comprised 100% of the isolates from leaves, with around 90% of the species being *S. vesicarium* and around 10% being *S. solani*. The highest number of fungal endophytes were isolated from the unamended regime, for a total of 27 isolates, and the lowest number from the NPK regime, for a total of 16 isolates (Figure 2.3).

Cotton Leafroll Dwarf Virus Results 2023

Three out of fifty samples tested positive for CLRDV presence, one each from Plot B: No nitrogen/no legumes, Plot 2: No phosphorous, and Plot 6: No potassium. The results were insufficient to further explore a relationship between CLRDV and nutrients.

Discussion

The objective of this work was to determine whether the fungal endophyte diversity and number of isolates in foliage of *G. hirsutum* differed based on applied nutrient regimes. Overall, the number of isolates increased from 2022 to 2023, therefore increasing the isolate number per sampled regime. Despite this, the number of isolates from the no potassium regime were still low for both sampling years.

Similarities between sampling years can be seen in the environmental conditions for both growing seasons. The optimal temperature range for cotton grown in the United States is 28±3°C, and exposure to higher temperatures can negatively impact the crop's growth and yield (Raper et al. 2020). In 2022, the maximum temperature in Auburn, AL during the growing season was 35°C, and in 2023 the maximum temperature reached 36°C (NOAA's National Weather Service). In both years, the maximum monthly temperature was higher than the optimal temperature for almost the entirety of the growing season (NOAA's National Weather Service).

Endophytes have the ability to protect their hosts against temperature fluctuations (Baron and Rigobelo 2021) and may also avoid the effects of temperature stress through plant colonization (Rodriguez et al. 2009). Redman et al. (2002) studied the effect of soil temperatures between 20° to 50°C on *Curvularia* sp. and the plant *Dichanthelium lanuginosum* and found that, alone, neither plant nor endophyte could survive temperatures above 40°C. However, plants

infected with *Curvularia* sp., not only were they able to survive temperatures over 40°C, but protection from high temperatures was also provided to the endophyte.

Environmental conditions may have also contributed to the differences between sampling years. Even though cotton is a relatively drought tolerant crop, the plant requires between 50.8 and 63.5 centimeters of rainfall throughout the growing season in the Southeastern United States (Farahani and Munk 2012). An increase in precipitation has been found to increase the frequency of fungal endophytes, in particular for those transmitted horizontally. This is because moisture can be important for spore germination and host colonization (Grabka et al. 2022). The total rainfall in Auburn, AL, for the 2022 growing season was around 63.60 centimeters and in 2023 around 74.09 centimeters (NOAA's National Weather Service). The amount of water required can vary based on growth stage and other environmental conditions (Datta et al. 2020). Elamo et al. (1999) studied the effect of environmental conditions on endophytic fungi in the leaves of plants in the Birch family. A higher endophyte frequency was observed during the sampling period with more rainfall and higher temperatures versus the period with less precipitation and cooler temperatures. Researchers theorized that this may be due to the fact that rain promotes spore dispersal and also increases the relative humidity in the area. These two factors have been regarded as important requirements for a majority of fungi to germinate and continue developing.

As the Cullars Rotation is unirrigated, drought or excess rain can have a major impact on the crop yields (Mitchell et al. 2012). Even though crops received adequate rainfall in both years, there was a 10.49 centimeter difference in the total rainfall between years. The combination of greater rainfall and high temperatures may have been a cause for the increase in the number of isolates in the 2023 sampling. In contrast, higher temperatures and lower precipitation may have had a role in the lower number of isolates for the 2022 sampling.

Laboratory conditions could have influenced fungal growth, therefore impacting number of isolates. Fungi are often cultivated at temperatures between 25°C and 28°C (Reis et al. 2022). In both years, fungi were cultured at ambient room temperature. However, room temperature fluctuations may have occurred during these periods, which could have possibly influenced growth. Additionally, in 2023, fungi were cultivated in the dark, while in 2022 petri dishes were exposed to light. While fungi can be cultured with or without light (Reis et al. 2022), the difference in light conditions may have impacted fungal growth.

The change in sampled nutrient regimes between 2022 and 2023 can be attributed to a shift in the focus of the research. In 2022, nitrogen plus winter legumes, nitrogen without winter legumes, and NPK + micronutrients were sampled. In 2023, these regimes were not sampled, due the primary research focus being on the presence and absence of the three macronutrients, nitrogen, phosphorous, and potassium.

In 2022, no significant differences were observed between nutrient regimes in relation to endophyte diversity. In both years, no significant differences were observed between nutrient regimes in relation to number of isolates. That being so, in 2023 the greatest number of isolates was in the unamended regime, and the lowest number in the NPK regime. This aligns with the hypothesis that the number of fungal endophytes will be greatest in plants experiencing nutrient deficiencies.

In the current work, fungal endophytes from cotton foliage included the genera *Alternaria, Cladosporium, Diaporthe, Fusarium, Phoma,* and the species *Stemphylium solani* and *S. vesicarium*. A study by Ek-Ramos et al. (2013) identified several fungal endophytic genera in *G. hirsutum* that were also isolated in the current work. A few of these include *Alternaria*, *Colletotrichum, Fusarium,* and *Phoma.* Vieiraa et al. (2011) isolated the fungal

genera *Curvularia*, *Cladosporium*, and *Nigrospora* from various tissues of *Gossypium* plants. These genera were also observed in the current work.

Differences in endophyte diversity based on nutrient regimes were observed. In 2022, *Diaporthe* and *Alternaria* were isolated from all plots except no potassium, and *Diaporthe*'s relative abundance was high in the no phosphorous regime. However, in 2023 the diversity in sampled foliage differed. *Alternaria* was only isolated from the no nitrogen and no phosphorous regimes. *Diaporthe* was again isolated from all regimes except the no potassium regime. *Colletotrichum truncatum*'s relative abundance was high in the no potassium regime in 2022, whereas in 2023 *Colletotrichum* sp. were only isolated from the unamended regime, with insignificant abundance. In 2023, the relative abundance in the no potassium regime is important, as the only endophytic species isolated were *Stemphylium vesicarium* and *S. solani*. The diversity of endophytes across nutrient regimes in 2023 may be attributed to the higher number of isolates.

In the current work, the impact of isolated and identified fungal endophytes on sampled *G. hirsutum* growth and nutrient uptake is unknown. However, previous research has described the beneficial role of endophytes in the same genera as the ones isolated here. An in vitro study by Suebrasri et al. (2020) demonstrated the ability of *Diaporthe phaseolorum* to solubilize inorganic phosphorous as well as produce the plant phytohormone indole-3-acetic acid (IAA). Species of *Penicillium* and *Fusarium* have been found to produce the phytohormone gibberellin (Leitao and Enguita 2016). Mauricio-Castillo et al. (2020) inoculated *Capsicum annuum* roots with *Alternaria solani* strain IA300 and observed an increase in plant growth of inoculated plants as time post inoculation increased. *Mucor* sp. have been found to increase phosphorous concentration when inoculated in *Arabidopsis arenosa* under heavy metal stress (Rozpadek et al.

2018). In vivo tests of tomato plants inoculated with two isolates of *Colletotrichum siamense* and one isolate of *Diaporthe masirevicii* apparently contributed to an increase in the plant's biomass. All three isolates were also found to be able to solubilize phosphorous (Silva Santos et al. 2022).

Oono et al. (2020) studied fungal endophyte association in a natural environment using the nutrient content of leaves from two species, *Pinus muricata*, the Bishop Pine, and *Vaccinium ovatum*, the California Huckleberry. The optimal nitrogen to phosphorous ratio for most plants is 15:1 (Luo et al. 2016). The results of the experiment found an increase in fungal endophyte diversity in plant leaves with a smaller nitrogen-to-phosphorous ratio and a decrease in endophytic diversity plant leaves with a higher ratio. Oono et al. (2020) stated that plant stress impacts fungal endophytes.

