The diversity of oomycetes associated with cotton seedlings in Alabama and the assembly of the spermosphere microbiome of cotton and soybean

by

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A thesis submitted to the Graduate Faculty of Auburn University
in partial fulfillment of the requirements for the Degree of Master of Science

Auburn, Alabama
December 10, 2022

Keywords: Oomycetes, species richness, spermosphere, microbiome, virulence

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Abstract

Cotton and soybean are major crops in the US grown for fiber and protein. Fungal and oomycete pathogens can affect cotton and soybean seedlings resulting in seed and seedling damping-off. Many different oomycete species may be associated with seedling disease, but the species diversity is not as well characterized in cotton compared to soybean. Several surveys of the oomycetes associated with cotton seedlings in Alabama and the US precede the development of molecular tools for species identification and recent changes in oomycete taxonomy. Chapter 1 reviews the relevant literature for seedling diseases, management, and oomycete taxonomy. Then in chapter 2, we seek to identify the diversity of oomycete species associated with cotton seedlings in Alabama using molecular tools, determine the pathogenic species using a seed virulence assay and correlate oomycete diversity with edaphic factors. We hypothesize the precise identification of the oomycete pathogens and their correlation with field properties will help inform better management strategies to maximize yield.

We identified a total of 339 oomycete isolates associated with cotton seedlings in North, Central and South Alabama in 2021 and 2022. The identified oomycetes included 28 different species of which 25, including an unnamed species, and species with diverse ecological roles like mycoparasites that have not been previously reported to be associated with cotton seedlings in Alabama and the US as confirmed by the USDA ARS fungal database. Surprisingly, we did not collect any *G. ultimum* isolate contrary to previous studies. Although *Globisporangium irregulare* and *G. sylvaticum* have been previously reported in past studies. Six species were pathogenic to cotton seeds and those isolated in frequencies from cotton seedlings in both years were *G. irregulare* and *P. nicotianae.* Species composition and richness varied by soil type. Northern fields with higher cation exchange capacity had higher oomycete richness, which reduced as we sampled in southern soils with lower cation exchange capacity. Some species like *P. nicotianae* prefer soils with low sand content while other species like *G. irregulare* were isolated across all soil types irrespective of the sand content.

Additionally, many damping-off pathogens residing in soil are stimulated by exudates released by seeds shortly after planting in an environment called the spermosphere. Pathogens move by chemotaxis towards exuding seeds, colonize the seeds and cause disease in a few hours.

Therefore, understanding microbial interactions in a spermosphere may give insight into seedling disease control and identification of native microbes that may be potential biocontrol. Interestingly, microbial interactions in the spermosphere are not well understood compared to later phases and plant-associated environments after which seedling disease must have occurred. A major challenge linked with this could be the absence of an easy and reliable method to collect spermosphere samples for high throughput sequencing like we have for the rhizosphere or phyllosphere. In chapter 3, we developed a simple reliable sampling method to collect the spermosphere and used this method to determine the changes in microbial diversity in the spermosphere of cotton and soybean.

We developed a sampling method that sufficiently collects spermosphere samples as defined in space and time for high throughput sequencing. Spermosphere microbial communities differed between soybean and cotton and from bulk soil. These communities develop as early as twelve hours after seeds are sown. Major changes observed in microbial communities was a reduction in the evenness of taxa from time point 0 to 18 after seeds were planted. Particularly, soybean spermosphere had the greatest reduction in taxa evenness, followed by cotton and bulk soil, which only had a slight reduction in taxa evenness over time. Significant indicator taxa enriched in soybean spermosphere included Bacilli and Gammaproteobacteria while cotton spermosphere was enriched in Planctomycetes compared to bulk soil. Additionally, we identified some genera with long history of plant growth promotion such as *Paenibacillus* and *Brevibacillus* to be enriched in soybean spermosphere.

Overall, this study demonstrates the need for prescriptive control strategies tailored to fields based on apparent soil properties and disease pressure history. It serves as a baseline study for future application in developing or determining better seed treatments and resistant varieties for oomycete pathogens in cotton seedlings. The method developed for spermosphere microbiome sequencing could be utilized for various future studies and some of the microbes enriched in soybean spermosphere could be further explored for improved plant growth. In chapter 4 we discuss the impacts and significance of these experiments. Thus, this thesis facilitates precision agriculture for a more sustainable control of seedling diseases caused by oomycetes and improves knowledge of the plant microbiome.

Acknowledgements

First, I am grateful to God, my source, for His unending grace, strength, goodness, guidance, and help. I would never have achieved this without Him!

I sincerely appreciate my major advisor, Dr. Zachary Noel, for his excellent mentorship and opportunity to thrive. I am thankful to my committee members, Dr. Kathy Lawrence and Dr. Mark Liles for their advice and contributions towards the successful completion of my program. My gratitude to the directors of the Alabama Experiment Stations, John McInroy, Tyler Sandlin for their help with the collection of diseased cotton samples and associated metadata. I am also thankful to Dr. Martin Chilvers and Dr. Alejandro Rojas for their prompt response and help with figuring out the best vials for oomycete growth. I appreciate every funding organization including the College of Agriculture, the ALFA farmers, the Alabama soybean producers, and Citrus Extracts. My sincere gratitude to the members of the Noel lab including Laura Rodriguez, Emily Munroe, and Beatrice Severance for their help with sample collection and for being such an amazing team.

I appreciate my parents, Mr. Sunday I. Olofintila and Mrs. Deborah Olofintila for their consistent prayers and unconditional love. I am grateful for my fiancé, Omolayo Olorunwa, for being a strong support system, an ear that listens and for always relieving my stress- thank you for everything! I appreciate Ms. Cassandra Labor for her care, prayers, love, and financial support. My gratitude to Festus Ajibefun for always taking me to school and everywhere else before I got my car and Dr. Seun Oladipupo for his guidance in navigating graduate school. I also appreciate every member of the African Students Association for a home away from home.

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1. CHAPTER ONE

Introduction and Literature Review

Oomycete pathology

With the world population projected to increase by over 2 billion by year 2050 and food demand exceeding production, the optimization of crop yield becomes important to achieve food security (United Nations 2022). Currently, crop yield is limited by a number of factors including diseases that have been estimated to account for $220 billion loss in global crop production by the Food and Agricultural Organization of the United Nations (FAO 2019). These diseases are caused by various microorganisms such as oomycetes that have a wide distribution across diverse habitats and consist of some notorious plant pathogens of historical and economic significance (Kamoun et al. 2015). An example is *Phytophthora infestans*, the primary pathogen responsible for potato late blight that was a leading factor for the 1800's Irish potato famine, which instigated pathogen population studies and collaborations between nations to track and control pathogen races (Cantwell 2017; Goodwin et al. 1992; Turner 2005). Among many others, oomycetes are also responsible for root and stem rot, fruit rot, wilt, seedling diseases (pre- and post-emergence damping off) of vegetables, turf grasses and field crops (Koike 2015; Ho 2018). This thesis focuses on the oomycetes responsible for seedling diseases.