Henning et al. (2020) explored the relationship between nutrient application to field grown *Andropogon gerardii* and the foliar fungal endophyte community. Researchers predicted nutrient application would directly lower endophyte diversity. In contrast, the experiment found that nutrient application had a minimal impact on endophyte diversity. As foliar fungal communities can be highly variable, plant diversity and time of sampling may have also had an impact on endophyte composition (Henning et al. 2021). While the hypothesis of the work herein was supported by the lowest number of endophytes isolated from the NPK regime and the highest isolated from the unamended regime, outside factors, such as temperature, rainfall, or sampling time may have also influenced endophyte colonization in the sampled foliage.

Fungal endophytes have been found to become pathogenic during a state of stress, conducive environmental conditions, or due to host age (Bamisile et al. 2018). However, endophytes generally exist in an asymptomatic state (Faeth and Fagan 2002). *Colletotrichum* has been detected as an endophyte with potential pathogenicity (Alam et al. 2021). Cotton is a host

of *Colletotrichum truncatum*, which can have a pathogenic and endophytic phase in plants. The fungus has the ability to enter an asymptomatic, or endophytic phase once it has colonized the tissue of its host (Ranathunge and Sandani 2016). This may explain its presence in the sampled *G. hirsutum* leaves.

In 2023, the isolation of *S. solani* from the no potassium regime may potentially be linked to Stemphylium leaf spot of cotton, a secondary fungal disease brought on by potassium deficiency. *Stemphylium solani*, the pathogen associated with this disease, is a weak pathogen, mostly infecting the plant during periods of nutrient or environmental stress (Strayer-Scherer et al. 2023). Disease symptoms appear from mid- to late-season, due to an increase in potassium requirements during boll and fruit development (Strayer-Scherer et al. 2023). Both the environmental conditions and time of sampling align with the requirements needed for disease occurrence, indicating the possibility of *S. solani* being a latent pathogen in the collected samples.

One limiting aspect of this work is that culture-dependent methods of fungal endophyte isolation do not provide a full picture of the fungal diversity, as some microorganisms cannot be cultured in a laboratory (Reis et al. 2022). Classification to species level for isolates may also be difficult, as the internal transcriber placer (ITS) may only be able to identify to the genus level (Bhunjun et al. 2021). Another limitation of this study is the lack of a control for PCR, due to error during processing methods. In future work, refined molecular techniques will enhance the findings of this research.

This study holds significance as it contributes to the understanding of fungal endophytes in *G. hirsutum* foliage based on varying nutrient regimes. Endophytes considered to be plant growth promoters, nutrient uptake enhancers, and phytohormone producers were isolated in the

current work. *Diaporthe* has been observed to solubilize phosphorous (Silva Santos et al. 2022), and in the 2022 sampling a high abundance of *Diaporthe* was isolated from the no phosphorous regime. Gibberellin producing endophytes, *Penicillium* and *Fusarium,* were also isolated in the current work (Leitao and Enguita 2016).

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Tables

Table 2.1 Treatments applied to sampled *Gossypium hirsutum* in 2022 and 2023 at the Cullars Rotation

		Treatments[1]			
Year	Description	Ammonium nitrate (34-0-0)	Concentrated superphosphate (0-46-0)	Muriate of potash (0-0-60)	Other
2022	No Nitrogen/+legume	0	Yes	Yes	Legume
Both	No Nitrogen/no legume	0	Yes	Yes	No legume
2023	No soil amendments	0	0	0	
2022	No winter legumes/ + Nitrogen	Yes	Yes	Yes	No legume
Both	No Phosphorous	Yes	0	Yes	
Both	NPK[2]	Yes	Yes	Yes	No micronutrients
Both	No Potassium	Yes	Yes	0	

(Mitchell et al. 2012)

[1] Standard fertilizer treatments:

- 90 lb. P_2O_5 per acre per 3-yr rotation
- 240 lb. K_2O per acre per 3-yr rotation
- 90 lb. NH_4NO_3/acre on cotton
- [2] NPK is Nitrogen, Phosphorous, and Potassium

Table 2.2 Primers used for polymerase chain reaction (PCR)

Sequence	Primer Name
CTTGGTCATTTAGAGGAAGTAA	ITSIF
TCCTCCGCTTATTGATATGC	ITS4

(White et al. 1990)

Figures

Figure 2.1 Fungal endophyte relative abundance in various nutrient regimes for 2022

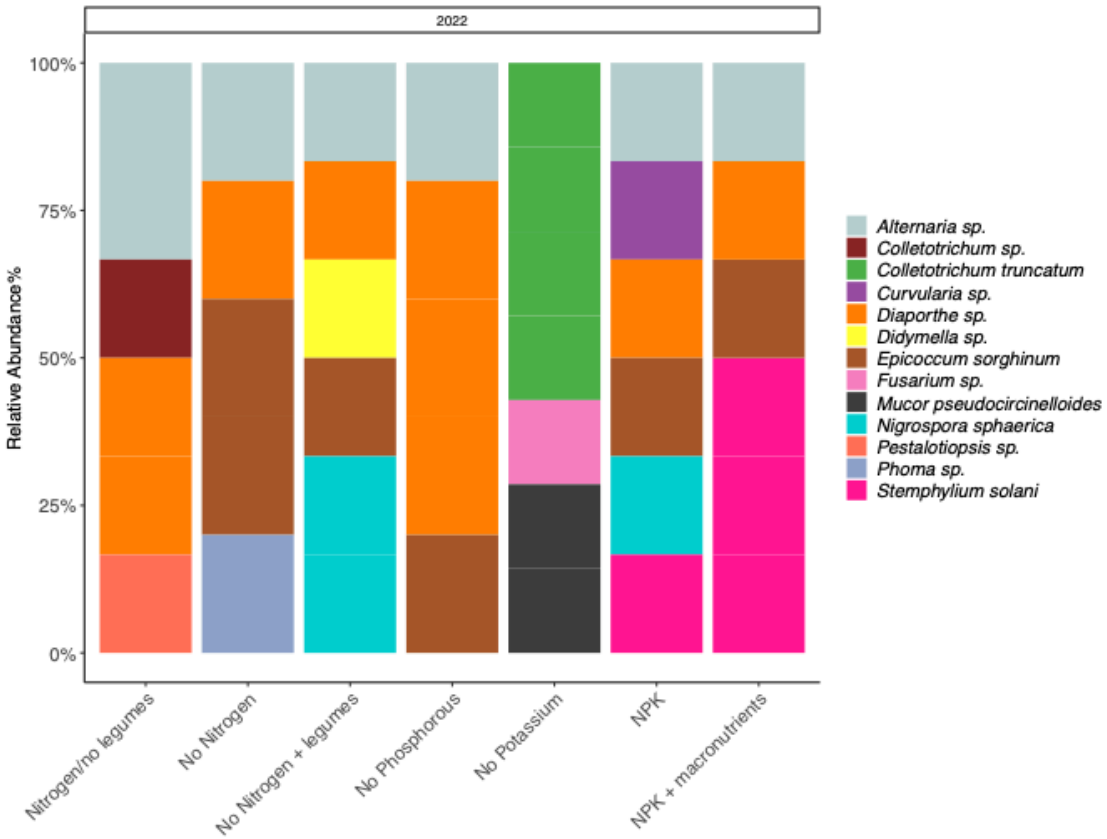


Figure 2.2 Fungal endophyte relative abundance in various nutrient regimes for 2023

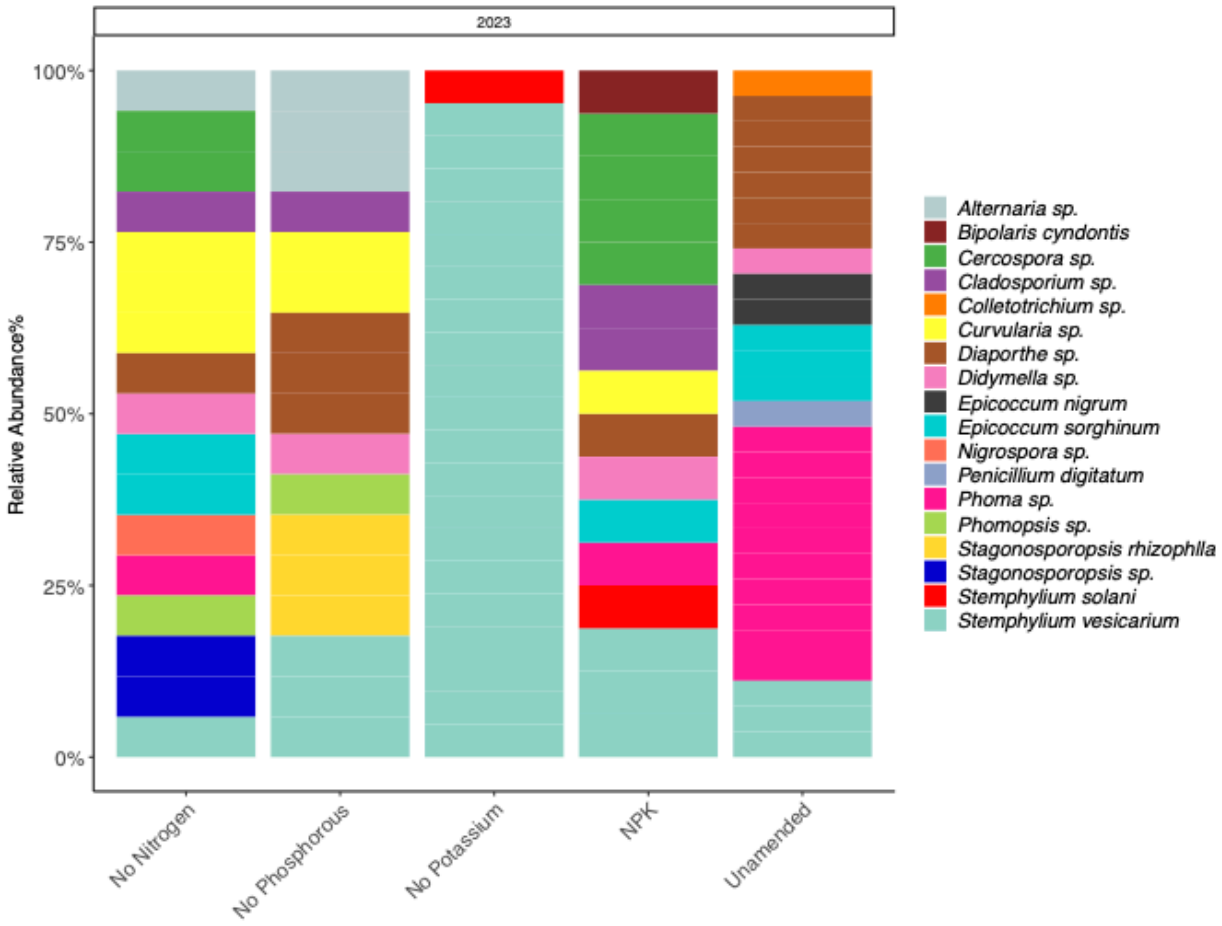
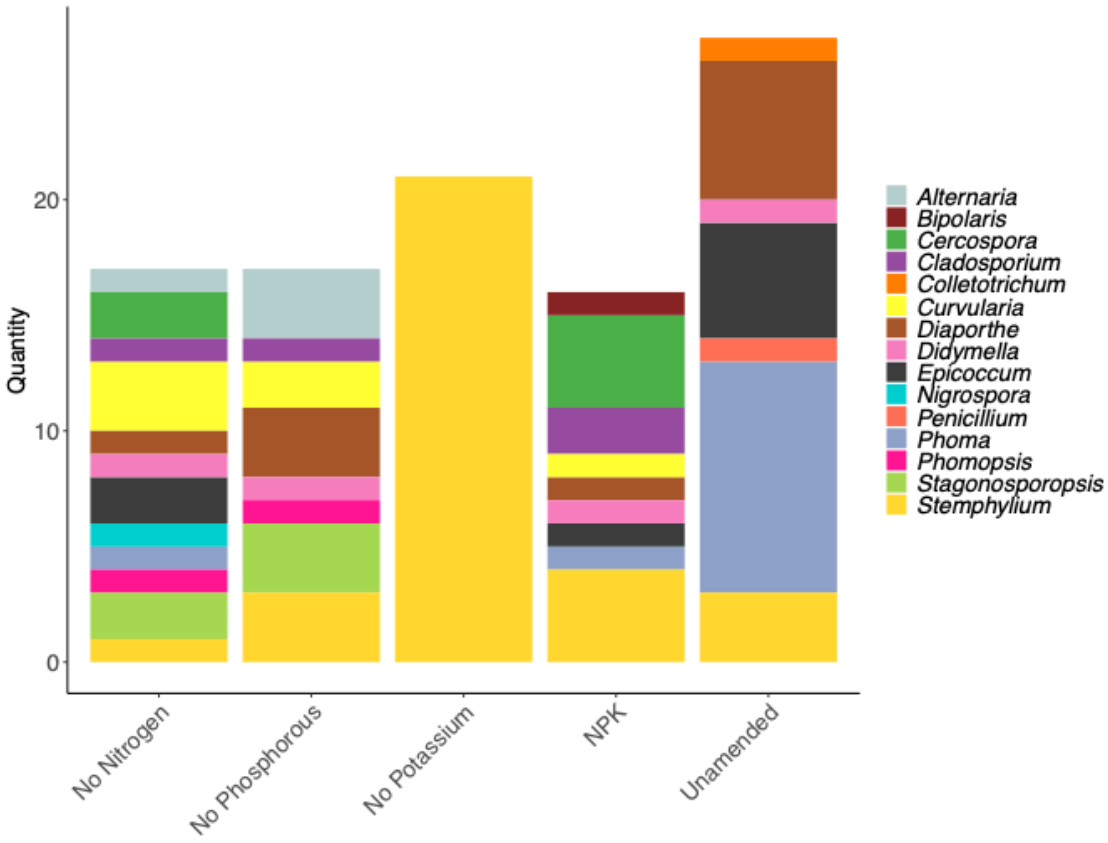


Figure 2.3 Number of fungal genera isolated from sampled nutrient regimes in 2023



III. The fungal endophyte microbiome in the leaves of *Gossypium hirsutum* grown under various soil nutrient regimes

Introduction

The fungal endophytic microbiome is comprised of fungal communities living in the tissue of a plant (Porras-Alfaro and Bayman 2011). A relationship is formed between the endophytic community and its host plant, with endophytes aiding in abiotic and biotic stress tolerance such as drought, temperature fluctuations, salinity, and plant pathogens (Baron and Rigobelo 2021). Endophytes may also improve plant growth and nutrient uptake. In return, nutrients are provided by the host plant to the endophytes (Baron and Rigobelo 2021). Diversity in the endophyte microbiome can be attributed to factors such as environmental conditions, host plant species, and nutrient availability (Porras-Alfaro and Bayman 2011). Fungal endophytes colonize a plant through horizontal and vertical transmission. Vertical transmission is from the seed to the plant, while horizontal transmission is from external sources such as soil, air, and rain (Bard et al. 2024).

Gossypium hirsutum, upland cotton, is the most commonly cultivated species of cotton, accounting for 90% of the world's cotton production (Aslam et al. 2020). Application of nutrients, such as nitrogen, phosphorous, and potassium, is vital for successful growth and yield of *Gossypium* species. Nitrogen is needed for photosynthesis and prevention of square and boll abscission (Sawan 2013). Phosphorous is essential for plant reproductive development and yield (Iqbal et al. 2020) and is required throughout the entire growing season (Malik 1998). Potassium is needed for boll development and impacts fiber quality (Fang et al. 2016). Due to the high

uptake rate and poor ability of cotton to absorb potassium, a continuous supply is required during the growing season (Makhdum et al. 2007).