Seedling diseases caused by oomycetes

Seedling disease is a widely distributed disease caused by oomycetes and fungi in several hosts and across various locations. Yield losses have been estimated for crops including soybean with over 525 million USD in a decade (Crop Protection Network 2019), cotton with over 40 million dollars lost yearly (Strayer-Scherer 2021) and corn with 4.2 billion USD lost in four years (Mueller et al. 2020). Seedling disease caused by oomycetes are favored by wet, compacted soils with poor drainage (Wise et al. 2015) observed as skips in the field of dead seeds that never emerged from soil (pre-emergence damping-off) or dead emerged seedlings (post-emergence damping-off). Even if the plant survives initial infection, stunted plants with water-soaked lesions along the roots can limit yield. Additionally, recent shifts towards agricultural practices such as reduced or no tillage, earlier planting dates, and cover cropping have been reported to increase pathogen populations (Bakker et al. 2016; Chilvers et al. 2020a). Although fungi and oomycetes

are different phylogenetically they both may be responsible for seedling disease, but oomycetes are often reported as the most important members in terms of frequency of isolation (Rizvi 1996; Griffin 1990). Rizvi (1996) reported the oomycetes, *Pythium* and *Phytophthora* as the most frequently isolated species (over 56%) from diseased soybean seedlings in Iowa across multiple years. *Pythium* species were also the most abundant cotton seedling pathogen isolated in Tennessee (Johnson 1978). More than one oomycete species may be recovered from a diseased crop sample (Noel et al. 2021) and correct species identification can aid the determination of primary pathogens (Broders et al. 2007). Thus, proper identification of associated oomycete species is critical for the characterization and management of seedling pathogens.

Oomycete biology and taxonomy

Oomycetes are filamentous eukaryotes sharing similar morphology and mode of nutrition with fungi and once classified as Phycomycetes with other fungal groups (Bessey 1950). Before the advent of molecular technologies, they were classified and identified solely based on morphology. Diploid vegetative nuclear state, presence of cellulose cell wall, asexual biflagellate zoospores and sexual oospores distinguished them from other fungal groups (Dick 1969; Paul et al. 1995). Generally, their hyphae are usually hyaline (colorless) and coenocytic (no cross-walls or septa), and sometimes bearing zoospore-containing sporangia that are either filamentous or globose. Direct germination and growth of hyphae from sporangium is possible for some species. However, they mainly reproduce asexually by a sporangium that releases biflagellate zoospores that is differentiated in some species from the undifferentiated protoplasm within vesicles at the tip of the discharge tube emerging from the sporangia (Uzuhashi et al. 2010; Bessey 1950). Particularly, the formation and differentiation of zoospores within vesicles outside the sporangium is typical of *Pythium* species and distinguishes it from the morphologically similar genera, *Phytophthora* that forms and differentiates zoospores within the sporangium (Jeger and Pautasso 2008; Bessey 1950). When applied for phylogeny purposes, the morphology of sporangia and ultrastructure of zoospores produced results consistent with molecular tools, but the complexity of obtaining zoospore sections and recovering sporangium make molecular phylogeny easier to pursue (Lévesque 2011; Sekimoto et al. 2008).

Sexual reproduction is by fusion of an oogonium (female gametangium) and the antheridium (male gametangium). Together they form a mature oospore, which is very hardy and

can last in soils for many years (Fry and Grünwald 2010). In terms of morphology, antheridia can be either hypogynous or monoclinous/diclinous. The oogonium may be ornamented or smooth and terminal or intercalary with varying numbers of associated antheridia. The oospore formed may fill the entire oogonium in which it is termed plerotic and space may exist between oospore and oogonium wall in aplerotic species (Dick 1969; Bessey 1950). The oospore morphology has been used in the taxonomy of oomycetes. However, compared to sporangia and zoospore morphology, they have been reported to show little correlation with phylogeny (Lévesque 2011). With the advent of molecular phylogenetic tools like 18S ribosomal DNA (rDNA) sequencing, oomycetes were grouped within the Kingdom Stramenopila as results reveal their close relationship with some photosynthetic eukaryotes such as brown algae and diatoms (Sogin and Silberman 1998; Uzuhashi et al. 2010). For ease, Levesque and de Cock (2004) organized the genus *Pythium* into 11 clades (A-K) based on molecular and morphological differences. However, the taxonomy of *Pythium* in particular has seen significant changes in recent years including the grouping of *Pythium* clade K into the new genus *Phytopythium* (Bala et al. 2010), and the more recent split of the clades A through J into four new genera, based on phylogenomics (Nguyen et al. 2022). This split resulted in four different genera, namely, *Pythium* (Clades A, B, C, D), *Globisporangium* (Clades E, F, G, I, J), *Elongisporangium* (Clade H) and *Pilasporangium* (a monotypic genus). The major challenge associated with the split is the renaming of some of the most important oomycete species in the world (Kamoun et al. 2015) like *Pythium ultimum* to *Globisporangium ultimum.* It is thus crucial to apply modern taxonomic information for the precise identification of isolated oomycetes, but it is also important for communication that the genus *Pythium* remains part of our active vocabulary. In this thesis, we used the new taxonomic names reported by Nguyen et al. (2022).

Diversity of oomycetes associated with seedling diseases

Various species of *Globisporangium* (*G.*), *Pythium* (*Py.*), *Phytopythium* (*Phy.*), and *Phytophthora* (*P.*), may be associated with seedling disease. Therefore, it is important to accurately identify associated pathogens to effectively manage these seedling diseases. Before the advent of improved taxonomic tools, spore morphology was the basis for the identification of oomycetes associated with crops, particularly sexual spores. For example, Howell (2002) identified *Py. aphanidermatum* and *G. ultimum* as cotton seedling pathogens, based on the morphology of their sexual structures. However, isolates that did not produce sexual structures could not be reliably

identified to species level. In 2000 and 2001, *G. ultimum and Py. aphanidermatum,* identified by reproductive structures*,* were the two oomycete species isolated from cotton seedlings in Alabama with *Py. aphanidermatum* having a lower isolation frequency compared to *G. ultimum* (Palmateer et al. 2004). Similarly, DeVay (1982) reported *G. ultimum, Py. aphanidermatum and G. irregulare* as the most frequently isolated oomycetes from diseased cotton seedlings in California, identified majorly by oospore morphology. *G. ultimum*, *G. sylvaticum, G. irregulare*, and *G. heterothallicum* were pathogenic to cotton seedlings and identified by sexual spores in Tennessee (Johnson 1978). A common challenge faced in the morphological identification of oomycetes is the difficulty in identifying species that do not sporulate easily.

The availability of improved sequencing technologies has increased the discovery of oomycetes with over 1,500 species known today (Judelson and Ah-Fong 2019) and also improved taxonomy. These technologies are gaining grounds in the study of oomycete diversity across seedlings of crops in different locations. Broders et al. (2007) identified *Py. dissotocum, G. sylvaticum* and *G. irregulare* as the three most frequently isolated species from soybean and corn seedlings in Ohio with variation in seed pathogenicity between crops. However, previous reports based on morphological identification recovered *G. splendens, G. irregulare and Py. catenulatum* from Ohio soybean fields with disease pressure using the soil-baiting technique (Dorrance et al. 2004). Additionally, Rojas et al. (2016) reported higher recovery of *G. irregulare* and *Py. sylvaticum* from Arkansas soybean seedlings contrary to previous studies with solely morphological identification where *Py. irregulare* was the least recovered oomycete *and Py. sylvaticum* was not reported (Kirkpatrick et al. 2006). He also recovered the pathogenic oomycete, *Py. oopapillum* with highest frequency from Ohio soybean seedlings which was not recovered in previous studies by Rizvi (1996). It is likely that these species have always been present, and improved taxonomy and methods now enable us to find them.