Nutrient transfer is an example of interactions between endophytes and their host plant (Garcia-Latorre et al. 2021). The plant provides carbohydrates to the endophytes, and in return the endophytes aid in nutrient absorption (Garcia-Latorre et al. 2021). Endophytes may also have the ability to synthesize phytohormones, including auxin, gibberellin and cytokinin (Chaturvedi 2022). Phytohormones play a major role in stimulating plant growth, particularly by increasing root surface area, which allow plants to absorb greater amounts of nutrients (Sabagh et al. 2022). Auxin assists in shoot and root development, gibberellin aids in fruit formation, stem elongation, and seed development (Baron and Rigobelo 2021), and cytokinin impacts seed dormancy and apical dominance (Rana et al. 2020).

Next generation sequencing techniques, such Illumina based sequencing, are commonly used to study the endophytic microbiome (Reis et al. 2022). These techniques provide information on the diversity and abundance (Satam et al. 2023) of culture-dependent and culture-independent fungal endophytes (Reis et al. 2022). The objective of this work is to observe the fungal endophyte microbiome in *G. hirsutum* leaves in relation to various nutrient regimes using Illumina based sequencing.

Materials and Methods

Gossypium hirsutum Sampling

Sampling of *Gossypium hirsutum*, growing in varying fertility regimes at the Cullars Rotation (circa 1911) located in Auburn, Alabama, was done at three dates from June 2023 to August 2023. The rotation consists of 14 nutrient regimes which allow researchers to observe the

long-term effects of specific nutrient deficiencies in soybean, cotton, corn, and small grain (Mitchell et al. 2012). The crops are on a three-year rotation and the rotation is unirrigated (Mitchell et al. 2012). The growing season for *G. hirsutum* begins in April or May and extends into September or October (Rehman and Farooq 2005). Early season sampling took place on June 8, 2023 when plants were in the vegetative growth stage, mid-season sampling on July 11, 2023 during the early bloom stage, and late season sampling on August 14, 2023 during peak bloom. The selected treatments were Plot B: no nitrogen/no legume, Plot C: no soil amendments, Plot 2: no phosphorous, Plot 3: NPK and Plot 6: no potassium (Table 3.1). Eight leaf cuttings were collected from each predetermined plot for a total of forty samples. The cuttings were stored on ice and transported to the laboratory for sterilization.

For each of the three sampling dates, 6 leaves were selected at random for analysis for a total of 18 samples per plot. Leaf samples were soaked in a 10% bleach solution (0.75% sodium hypochlorite) for three minutes followed by a three-part sterile deionized water bath for sterilization. An ethanol and flame-sterilized hole punch was used to extract seven to ten circular 0.95 cm diameter tissue samples from each of the sterilized leaves. The tissue samples were placed in 2 ml Eppendorf tubes containing 700 µL CSPL buffer from the Mag-Bind® Plant DNA Kit (Omega Bio-tek, Norcross, GA) and stored in a -28° C freezer.

DNA Extraction

A TissueLyser (Qiagen, Hilden, Germany) was used to break apart plant tissue by shaking Eppendorf tubes containing one 4 mm stainless steel bearing ball (Amazon, Seattle, WA) for 30 seconds at thirty hertz. The process was repeated as needed to ensure proper tissue disruption. The Mag-Bind® Plant DNA Plus 96 Kit protocol was used to extract DNA from a

total of 82 samples. A negative control, without the addition of leaf samples, was included. All samples were stored in a -28° C freezer until fungal library preparation began.

Fungal Library Preparation

The protocol involved three steps of polymerase chain reaction (PCR). The PCR steps, cycling conditions, and primers, are found in Tables 3.2 and 3.3. The negative control was included and sequenced with the fungal DNA. A mock community containing 12 fake ITS sequences was used as a positive control (Palmer et al. 2018). Following PCR, the samples were normalized using the SequalPrep™ Normalization Plate Kit, 96-well (ThermoFisher Scientific, Waltham, MA) and pooled and concentrated using Amicon® Ultra 0.5 mL filters (MilliporeSigma, Burlington, MA). The pooled DNA was cleaned using AMPure beads at 0.7X (Beckman Coulter, Brea, CA). Gel electrophoresis and a Qubit 3.0 fluorometer (ThermoFisher Scientific, Waltham, MA) were used to determine the cleaned library concentration. The total concentration of the library measured 7.32 ng/uL. A total of 82 samples were sequenced at SeqCenter (Pittsburgh, PA) for using an Illumina MiSeq 2x300 bp.

Bioinformatics

Sequences were uploaded into FastQC v.0.12.0 for quality analysis. Only forward reads were used, as the area of interest is the ITS1 region. Fungal primers were stripped with cutadapt v.2.10 (Martin 2011). Sequences were trimmed to 265 bp length and filtered with a < 1.0 expected errors threshold with vsearch v.2.22.1. Chimeras were removed using usearch v.11.0.677 (Edgar 2010) after quality filtering and dereplication using vsearch (Rognes et al. 2016). Sequences were clustered into Operational Taxonomic Units (OTUs) at traditional 97%

identity using usearch. Taxonomy was predicted using SINTAX and Naïve Baysian Classifier (NBC) (Taheri and Mammadov 2013). The NBC was implemented through dada2 v3.18 programs (Davis et al. 2018) against the UNITE version 9 database (Abarenkov et al. 2023).

The OTU tables, taxonomy tables, and metadata file were imported into R v.4.3.2 and combined with the package 'phyloseq' v.1.46.0. Samples with low sequencing coverage, less than 5,000 reads, were removed from the analysis using the 'decontam' package v.1.22.0 (Davis et al. 2018). Negative control OTUs, mock community OTUs, and contaminant OTUs were also removed using the 'decontam' package. The package 'ranacapa' v.0.1.0. was used to run a rarefaction analysis on the clean phyloseq object. The alpha diversity was measured with the 'phyloseq' v.1.46.0 package using a linear model. Alpha diversity is defined as the richness, or number of taxa, and evenness, or relative abundance of taxa (Walters and Martiny 2020). The sequences were normalized using the 'metagenomeSeq' function (Paulson et al. 2013).

Beta diversity assesses the differences in the fungal composition between different samples (Walters and Martiny 2020). Beta diversity was tested by Principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) in R v.4.3.2 using the Bray-Curtis distance metric and the 'adonis2' function in the package 'vegan' v.2.6.4. A beta dispersion test was performed using the 'permutest' function between nutrient regime and relative abundance. A beta dispersion test was also performed between time of season and relative abundance. This test is used to determine if there is a difference in the microbiome composition and selected variable (Kelly et al. 2015). Rarefaction was analyzed and determined the read depth to be sufficient (Figure 3.1). The most abundant indicator OTUs in nutrient regimes were determined using the 'indicspecies' package v.1.7.14 in R v.4.3.2.

Results

The total number of reads from the cleaned and filtered data were 1,130,477, the mean read depth was 17,663, and the median read depth was 5,204. Alpha diversity of richness and evenness over time of season and nutrient regime was analyzed. Significance ($P \leq 0.05$) was observed for richness over time of season and nutrient regime for late season sampling and the no potassium regime (Table 3.4). Significance ($P \leq 0.05$) was observed for evenness over time of season and nutrient regime for the no potassium regime (Table 3.4). The PERMANOVA test showed significance for regime and time of season (Table 3.5). The PERMANOVA test also found that interactions between regime and time of season were significant (Table 3.5). The Pairwise Adonis test revealed the no potassium regime was significantly different from each of the four other regimes (Table 3.6). The no nitrogen regime was found to be significantly different from the unamended regime (Table 3.6). The beta dispersion test showed that the *P*-value was not significant between nutrient regime and relative abundance (Table 3.7). The permutation test showed that the *P*-value was not significant between time of season sampled and relative abundance (Table 3.7).

Relative Abundance

The most abundant fungal phylum across all sampled regimes was Ascomycota (Figure 3.2). The most abundant fungal genus was *Stemphylium*, comprising about 60% of the community in the no potassium regime. *Colletotrichum*'s relative abundance was also high in the no potassium regime (Figure 3.3). The relative abundance of *Diaporthe* was high in the no nitrogen and NPK regimes (Figure 3.3).

Beta diversity

The Bray-Curtis dissimilarity index tested the dissimilarity of the fungal communities in relation to differences in the species abundance (Hao et al. 2019). The similarity and dissimilarity is based on the distance between clusters (Galimanas et al. 2014). Axis 1 explained 12.1% of variance in fungal communities and Axis 2 explained 8.5% of the variance (Figure 3.4). The results showed over 75% of the no potassium regime during the mid-season and late-season were clustered away from all other regimes.

Indicator Species Analysis

Indicator species are species used as ecological indicators of community types, changes in the environment, or environmental conditions (De Caceres et al. 2010). The most abundant indicator OTUs in nutrient regimes were plotted on a heatmap (Figure 3.5). The fungi with the most abundance across nutrient regimes were *Colletotrichum truncatum* and *Stemphylium* spp., indicating a difference in the community based on the applied nutrient regime (Figure 3.5). A plot of the abundance of the *Stemphylium* indicator species throughout the time of season revealed high abundance of the fungal community during mid-season and late season sampling (Figure 3.6). There was low *Stemphylium* presence in the early season sampling (Figure 3.6).

Discussion

The objective of this work was to observe whether the fungal endophyte microbiome in *G. hirsutum* leaves was possibly impacted by various nutrient regimes. The significance of

richness over time suggests that as the growing season progressed, the abundance of endophytes in the plant's leaves increased. This may be attributed to leaf age. Nascimento et al. (2015) studied the colonization of naturally occurring fungal endophytes at different leaf ages in *Calotropis procera* and found that the number of isolates and endophyte diversity increased with leaf age.

Foliar fungal communities can be highly variable (Henning et al. 2020), and environmental conditions, such as temperature and rainfall, can also influence endophyte colonization. High relative humidity (Elamo et al. 1999) and moisture can be important for spore germination and host colonization (Grabka et al. 2022). As stated in Chapter II, the total rainfall for the 2023 growing season met the necessary requirements, between 50.8 and 63.5 centimeters (Farahani and Munk 2012). The temperatures throughout a majority of growing season were above the optimal range of 28±3°C (Raper et al. 2020). These factors, combined with the fact that rainfall is the only source of irrigation at the Cullars Rotation (Mitchell et al. 2012), could have potentially impacted host plant colonization by fungal endophytes.

High-throughput sequencing techniques can provide information on the alpha and beta of fungal communities (Baldrian et al. 2021). Significance for evenness over time implies that the community distribution may be correlated to the community diversity. This can be observed in Bray-Curtis dissimilarity index, as the clustering of a majority of the no potassium regime away from all other regimes indicated a difference in the community. The no potassium regime was also found to be significantly different ($P \leq 0.05$) when compared to all other sampled regimes in relation to endophyte diversity.

Similar results to Chapter II were seen for relative abundance and significance of fungal endophyte diversity when compared to nutrient regimes. As with the current chapter, the no

potassium regime was observed to be significantly different ($P \leq 0.05$) from all other sampled regimes. In regard to relative abundance, *Stemphylium solani and S. vesicarium* were the only endophytes extracted from leaves in the no potassium regime in Chapter II. In the current chapter, *Stemphylium* was identified as an indicator species with a high relative abundance in the no potassium regime. While the species level is unknown for the isolates in the current chapter, the presence of *Stemphylium* in the no potassium regime may potentially be linked to Stemphylium leaf spot of cotton. This is a secondary fungal disease brought on by potassium deficiency (Strayer-Scherer et al. 2023), and caused by *Stemphylium solani*, a weak pathogen. Disease symptoms typically appear from mid- to late-season (Strayer-Scherer et al. 2023). This hypothesis is supported by the significance of the no potassium regime ($P \leq 0.05$) in relation to all othered sampled regimes, the clustering of the no potassium regime in the Bray-Curtis indices, and the high abundance of the *Stemphylium* indicator species 'FOTU_2' during mid- and late-season sampling. Based on the rarefaction curve, the high abundance of the 'FOTU_2' was not influenced by the sequencing depth.

In this chapter, *Colletotrichum truncatum* was identified as an indicator species, with high abundance in the no potassium regime. In Chapter II, this fungal endophyte was also observed to have high abundance in the no potassium regime. Cotton is a host of *C. truncatum*, which has the ability to enter an asymptomatic, or endophytic, phase once it has colonized the tissue of its host (Ranathunge and Sandani 2016). Fungal endophytes can exhibit a prolonged latency period (Ek-Ramos et al. 2013), and latent pathogens may become active during a state of stress, conducive environmental conditions, or due to host age (Bamisile et al. 2018). That being so, endophytes tend to remain in an asymptomatic state (Faeth and Fagan 2002). This information may explain the presence of *C. truncatum.*

Several fungal genera identified in the current chapter were previously isolated in Chapter II. A few of these include *Alternaria*, *Cercospora*, *Colletotrichum*, *Bipolaris, Cladosporium*, *Diaporthe*, *Fusarium*, *Nigrospora*, *Penicillium* and *Stagonosporopsis. Diaporthe* was found to have a high relative abundance in the no nitrogen and NPK regimes. *Stagonosporopsis* abundance was also high in the no nitrogen regime. While the role of identified fungal endophytes in regard to *G. hirsutum* growth and nutrient uptake is unknown in the current work, previous studies have observed the benefit of endophytes in the same genera through in vitro analysis and plant inoculation. Co-culture in vitro tests of *Stagonosporopsis cucurbitacearum* with *Glycyrrhiza glabra* showed a visible increase in the plant root and shoot growth in comparison to control plants (Arora et al. 2019). Sodhi and Saxena (2023) inoculated *Oryza sativa* with *Nigrospora oryzae* and observed that inoculated plants under normal conditions, salinity stress, and drought stress exhibited greater shoot and root growth than uninoculated plants under the same conditions. A study on the effect of *Bipolaris* sp. inoculated in soybeans showed an increase in the plant's growth under both salinity stress and normal conditions in comparison to uninoculated plants (Lubna et al. 2022). In vitro analysis also found *Bipolaris* sp. capable of producing indole-3-acetic acid (IAA) and gibberellin (GA) (Lubna et al. 2022).

Yang et al. (2023) studied the growth promotion and phytohormone production capabilities of a species of *Cladosporium*. In vitro experimentation showed an increase in plant height as well as the number of roots and leaves in *Sesuvium portulacastrum* plants inoculated with the *Cladosporium* species. *Arabidopsis thaliana* seedlings inoculated with *Cladosporium* sp. had increased nitrogen uptake. The endophyte was also observed to produce indole-3-acetic acid (IAA). In vitro tests by Silva Santos et al. (2022) of two isolates of *Colletotrichum siamense*

and one isolate of *Diaporthe masirevicii* inoculated in tomato plants revealed an increase in the plants' biomass. All three endophytes were also found to successfully solubilize phosphorous. In an in vitro experiment by Suebrasri et al. (2020), *Diaporthe phaseolorum* was observed to produce the plant phytohormone indole-3-acetic acid (IAA) as well as solubilize inorganic phosphorous. The endophyte *Penicillium* has also been identified as one of the most prevalent phosphorous solubilizing endophytes (Mehta et al. 2019).