Since there has been no in-depth study on the diversity of oomycetes associated with crop seedlings in Alabama, we focused on the use of molecular techniques, particularly the Internal Transcribed Spacer (ITS) Sequencing to identify isolated oomycete species (Noel et al. 2020; Rojas et al. 2016). For this survey of oomycete diversity, we selected cotton because it currently ranks number one crop in terms of field production area and generated revenue estimating $288 million in Alabama (Meyer 2022) with yield affected by oomycete seedling disease (Strayer-Scherer 2021).

Previous reports have confirmed that the recovery of oomycete varies depending on soil types and locations (Hoppe and Middleton 1950). For instance, Broders et al. (2009) reported a positive correlation in the frequencies of *Pythium species* isolated and soil silt content in Ohio soybean and corn fields. Because previous studies (Rojas et al. 2016; Broders et al. 2007) have reported close connection with edaphic factors and not much is known about the distribution of oomycetes associated with cotton in Alabama, we set out to improve this knowledge in Chapter 2. This knowledge can help inform regional patterns in oomycete species and help prioritize future research on specific prevalent species.

Management of seedling diseases caused by oomycetes

The oospore and sporangia are major resting structures produced by oomycetes. These structures are triggered to germinate by environmental cues such as exudates from germinating seeds or seedlings after which they produce zoospores or hyphae that infect seed and seedlings, thus resulting in pre- and post-emergence damping off, respectively (Agrios 2005). Stanghellini and Hancock (1971) confirmed 78% of *G. ultimum* sporangia germinate within four hours in soil containing common bean (*Phaseolus vulgaris*) seed and colonization within 24 hours. Similarly, *G. ultimum* has been reported to colonize 100% of cotton seeds within the first 12 hours after seeds are sown (Nelson 1988).

Various methods have been applied in managing seedling damping-off including fungicide and biopesticide seed treatments and applications, deployment of cultural practices such as planting on raised beds or at higher soil temperatures that improve seedling emergence (Isakeit 2016), and an integrated approach including all aforementioned measures (Lamichhane et al. 2017). However, many of these strategies are either variable in activity or excessive use can be problematic if misused. Agriculture accounts for 66% of pesticide use in the US of which fungicides take up 16% (US EPA 2015a). Some crops with the highest pesticide usage include corn, soybeans, potatoes, and cotton (Fernandez-Cornejo et al. 2014). The major chemicals used to control oomycete plant pathogens include the enantiomers, metalaxyl and mefenoxam which act by disrupting the RNA polymerase 1 complex of oomycetes thereby inhibiting ribosomal RNA synthesis. However, in some agricultural production environments, *Pythium ultimum* was reported to express ABC transport proteins of the G subfamily in response to metalaxyl and release ubiquitin-protein ligase which functions to denature proteins so that rDNA, the target of

mefenoxam is reduced or modified. This way, the pathogen develops resistance to the fungicide (Lévesque et al. 2010). This fungicide resistance problem, coupled with rising concerns of toxicity to non-target aquatic and terrestrial organisms (Zubrod et al. 2019; Iyaniwura 1991) has led to exploration of naturally occurring biopesticides (US EPA 2015b). Of all biopesticides, microbial biopesticides derived from microorganisms dominate the market (Chandler et al. 2011; Arakere et al. 2022) and have been applied in crop production with reported prospects to reduce the reliance on and excessive use of chemical fungicides (Adesemoye et al. 2009).

Plant Growth Promoting Rhizobacteria (PGPR) are bacteria capable of producing microbial biopesticides that have been isolated from soil surrounding plant roots (rhizosphere) and applied for crop protection from pathogens and improved plant growth (Hassan et al. 2019). For example, spore-forming *Bacillus subtilis* strain GBO3 registered as Kodiak® and *Bacillus subtilis* MBI 600 is used commercially as seed-treatment biopesticides for seedling disease suppression and plant growth promotion in cotton (Brannen and Kenney 1997; Samaras et al. 2021). Another PGPR utilized commercially in crop protection from seedling diseases caused by oomycetes is Serenade ASO, *Bacillus subtilis* strain QST 713 which could be applied at planting with the intention of improving seedling emergence or afterwards to protect against post-emergence damping-off (Jo et al. 2014). However, sensitivity to edaphic factors and performance variation in the field makes the application of microbial biopesticides challenging (Fenibo et al. 2021). This variation in performance may be due to poor competence of the biocontrol agents to outcompete native soil microbes (Whipps 2001; Schreiter et al. 2018). Taking a more detailed approach to understand the initial series of interactions within soil microbial community in the presence of a germinating seed could offer answers to some challenges in biological control applications. Therefore, in Chapter 3, I explored the microbiota associated with a plant environment called the spermosphere so that we may understand better how to tip the scales in favor of beneficial microbiota rather than seedling pathogens. Below I describe the spermosphere plant environment in more detail.

The spermosphere and seed exudates' influence on the microbiome

The transient, immediate five to ten-millimeter zone of soil surrounding an imbibing and germinating seed is termed the spermosphere (Nelson 2004a; Stanghellini and Hancock 1971). It is critical to plant development (Windstam and Nelson 2008) and can be considered the basis of

microbial establishment with crops. This environment is formed pre-germination as seeds imbibe water, leading to the saturation of nutrient reserves and leakage of stored nutrients (as exudates) through damaged cell walls and membranes. These released exudates including nutrients and an array of defensive antimicrobials select microbial communities that influences seedling and plant establishment (Nelson 2018; Scarafoni et al. 2013). For example, the release of long-chain unsaturated fatty acids in seed exudates of corn and cucumber has been reported to promote the development of sporangia of *G. ultimum* leading to a complete colonization within the first 24 hours after sowing the seeds (Nelson 2004). However, when the bacterium *Enterobacter cloaceae* is applied as seed treatments on corn and cucumber seeds, differential responses were reported (Windstam and Nelson 2008a). It was observed that even though germinating corn seeds released more unsaturated fatty acids than cucumber seeds, the high percentage of simple sugars including glucose, sucrose, and fructose in exudate content of corn inhibited the ability of the bacterium to compete with the oomycete pathogen for nutrients because the bacterium preferred to utilize the abundant simple sugars rather than more complex fatty acid carbon substrates leaving enough for the oomycete. The bacterium was observed to outcompete the pathogen in cucumber seeds with lower exudate sugar content. Results similar to cucumber was also observed in cotton (Kageyama and Nelson 2003). Additionally, Scarafoni et al. (2013) identified chitinase secreted in seed exudates of lupin that inhibited conidia germination and hyphal elongation of pathogenic fungal species. However, as important and insightful spermosphere research can be, fewer studies are recorded compared with the later phases such as the rhizosphere after seedling pathogens have fully colonized the host (Shade et al. 2017; Schiltz et al. 2015). For example, a search of publications with “spermosphere” in the title on the publons database (Accessed 9/26/2022) yielded only 52 results while “rhizosphere” yielded 9807 results (Publons 2022).

Ecology of microbes in the spermosphere

Slykhuis (1947) was the first to report microbial interactions around a germinating seed when he observed three fungal species that reduced *Fusarium culmorum* disease severity by inhibiting the development of the pathogen around a germinating seed, an observation that was not recorded in bulk soil. He termed this environment, “The Spermatosphere”. In the late 1950s, this environment became well defined as a “zone of elevated microbial activity” influenced by seed exudates and named “The Spermosphere” (Verona 1958). Since microbes must compete for the

limited nutrients in seed exudates, their fitness or ability to colonize the spermosphere, expressed as spermosphere competency is largely determined by various traits. This includes the ability to form biofilms, adhere to seeds, efficiently scavenge and utilize nutrients, exhibit chemotaxis and antibiosis (Shade et al. 2017; Lemanceau et al. 2017).

Several studies involving microbial interactions within the early hours of planting have been previously conducted using the culture-dependent technique. The bulk of identified microbes include fungi and bacteria originating from the growth media, seed surface or within the seed itself, of which the native soil microbial communities constitute the major seed/seedling colonizers that differ from plant to plant (Buyer et al. 1999). The current study in this thesis (Chapter 3) focused on understanding spermosphere microbiome of two important crops in Alabama, soybean and cotton (USDA 2021c). The assembly of microbial communities in the spermosphere seems to be based on function instead of community structure (Buyer et al. 1999). Ota et al. (1991) showed increased populations of nitrogen-fixing bacteria in the spermosphere of different plant species compared to bulk soil. Also, fatty acid metabolizing bacteria in suppressive compost was observed to colonize the spermosphere of cotton, degrade exuded linoleic acids and suppress sporangia germination of *G. ultimum* (McKellar and Nelson 2003). By growing various seeds including soybean in autoclaved water for 48 hours, Johnston-Monje et al. (2021) identified *Fusarium, Pseudomonas* and *Bacillus* as major microbes in they termed the spermosphere, which would have majorly originated from the seed since soil was not used. The application of high throughput technologies to capture microbial diversity in the spermosphere is very limited. However, the majority of these studies fail to capture the very first hours when microbial communities are being established or focus on the seed microbiome by growing seeds in water, which excludes the soil (Moroenyane et al. 2021; Johnston-Monje et al. 2021). This may be owing to various reasons including the lack of a sampling method to collect the spermosphere for microbial analysis (Schiltz et al. 2015). Therefore, it is important to develop a method that enables quick and easy collection of spermosphere soils that is amenable to high-throughput sequencing. In the future, these data can be used to apply this knowledge in harnessing native spermosphere microbes for applications in biological control or better understanding the basis of variation in biocontrol performance.

Conclusions and thesis objectives

Overall, the literature reviewed herein suggests there is a gap in knowledge surrounding the oomycetes associated with cotton in Alabama and a need for improved knowledge on the spermosphere microbiome. Therefore, the objectives for this thesis were to 1) identify the diversity of pathogenic oomycetes associated with Alabama cotton seedlings and 2) develop a spermosphere sampling method that can be used to sequence the spermosphere microbiome to improve knowledge of spermosphere microbiome in cotton and soybean. In Chapter 2, I detail the diversity of oomycetes associated with cotton seedlings collected across Alabama regions, their virulence towards cotton seeds and richness across soil edaphic factors. In Chapter 3, I explain the development of a method to sample the spermosphere microbiome of soybean and cotton and as a proof of concept sequenced the bacterial communities present over the course of 18 hours after planting. The knowledge gained within these sets of experiments will be crucial for the translation into improved management of oomycete seedling diseases.

2. CHAPTER TWO

Diversity of Oomycetes Associated with Cotton Seedlings in Alabama

Abstract

Many oomycete species are associated with seedlings of crops, including cotton (*Gossypium hirsutum*) leading to annual production losses up to several million. The diversity of oomycete species across Alabama regions is not well known. This study aims to identify oomycetes associated with Alabama cotton seedlings, correlate diversity with soil edaphic factors and assess virulence toward cotton seeds. Thirty symptomatic cotton seedlings were collected from 13 fields each, across Alabama regions in 2021 and 2022. Oomycetes were isolated from symptomatic cotton roots two to four weeks after planting by plating onto a semi-selective agar medium. Internal transcribed spacer region sequencing identified the resulting isolates. A seed virulence assay was conducted *in vitro* to verify the pathogenic species. 182 and 157 oomycete isolates were obtained across 24 and 21 species respectively in 2021 and 2022. Northern Alabama soils had the richest oomycete diversity with higher cation exchange capacity compared to soils in central and southern Alabama coastal plains. *Pythium irregulare* was virulent and abundant across all Alabama regions in both years, *Phytophthora nicotianae* was virulent and more abundant in soils with lower sand content and was third and first most abundant across both years. To our knowledge, 25 of the obtained oomycete species, of which six are pathogenic, have not been previously reported in Alabama or Southern US. Altogether, this knowledge will help facilitate effective management strategies for cotton seedling diseases caused by oomycetes in Alabama and the US.

This chapter is written for the intended publication into *Plant Disease*

Olofintila, O.E., Lawrence, K.S., Noel, Z.A., 2023. Diversity of oomycetes associated with cotton seedlings in Alabama. *Plant Disease.*

Introduction

Cotton (Gossypium hirsutum L.) is crucial for our economy and everyday livelihood, generating an annual revenue of about 7 billion USD to the US and over 288 million USD to Alabama where it ranks first in terms of production area (USDA 2021c; Meyer 2022). However, cotton production is threatened by various diseases amongst which seedling disease accounts for 34% of total losses and estimates over 40 million dollars in production loss each year, excluding replanting costs (Blasingame, D 2005; USDA 2021a; Strayer-Scherer 2021). Cotton seeds are the second most expensive operating cost for farmers estimating about $1 billion annually (Blair McCowen 2022; USDA-ERS 2022) thus, protecting the seed from seedling diseases is crucial. Seedling diseases are characterized by death or decay of seeds and seedlings before and after emergence termed pre- and postemergence damping-off. Necrotic lesions along the hypocotyl and roots of seedlings are also distinctive of cotton seedling diseases (Rothrock and Buchanan 2007). In cases when disease does not result in death, seedlings can be stunted, chlorotic, and may not yield fully. Several studies in the US have reported major cotton seedling pathogens to include the fungi *Rhizoctonia solani, Thielaviopsis basicola* and *Fusarium spp.* and oomycetes *Pythium spp.* and *Phytophthora spp.* (Hu and Norton 2020; Rothrock and Buchanan 2007; Wrather et al. 2002).

Oomycetes are fungal-like organisms due to similar morphology and method of obtaining nutrients. Though previously grouped with true Fungi, molecular phylogenetic studies have revealed their close relationship with some photosynthetic eukaryotes such as brown algae and diatoms (Paul et al. 2005; Uzuhashi et al. 2010), thus, their current classification within the Kingdom Stramenopila. With respect to crop production, they include some of the most devastating pathogens responsible for significant losses in economically important crops such as potatoes, tomatoes, soybean, corn and cotton (Kamoun et al. 2015; Thines and Kamoun 2010; Rojas et al. 2019; Alejandro Rojas et al. 2016; Rothrock and Buchanan 2007). Many species of oomycetes can associate with crop seedlings. For example, Rojas et al. (2017ab) revealed over 80 species associated with soybeans and over half were pathogenic towards seed and roots. Therefore, the diversity of species associated with seedlings and factors that drive that diversity are important for improved management. However, the diversity of species associated with cotton seedlings is not as well characterized.