A limiting factor of the current work is the indicator species analysis did not identify *Stemphylium* spp. to the species level. This prevents knowledge of whether the causal agent for Stemphylium leaf spot was present in the sampled plants. In future work, a focus on the no potassium regime towards the end of the growing season would provide more information on the potential presence of *Stemphylium solani* in the plot.

This experiment provided insight on the fungal endophyte community in leaf tissue of *G. hirsutum* plants growing in various nutrient regimes. Fungal endophytes previously identified as plant growth promotors were isolated in the current work. A few of these include *Penicillium* (Mehta et al. 2019), *Diaporthe* (Silva Santos et al. 2022), and *Cladosporium* (Yang et al. 2023). The potential for latent pathogenicity, due to nutrient stress, was also observed. Knowledge of possible pathogenic fungal endophytes is important, as their incorporation in agricultural practices, such as with the application of biofertilizers, is a promising method to supply plants with nutrients necessary for improved growth and yield (Fasusi et al. 2021). The abundance of *Diaporthe* and *Stagonosporopsis* were also important findings of this experiment, as it may be linked to their ability to assist plants with nutrient uptake.

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Tables

Table 3.1 Treatments Applied to sampled *Gossypium hirsutum* plots in 2023 at the Cullars Rotation

		Treatments[1]			
Plot	Description	Ammonium nitrate (34-0-0)	Concentrated superphosphate (0-46-0)	Muriate of potash (0-0-60)	Other
B	No Nitrogen/no legume	0	Yes	Yes	No legume
C	No soil amendments	0	0	0	
2	No Phosphorous	Yes	0	Yes	
3	NPK[2]	Yes	Yes	Yes	No micronutrients
6	No Potassium	Yes	Yes	0	

(Mitchell et al. 2012)

[1] Standard fertilizer treatments:
90 lb. P_2O_5 per acre per 3-yr rotation
240 lb. K_2O per acre per 3-yr rotation
90 lb. NH_4NO_3/acre on cotton

[2] NPK is Nitrogen, Phosphorous, and Potassium

Table 3.2 Three-step polymerase chain reaction (PCR) process for fungal library preparation

Reagent	Volume per reaction (uL)	Time	Temperature (°C)	Cycles
Step 1				
Dream Taq Green PCR Master Mix (ThermoFisher, USA)	6.25 uL	5:00	95	
ITS 1F Primer	0.375 uL	0:30	95	10x
ITS4 Primer	0.375 uL	0:30	50	
Bovine Serum Albumin (BSA 3%)	3 uL	1:00	72	
H2O	1 uL	7:00	72	
Extracted DNA		Infinite	10	
Step 2				
Dream Taq Green PCR Master Mix (ThermoFisher, USA)	6 uL	5:00	95	
Primer2 Mix uM	0.6 uL	0:30	95	
Bovine Serum Albumin (BSA 3%)	3 uL	0:35	50	10x
H2O	0.4 uL	0:35	72	
Step 1 Product		7:00	72	
		Infinite	10	
Step 3				
Dream Taq Green PCR Master Mix (ThermoFisher, USA)	8 uL	5:00	95	
10 uM Forward Primer F	0.5 uL	0:30	95	
H2O	1.5 uL	0:35	63	15x
10 uM Barcode Primers R	1 uL	1:10	72	
Step 2 Product		7:00	72	
		Infinite	10	

(Noel et al. 2022)

Table 3.3 Primers used for fungal library preparation

Sequence	Primer Name
CTTGGTCATTTAGAGGAAGTAA	ITSIF
AGCCTCCGCTTATTGATATGCTTAART	ITS4
	Frameshifts (combination of 6)
NNNNNNNN TT CTTGGTCA TTTAGAGGAAGTAA	ITS IF FI
NNNNTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS IF F2
NNNNCTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS IF F3
NNNNACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS IF F4
NNNNGACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS IFFS
NNNNTGACTNNNN TT CTTGGTCATTTAGAGGAAGTAA	ITS IF F6
NNNNN AG AGCCTCCGCTTA TTGATA TGCTT AART	ITS IF FI
NNTNNN AG AGCCTCCGCTTATTGATA TGCTT AART	ITS4 F2
NNCTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F3
NNACTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F4
NNGACTNNN AG AGCCTCCGCTTATTGATATGCTTAART	ITS4 F5
NNTGACTNNN AG AGCCTCCGCTT A TTGA TA TGCTT AART	ITS4 F6
AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGC GCCATCAGAGATGTG	PCR F

*Frameshift primers are used in PCR reactions at an equal molar ratio of forward and reverse primers (Noel et al. 2022)

Table 3.4 Alpha diversity of richness and evenness over time of season and nutrient regime

	Richness			Evenness		
Predictors	Estimates	CI	*P*-value	Estimates	CI	*P*-value
(Intercept)	18.1	11.02 – 25.19	<0.001	0.55	0.38 – 0.71	<0.001
Time of season [Late-season]	7.13	1.21 – 13.05	0.019[1]	0	-0.14 – 0.14	0.985
Time of season [Mid-season]	2.83	-2.96 – 8.61	0.332	-0.04	-0.17 – 0.10	0.576
Regime [No Phosphorous]	3.26	-3.34 – 9.86	0.327	0.05	-0.10 – 0.21	0.484
Regime [No Potassium]	6.45	0.22 – 12.67	0.043[1]	-0.18	-0.33 – -0.04	0.015[1]
Regime [NPK]	-1.91	-8.98 – 5.17	0.591	0.03	-0.13 – 0.20	0.687
Regime [Unamended]	0.91	-5.28 – 7.10	0.769	-0.01	-0.16 – 0.13	0.864
(Intercept)	18.1	11.02 – 25.19	<0.001			
Observations	64			64		
R^2 / R^2 adjusted	0.212 / 0.129			0.202 / 0.118		

[1] P-value determined to be significant if ≤ 0.05

Table 3.5: Permutational Multivariate analysis of variance (PERMANOVA) of differences between fungal communities based on time of season sampled and nutrient regime

	Degrees of Freedom	Sum of Squares	R^2	F-value	Pr(>F)
Regime	4	3.759	0.137	2.539	0.0001[1]
Time of season	2	1.734	0.063	2.342	0.0001[1]
Regime:Time of season	7	3.488	0.127	1.346	0.0014[1]
Residual	50	18.505	0.673	NA	NA
Total	63	27.486	1.000	NA	NA

[1] P-value determined to be significant if ≤ 0.05

Table 3.6 Pairwise Adonis test to determine significance between nutrient regimes

Regime	Df	Sum of Squares	R^2	F-value	P-value
No potassium vs. No nitrogen	1	1.458	0.135	3.913	0.001[1]
No potassium vs. Unamended	1	1.850	0.145	4.899	0.001[1]
No potassium vs. No phosphorous	1	1.277	0.116	3.292	0.001[1]
No potassium vs. NPK	1	1.132	0.122	3.046	0.002[1]
No nitrogen vs. Unamended	1	0.668	0.059	1.629	0.034[1]

[1] P-value determined to be significant if ≤ 0.05

Table 3.7 Beta dispersion test of similarity between nutrient regime and relative abundance of fungal communities and time of season and relative abundance of fungal communities

	Degrees of freedom	Sum of Squares	R^2	F-value	N.perm	P-value
Nutrient regime						
Groups	4	0.092	0.023	1.324	999	0.286
Residuals	59	1.028	0.017			
Time of season						
Groups	2	0.006	0.003	0.782	999	0.469
Residuals	61	0.251	0.004			