Past studies identified oomycete isolates from diseased cotton seedlings by morphological features such as the structure of the sporangia or oospores (Howell 2002; Palmateer et al. 2004;

Wrather et al. 2002). Howell (2002) identified *Pythium aphanidermatum* and *Globisporangium ultimum* as cotton seedling pathogens, based on the morphology of their sexual structures and isolates that did not produce sexual structures could not be reliably identified to species. In Alabama, *G. ultimum* and *Py. aphanidermatum* were the two oomycete species isolated from cotton seedlings and identified by reproductive structures (Palmateer et al. 2004). Similarly, DeVay (1982) reported *G. ultimum, Py. aphanidermatum* and *G. irregulare* as the most frequently isolated oomycetes from diseased cotton seedlings in California, identified majorly by oospore morphology. *G. ultimum*, *G. sylvaticum, G. irregulare*, and *G. heterothallicum* were pathogenic to cotton seedlings and identified by sexual spores in Tennessee (Johnson 1978). Although useful, similarity in spore morphology and difficulty in spore recovery for some oomycete members can make species separation based purely on morphology challenging (Nam and Choi 2019). Since previous surveys of oomycetes on cotton were conducted, molecular barcoding tools have been developed to support morphological identification for better species separation including the internal transcribed spacer (ITS) and Large-subunit (LSU) rDNA D1/D2 region of the nuclear ribosomal DNA (rDNA), cytochrome oxidase II (coxII) gene, ribosomal protein in the S10 family of the mitochondria, and beta-tubulin gene (André Lévesque and De Cock 2004; Uzuhashi et al. 2010; Foster et al. 2021; Villa et al. 2006). Currently, ITS and the coxII loci are the recommended barcodes to identify most oomycetes to species level, and ITS has the most representation across oomycetes (Robideau et al. 2011). Additionally, the taxonomy of oomycetes has been refined with new species descriptions like the grouping of some previous *Pythium* members into the new genus, *Phytopythium* (Cock et al. 2010; de Cock et al. 2015; Bala et al. 2010) and the recent split of the genus *Pythium* into four different genera, namely *Pythium* (Clades A, B, C, D), *Globisporangium* (Clades E, F, G, I, J), *Elongisporangium* (Clade H) and *Pilasporangium* (a monotypic genus) (Nguyen et al. 2022). The major challenge associated with this split is the renaming of some important oomycetes like *Pythium ultimum* to *Globisporangium ultimum* (Kamoun et al. 2015). It becomes important to adapt these changes in oomycete taxonomy and improved molecular tools, to accurately identify oomycetes associated with cotton seedlings.

Additionally, species could differ across locations. In North America, Rojas et al. (2016) isolated *G. sylvaticum* from North Central states; *G. heterothallicum* and *G. ultimum* from states that were further north and *G. irregulare* from southern states. Species can also vary in their virulence, sensitivity to seed treatment fungicides (oomicides) (Noel et al. 2020; Radmer et al.

2017), and conditions which they prefer to cause disease (Matthiesen et al. 2016). Therefore, it is prudent to periodically perform surveys to know the pathogens present and factors that drive diversity, especially in regions that are under-sampled like Alabama.

Weakly pathogenic or saprobic species may also contribute to seedling disease as secondary pathogens. Furthermore, non-pathogenic oomycetes associated with cotton seedlings may also be important since some can be mycoparasitic or competitors with pathogenic species (Bro 2002; Lifshitz et al. 1984). Therefore, this study focuses on the entirety of species associated with cotton seedlings and the geographic range of these species in Alabama. The objectives of this manuscript were threefold: 1) to isolate oomycetes associated with cotton seedlings in Alabama and identify them using ITS sequencing 2) correlate oomycete diversity with soil edaphic factors and 3) test the virulence of isolated oomycetes towards cotton seeds *in vitro* using a seed virulence assay.

Materials and Methods

Sample collection. Seedlings that were stunted, slow to emerge or had necrotic lesions on roots and hypocotyl were collected from fields distributed equally across seven counties in Alabama; Northern: Limestone and Lawrence counties; Central: Autauga and Macon counties; Southern: Baldwin, Escambia, and Henry Counties (

Table 1). Information on variety and seed treatments associated with collected samples were documented (Table 2). Two fields in each county (except Henry County) were sampled for a total of thirteen locations in 2021 and 2022 (Figure 1). The fields sampled were chosen based on historic cotton production and across a diversity of soils representing four of six soil lineages present in Alabama, representing the major agricultural soils in Alabama (Mitchell and Loerch 2008). For example, Northern soils consist of Appalachian plateau and Limestone valley soils. Central and Southern Alabama consists of a mix of Blackbelt and Coastal plain soils. A total of 30 symptomatic seedlings were randomly collected from each field. Bulk soil was also collected in a W-shaped transect from each field and submitted to the Soil, Forage, and Water Testing Laboratory in Auburn University for physical and chemical analysis. Locations and soil characteristics are listed in

Table 1.

Oomycete isolation. Collected seedlings were placed in sterile labelled coin envelopes and transported to the laboratory where the seedlings were processed within 24 hours after collection.

Seedling roots were washed under running tap water for 30 minutes until adhering soils were removed and 10 to 20 mm root pieces with lesions were placed on Corn Meal Agar (HiMedia, India) medium amended 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 (Jeffers 1986). Petri dishes were observed daily for a week for mycelial growth. Hyphal tips of coenocytic hyphae were transferred to a fresh CMA+PARPB agar medium.

DNA extraction and identification. Crude genomic DNA was isolated using a fast extraction method (Noel et al. 2021). The cells were lysed by placing hyphae of each isolate into extraction solution (1M Tris solution, pH-8.0) and incubating at 95°C for ten minutes then diluting with 3% bovine serum albumin (Sigma-Aldrich, USA). The Internal Transcribed Spacer region (ITS) was then amplified by polymerase chain reaction (PCR), which included a final concentration of 1× DreamTaq green, 0.4 µM of ITS6 and ITS4 primers, and 1 µl of the extracted DNA. The thermal cycling program was performed as followed; 94°C for 3 min, 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, which was then followed by a final cycle at 72°C for 7 min. The amplification of ITS was confirmed through gel electrophoresis. Successfully amplified ITS amplicons were cleaned by adding 5 µl Exo-AP solution to 5 µl PCR product then incubated for 37°C for 30 minutes, 80°C for 15 minutes and final hold at 10°C. The Exo-AP solution consisted of Exonuclease 1 at 3.57U, Antarctic Phosphatase at 0.29U, buffers and ddH20 per µl reaction. Sequences from cleaned amplicons were obtained by Sanger sequencing with the ITS6 primer from Eurofins (Eurofins Genomics, USA). Sequences were trimmed for quality using Geneious Prime (Geneious Prime, New Zealand) to remove regions with over one percent chance of error per base. Trimmed reads were searched against a curated oomycete nucleotide database using BLASTn containing sequences from Robideau et al. 2011 and LéVesque and De Cock 2004. Oomycete sequences with query coverage higher than 98% and sequence identity higher than 97% were assigned to a species (Alejandro Rojas et al. 2016). Sequences that did not meet these criteria against the curated database were then used in BLASTn search against the genebank nucleotide database to determine if they matched sequences outside the database. Sequences were identified into respective clades by searching for Genbank accessions through the UPGMA (unweighted pair group method with arithmetic mean) tree of oomycete clades (Robideau et al. 2011). Following

recent changes to oomycete taxonomy, we retain genus names in this study as reported by Nguyen et al. (2022).

Virulence of oomycetes to cotton seeds. A seed virulence assay (Broders et al. 2009) was conducted by placing a 6 mm plug of each oomycete isolate at the middle of petri-dishes containing water agar (1.7%) and left to grow at room temperature (25 ± 2°C) until the colony reached about 40mm in diameter. Then five surface-disinfested nontreated cotton seeds (Delta Pine 1646 B2XF) free of cracks and discoloration were placed at equal distances from the center on the edge of a growing oomycete colony. Seed surface disinfestation was achieved by placing seeds in 6% sodium hypochlorite solution for ten minutes and rinsed three times with sterile deionized water. Each isolate was repeated three times and due to large number of isolates, the experiment was split into groups with each group having one isolate repeated (*Pythium irregulare* OEO-O28) as a positive control, which consistently had disease severity ratings between 95% and 100% throughout the experiment. The negative control was cotton seeds without any oomycete present. Each seed was given a disease severity score between zero and four (Broders et al. 2009; Rojas et al. 2017; Noel et al. 2019). Seeds that germinated without lesions present along the radicle were assigned the score zero, seeds with minimal discolorations along the radicle were assigned the score one, those with isolated lesions along the radicle were scored two, seed with coalesced lesions along radicle received a score of three and seeds that did not germinate and were completely colonized by an oomycete scored four. Seeds and corresponding disease severity ratings are shown in

Isolate Code	County Collected	Location	Region	Date Collected	Species
OEO_O26	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O27	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O28	Madison	TVREC1	North	2021	Globisporangium irregulare
OEO_O29	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O30	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O31	Madison	TVREC1	North	2021	Pythium longandrum
OEO_O32	Madison	TVREC1	North	2021	Phytophthora nicotianae
OEO_O33	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O34	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O35	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O36	Madison	TVREC1	North	2021	Pythium longandrum

OEO_O37	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O38	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O39	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O40	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O41	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O42	Shorter	EVS1	Central	2021	Globisporangium irregulare
OEO_O44	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O45	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O47	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O48	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O52	Shorter	EVS1	Central	2021	Globisporangium sylvaticum
OEO_O53	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O54	Madison	TVREC2	North	2021	Globisporangium heterothallicum
OEO_O55	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O56	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O57	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O58	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O59	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O60	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O61	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O62	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O63	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O64	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O65	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O66	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O67	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O68	Madison	TVREC2	North	2021	Globisporangium sylvaticum
OEO_O70	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O71	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O72	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O73	Madison	TVREC2	North	2021	Globisporangium sylvaticum
OEO_O74	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O75	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O77	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O78	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O81	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O82	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O83	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O84	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O85	Madison	TVREC2	North	2021	Phytophthora nicotianae

OEO_O86	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O87	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O88	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O89	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O90	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O91	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O93	Madison	TVREC2	North	2021	Pythium dissotocum
OEO_O94	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O95	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O98	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O99	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O103	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O105	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O106	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O109	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O111	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O116	Autauga	Prattville2	Central	2021	Phytophthora cactorum
OEO_O126	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O128	Shorter	EVS2	Central	2021	Globisporangium perplexum
OEO_O129	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O130	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O131	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O132	Shorter	EVS2	Central	2021	Pythium torulosum
OEO_O133	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O135	Escambia	Brewton1	South	2021	Pythium inflatum
OEO_O137	Escambia	Brewton1	South	2021	Lagenidium sp.
OEO_O138	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O139	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O140	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O143	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O144	Escambia	Brewton2	South	2021	Pythium aristosporum
OEO_O145	Escambia	Brewton2	South	2021	Phytopythium cucurbitacearum
OEO_O146	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O147	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O148	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O149	Escambia	Brewton2	South	2021	Pythium torulosum
OEO_O150	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O151	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O153	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O155	Escambia	Brewton2	South	2021	Globisporangium irregulare

OEO_O157	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O158	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O159	Escambia	Brewton2	South	2021	Phytopythium helicoides
OEO_O160	Escambia	Brewton2	South	2021	Globisporangium acanthophoron
OEO_O161	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O163	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O164	Escambia	Brewton2	South	2021	Phytopythium cucurbitacearum
OEO_O165	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O168	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O170	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O172	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O173	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O176	Autauga	Prattville1	Central	2021	Globisporangium spinosum
OEO_O177	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O178	Autauga	Prattville1	Central	2021	Globisporangium spinosum
OEO_O180	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O181	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O183	Autauga	Prattville2	Central	2021	Pythium torulosum
OEO_O184	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O186	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O188	Shorter	EVS2	Central	2021	Pythium longandrum
OEO_O194	Shorter	EVS2	Central	2021	Globisporangium spinosum
OEO_O195	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O201	Madison	TVREC1	North	2021	Globisporangium spinosum
OEO_O202	Shorter	EVS1	Central	2021	Globisporangium rostratifingens
OEO_O211	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O212	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O213	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O214	Baldwin	Gulfcoast1	South	2021	Globisporangium spinosum
OEO_O215	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O217	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O220	Baldwin	Gulfcoast1	South	2021	Globisporangium perplexum
OEO_O221	Baldwin	Gulfcoast1	South	2021	Pythium acanthicum
OEO_O222	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O223	Baldwin	Gulfcoast1	South	2021	Pythium acanthicum
OEO_O224	Baldwin	Gulfcoast1	South	2021	Phytopythium helicoides
OEO_O226	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O227	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O229	Baldwin	Gulfcoast1	South	2021	Pythium vanterpoolii
OEO_O231	Baldwin	Gulfcoast2	South	2021	Globisporangium acanthophoron

OEO_O232	Baldwin	Gulfcoast2	South	2021	Globisporangium irregulare
OEO_O233	Baldwin	Gulfcoast2	South	2021	Globisporangium acanthophoron
OEO_O235	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O238	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O239	Baldwin	Gulfcoast2	South	2021	Pythium inflatum
OEO_O242	Baldwin	Gulfcoast2	South	2021	Phytopythium cucurbitacearum
OEO_O243	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O244	Baldwin	Gulfcoast2	South	2021	Phytopythium cucurbitacearum
OEO_O246	Baldwin	Gulfcoast2	South	2021	Globisporangium irregulare
OEO_O249	Baldwin	Gulfcoast1	South	2021	Globisporangium rostratifingens
OEO_O254	Baldwin	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O255	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O257	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O259	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O262	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O264	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O265	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O266	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O267	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O268	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O269	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O272	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O274	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O275	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O276	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O279	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O280	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O281	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O284	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O286	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O287	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O288	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O290	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O291	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O292	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O293	Lawrence	Lawrence2	North	2021	Pythium periplocum
OEO_O294	Lawrence	Lawrence2	North	2021	Pythium dissotocum
OEO_O295	Lawrence	Lawrence2	North	2021	Globisporangium irregulare
OEO_O297	Lawrence	Lawrence2	North	2021	Pythium dissotocum
OEO_O298	Lawrence	Lawrence2	North	2021	Pythium inflatum