[1] P-value determined to be significant if ≤ 0.05

Figures

Figure 3.1 Rarefaction curves for sequenced samples

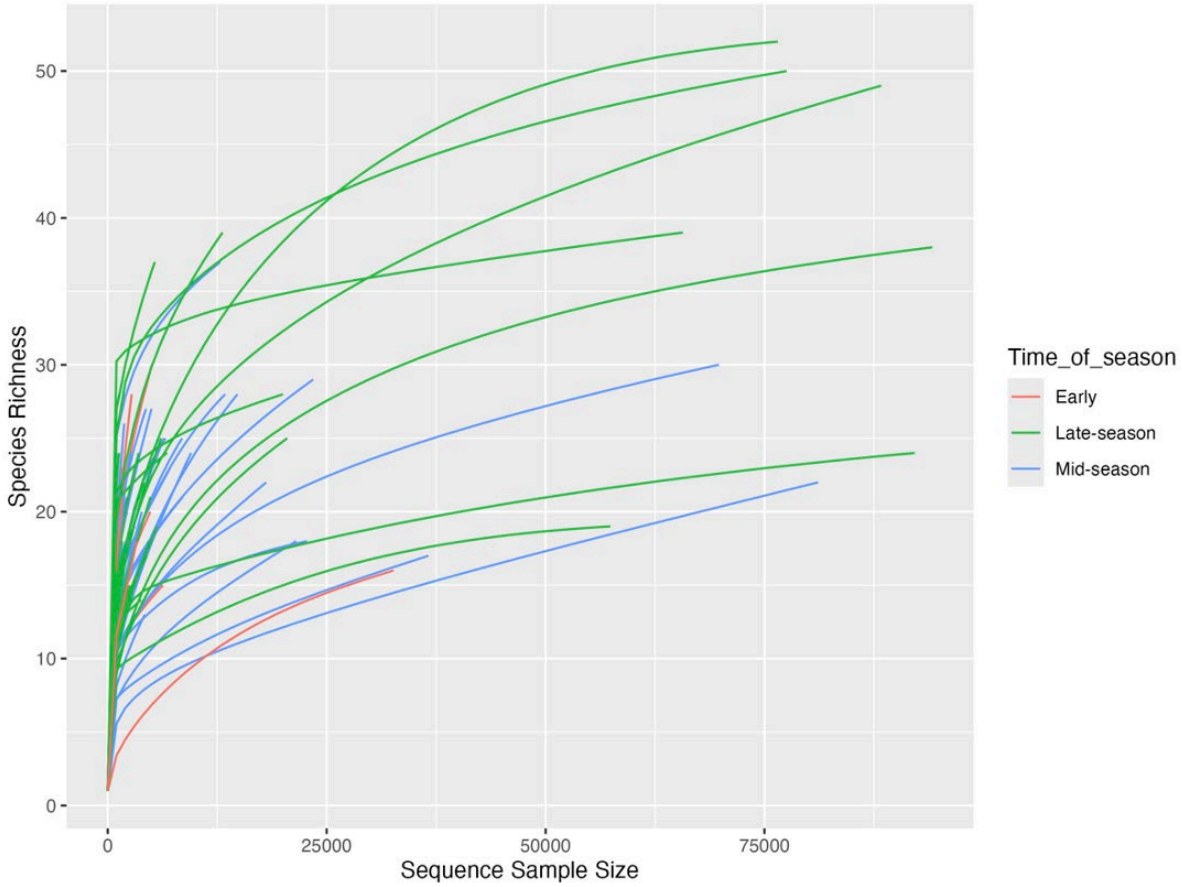

Figure 3.2 Relative abundance of fungal phyla across sampled nutrient regimes

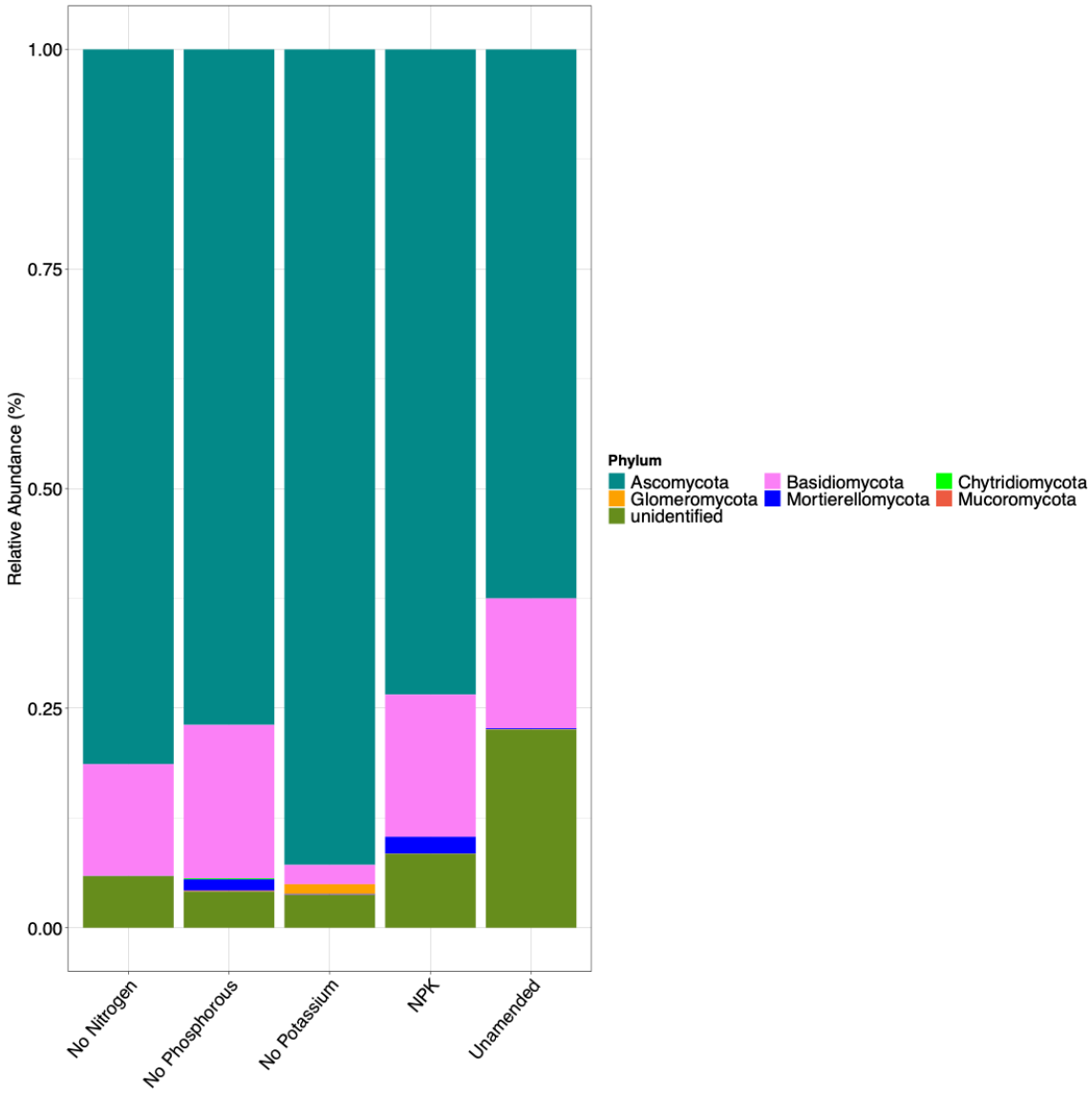

Figure 3.3 Relative abundance of fungal genera across sampled nutrient regimes