OEO_O303	Lawrence	Lawrence2	North	2021	Globisporangium sylvaticum
OEO_O306	Lawrence	Lawrence2	North	2021	Pythium oligandrum
OEO_O308	Lawrence	Lawrence2	North	2021	Globisporangium sylvaticum
OEO_O309	Lawrence	Lawrence2	North	2021	Globisporangium orthogonon
OEO_O310	Lawrence	Lawrence2	North	2021	Globisporangium perplexum
OEO_O311	Lawrence	Lawrence2	North	2021	Pythium inflatum
OEO_O314	Lawrence	Lawrence2	North	2021	Pythium sp.
OEO_O316	Lawrence	Lawrence2	North	2021	Globisporangium heterothallicum
OEO_O319	Lawrence	Lawrence2	North	2021	Globisporangium acanthophoron
OEO_O320	Henry	Wiregrass	South	2021	Pythium acanthicum
OEO_O321	Henry	Wiregrass	South	2021	Pythium acanthicum
OEO_O326	Henry	Wiregrass	South	2022	Globisporangium orthogonon
OEO_O327	Henry	Wiregrass	South	2022	Pythium torulosum
OEO_O332	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O333	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O334	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O335	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O337	Baldwin	GulfCoast1	South	2022	Pythium deliense
OEO_O338	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O339	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O340	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O341	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O342	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O343	Baldwin	GulfCoast1	South	2022	Pythium deliense
OEO_O344	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O345	Baldwin	GulfCoast1	South	2022	Phytopythium helicoides
OEO_O346	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O347	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O348	Baldwin	GulfCoast1	South	2022	Globisporangium spinosum
OEO_O349	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O350	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O351	Baldwin	GulfCoast1	South	2022	Globisporangium irregulare
OEO_O352	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O356	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O357	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O358	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O359	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O360	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O361	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O362	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae

OEO_O363	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O364	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O366	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O367	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O368	Escambia	Brewton1	South	2022	Globisporangium irregulare
OEO_O370	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O371	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O372	Escambia	Brewton1	South	2022	Pythium oligandrum
OEO_O373	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O376	Escambia	Brewton1	South	2022	Globisporangium irregulare
OEO_O381	Escambia	Brewton1	South	2022	Pythium oligandrum
OEO_O382	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O391	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O392	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O394	Escambia	Brewton2	South	2022	Pythium oligandrum
OEO_O395	Escambia	Brewton1	South	2022	Pythium acanthicum
OEO_O396	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O397	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O398	Lawrence	Lawrence1	North	2022	Globisporangium nunn
OEO_O399	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O400	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O401	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O402	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O403	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O404	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O405	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O406	Lawrence	Lawrence1	North	2022	Globisporangium pleroticum
OEO_O407	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O408	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O409	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O410	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O411	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O412	Lawrence	Lawrence1	North	2022	Pythium dissotocum
OEO_O413	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O415	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O416	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O418	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O419	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O420	Lawrence	Lawrence1	North	2022	Globisporangium sylvaticum
OEO_O421	Lawrence	Lawrence1	North	2022	Globisporangium acanthophoron

OEO_O422	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O424	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O426	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O427	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O428	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O430	Lawrence	Lawrence1	North	2022	Globisporangium acanthophoron
OEO_O431	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O432	Lawrence	Lawrence1	North	2022	Globisporangium rostratifingens
OEO_O433	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O434	Lawrence	Lawrence2	North	2022	Globisporangium nunn
OEO_O435	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O436	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O437	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O438	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O439	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O440	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O441	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O442	Lawrence	Lawrence2	North	2022	Globisporangium sylvaticum
OEO_O443	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O444	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O447	Lawrence	Lawrence2	North	2022	Globisporangium rostratifingens
OEO_O448	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O449	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O450	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O451	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O452	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O453	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O454	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O455	Lawrence	Lawrence2	North	2022	Globisporangium sylvaticum
OEO_O456	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O457	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O458	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O459	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O460	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O461	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O462	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O463	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O464	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O465	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O466	Madison	TVREC1	North	2022	Globisporangium sylvaticum

OEO_O467	Madison	TVREC1	North	2022	Phytophthora nicotianae
OEO_O468	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O469	Madison	TVREC1	North	2022	Pythium sp.
OEO_O472	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O473	Madison	TVREC1	North	2022	Pythium dissotocum
OEO_O474	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O475	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O476	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O477	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O479	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O480	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O482	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O483	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O484	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O485	Madison	TVREC1	North	2022	Pythium oopapillum
OEO_O486	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O487	Madison	TVREC1	North	2022	Globisporangium rostratifingens
OEO_O488	Madison	TVREC1	North	2022	Pythium dissotocum
OEO_O489	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O490	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O491	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O492	Madison	TVREC1	North	2022	Phytopythium cucurbitacearum
OEO_O494	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O495	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O496	Madison	TVREC2	North	2022	Globisporangium pleroticum
OEO_O497	Madison	TVREC2	North	2022	Globisporangium irregulare
OEO_O498	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O499	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O501	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O504	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O505	Madison	TVREC2	North	2022	Globisporangium acanthophoron
OEO_O507	Madison	TVREC2	North	2022	Pythium periplocum
OEO_O508	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O510	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O512	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O513	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O514	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O515	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O516	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O517	Madison	TVREC2	North	2022	Globisporangium orthogonon

OEO_O518	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O540	Shorter	EVS2	Central	2022	Phytopythium cucurbitacearum
OEO_O547	Shorter	EVS2	Central	2022	Globisporangium orthogonon
OEO_O552	Shorter	EVS2	Central	2022	Globisporangium orthogonon
OEO_O554	Autauga	Prattville1	Central	2022	Globisporangium irregulare
OEO_O555	Autauga	Prattville1	Central	2022	Pythium acanthicum
OEO_O557	Autauga	Prattville2	Central	2022	Globisporangium irregulare
OEO_O560	Escambia	Brewton1	South	2022	Globisporangium orthogonon

Table 4. A disease severity index (DSI) was calculated for each isolate by dividing the sum of seed scores by the total possible score multiplied by 100. The formula used in calculating DSI was adapted from Rojas et al. (2016) and shown below:

$$\frac{DSI = \sum(severity\,rating \times seeds\,per\,rating) \;\; X\;100}{(total\,seeds \times highest\,severity\,rating)}$$

The seed virulence assay was repeated twice for species that were isolated and identified in 2021 and 2022 with frequency above 2%. Mean of the DSI was calculated in R and species that had mean that was not significantly different from the positive control but different from the negative control were termed highly indicating that they are pathogenic towards cotton seeds. Those that were not significantly different from the non-inoculated negative control but different from the positive control were the non-virulent species. The mildly virulent species had mean DSI that was not significantly different from both negative and positive controls.