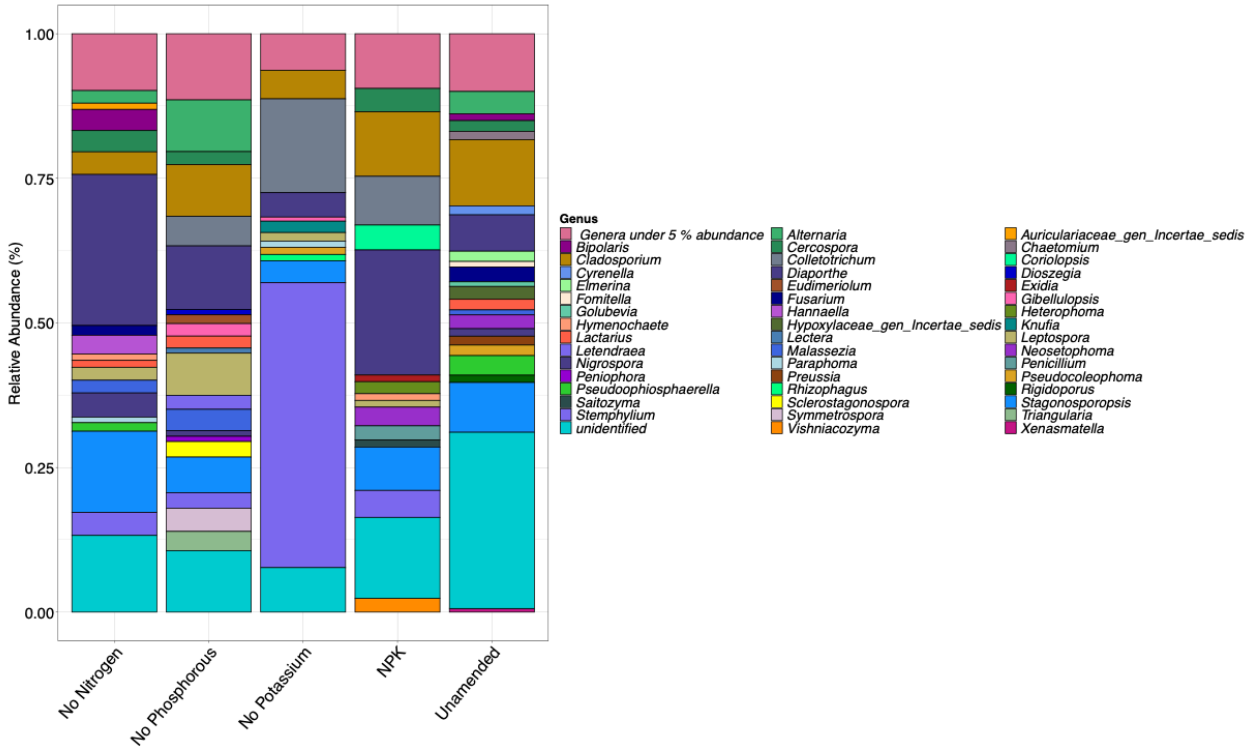

Figure 3.4 Bray-Curtis dissimilarity of fungal communities in nutrient regimes

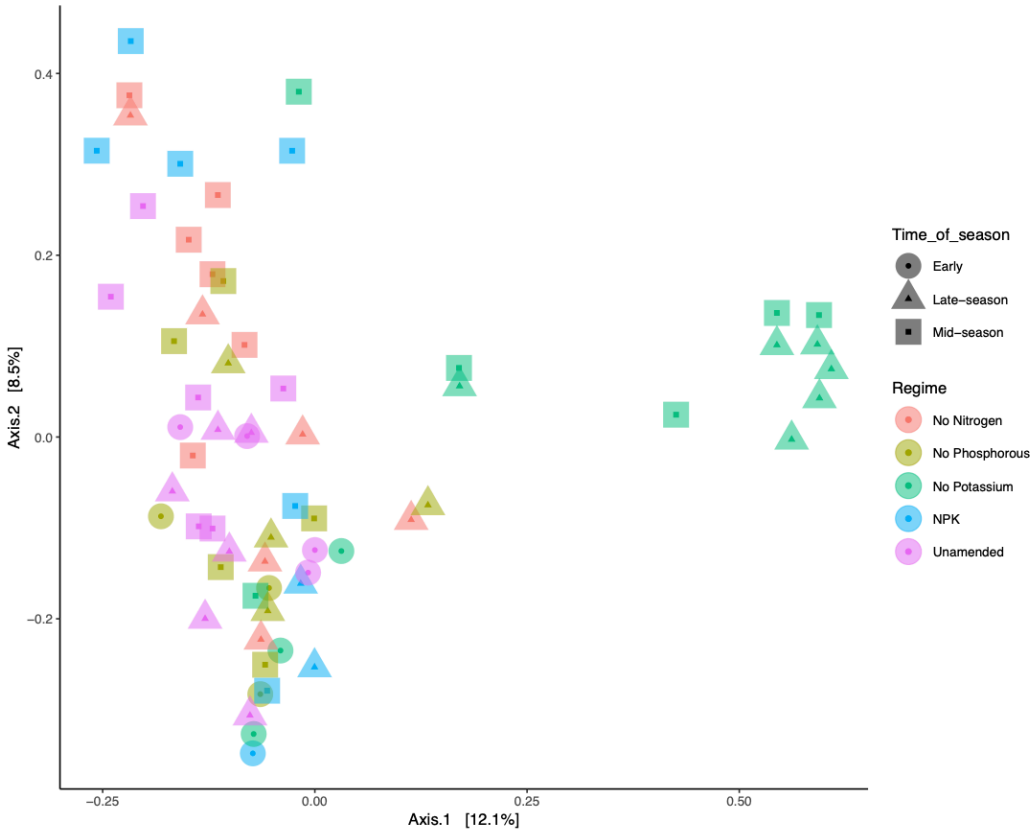

Figure 3.5 Heatmap of indicator species in sampled nutrient regimes

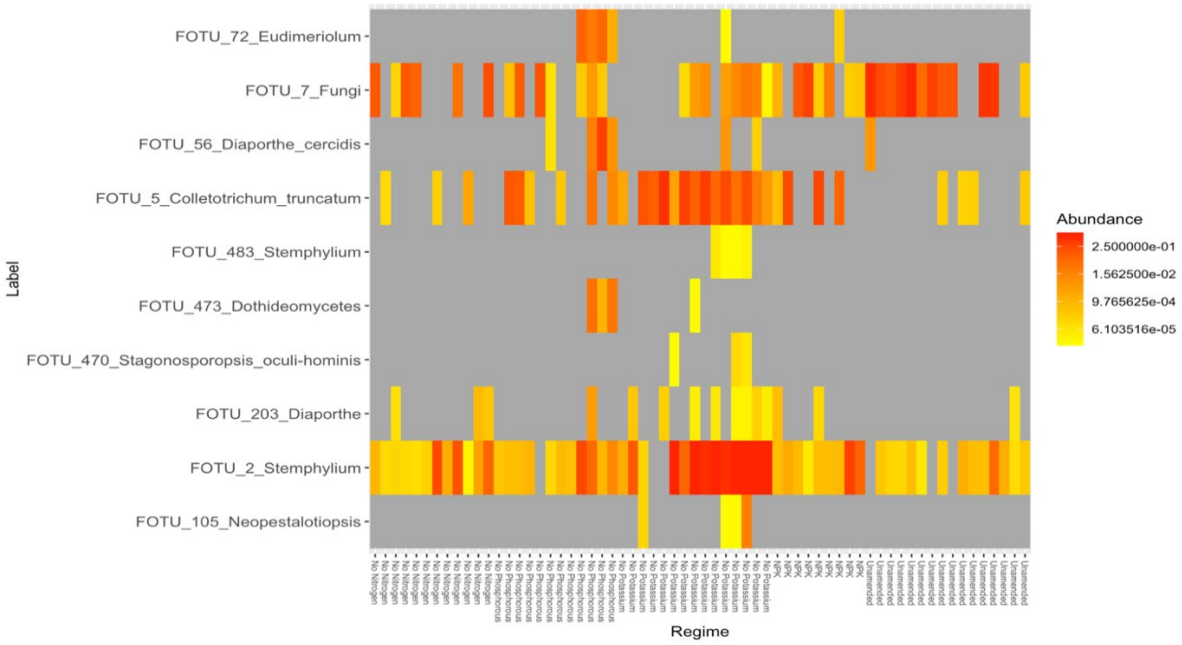

Figure 3.6 Abundance of the *Stemphylium* indicator 'FOTU_2' throughout the sampled growing season