Statistical analyses. A table containing taxonomic information, sample information (Table 1), and a species count table was generated manually for analyses using the R packages 'vegan' v. 2.5-7 (Oksanen et al. 2020) and 'phyloseq' v. 1.34.0' (McMurdie and Holmes 2013). Data were imported into R software version 4.0.4 (R core team 2013). Spearman's correlation coefficient was used to test for correlation between oomycete richness and soil edaphic factors such as cation exchange capacity (CEC), soil organic matter (SOM), percent sand, percent clay, and pH. A permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distances was used to evaluate differences in centroids (multivariate means) between communities of different regions, soil properties, and location. Species association to a region (North, Central, South) was analyzed using

indicator species analysis with the R package ‘indicspecies v. 1.7.9 (De Cáceres et al. 2010). Species that were pathogenic toward cotton seeds were determined by a linear mixed model in which isolates nested in species were the fixed effects, experiment sets were the random effect. P-values were adjusted using a Bonferroni correction and contrasts performed between species and the positive or the negative control (no pathogen present) determined which species were pathogenic. Contrasts were performed using the R package ‘lsmeans version 2.30-0’ (Lenth 2016). All plots were generated using the package ‘ggplot2 version 3.3.5’ (Wickham 2016). Data files and scripts used for analysis are available on GitHub (https://github.com/OLUWAKEMI-SOLA/OomyceteDiversityinAL).

Results

Oomycetes recovered from symptomatic seedlings. A total of 182 isolates in 2021 and 157 isolates in 2022 were identified from symptomatic seedlings. In 2021, Oomycetes were identified across 24 different species (

Figure 2a) of which *Globisporangium* species was the most abundant genus, followed by *Pythium, Phytophthora*, *Phytopythium*, and *Lagenidium* species. *Globisporangium irregulare* was recovered most often with 25.82% of total isolates, followed by *Globisporangium rostratifingens* (14.29%), and *Phytophthora nicotianae* (10.44%). One *Pythium* species was not identified to species level and had 99% identity and 98% query coverage to the undescribed *Pythium* sp. nov Lev 1523 with accession number, HQ643803 (Robideau et al. 2011). In this manuscript is referred to as *Pythium* species clade E, because of its close relationship with other clade E *Pythium* species. This species composed 2.20% of the total oomycetes isolated in 2021 and 0.64% in 2022. A species of *Lagenidium* was also identified in 2021, however in very low abundance (one isolate). 21 different species were recovered in 2022 (

Figure 2b) of which *Phytophthora nicotianae, G. irregulare* and *G. perplexum* and *G. acanthophoron* were the four most abundant species at 24.20%, 12.10%, 10.83% and 10.19% of total isolates.

Oomycete richness correlated with soil edaphic factors. In this study, oomycete diversity was defined by the number of observed species (richness) and compositional dissimilarity between samples (Bray-Curtis). In both years, oomycete richness was highest in Northern fields, followed by Southern and Central fields (Figure 3) but was only significant in 2022 (2021, $P = 0.12$; 2022, $P < 0.003$). In 2021, Oomycete richness (Figure 4a) was positively correlated with CEC (r = 0.594; $P = 0.032$) and negatively correlated with percent sand (r = -0.622, P=0.023). However, in 2022 (Figure 4b), oomycete richness only had a positive correlation with CEC of fields (r = 0.582, P=0.047). In 2021, oomycete species composition differed by region ($P = 0.008$), county (0.037), percent sand (P<0.017), CEC (P<0.025), and latitude (P=0.011). In 2022, oomycete composition differed by county (P=0.002), percent sand (P=0.018), clay (0.026), longitude (0.004) and SOM (P<0.007). Some oomycete species such as *Phytophthora nicotianae* showed specificity to North Alabama (P=0.027) and *G. irregulare* was found across all regions ($P > 0.05$).

Six oomycete species pathogenic towards cotton seeds. Virulence of oomycete species to cotton seeds was defined by significant differences in means of DSI from the controls (Table 3). Six species including *G. sylvaticum, G. irregulare, P. nicotianae, Phy. helicoides, G. acanthophoron* and *Py. acanthicum* were grouped as highly virulent. *G. spinosum, Phy. cucurbitacearum, Py. dissotocum* and *Py. torulosum* were classified as mildly virulent species while other species including *G. rostratifingens, G. perplexum, G. heterothallicum, G. orthogonon* and *G. species* Clade E were non-virulent (Figure 5).

Discussion

The objectives of this manuscript were to isolate and identify oomycetes associated with cotton seedlings in Alabama, how virulent species were against cotton seeds, and correlate oomycete diversity with soil edaphic factors. This study represents the most comprehensive survey of oomycetes associated with cotton seedlings in Alabama and the first in almost two decades, making it a significant advancement in the management of damping-off of cotton in Alabama and the Southeast. Of the 28 species isolated in both years, 25 species, including an unnamed species, and species with diverse ecological roles like mycoparasites were not previously reported to be associated with cotton seedlings in Alabama or the US as confirmed by the USDA ARS fungal database (https://nt.ars-grin.gov/fungaldatabases/index.cfm). Thus, we present the first report of the oomycete species, *Phy. curcurbitacearum, G. heterothallicum, Py. oligandrum, Py. periplocum, G. rostratifingens, G. spinosum, Py. vanterpoolii, G. perplexum, Py. dissotocum, Py. acanthicum, Py. torulosum, Py. aristosporum, Phy. helicoides, Py. inflatum, Py. longandrum, G. orthogonon, P. cactorum, G. acanthophoron, G. attrantheridium, Py. deliense, G. pleroticum, G. nunn, Lagenidium* species and *Py. oopapillum* associated with US cotton seedlings. *G. irregulare* and *G. sylvaticum* were not listed in the USDA ARS fungal database but have been found in previous reports (Johnson 1978; DeVay 1982). Six of these species were able to rot cotton seeds in the lab.

The diversity of oomycetes associated with cotton seedlings were identified across genera similar to previous reports in North America's soybean seedlings (Radmer et al. 2017; Rojas et al. 2016). The pathogenic and most frequently isolated oomycete species from cotton seedlings in the two years of this study were *Globisporangium irregulare* and *Phytophthora nicotianae.* Species composition and richness varied by soil type. Northern fields with higher Cation Exchange

Capacity (CEC) had higher oomycete richness (12 to 14 species) whereas southern and central soils had higher sand content and about half the number of species in most locations compared to North Alabama. We found that cation exchange capacity was a soil variable that consistently altered oomycete species richness while sand content consistently altered composition across both years. For example, *P. nicotianae* was isolated primarily from northern Alabama, whereas *G. irregulare* was isolated regardless of geography or soil conditions.

Surprisingly, no *G. ultimum* was isolated in 2021 and 2022 contrary to previous studies (Wrather et al. 2002; Howell 2002; Palmateer et al. 2004). This may have been due to differences in location or the fact that previous studies were not exclusive to oomycetes with the use of culture media that were more favorable for fungi or the sole use of morphology for species identification. Some known mycoparasites such as *Py. oligandrum, G. nunn* and *Py. periplocum* were also isolated from cotton seedlings, but in very low abundance (Bro 2002; Paul 1999; Lifshitz et al. 1984). Efforts to study and facilitate their abundance in soil could be novel control strategies. For example, *Py. oligandrum* was documented to control seedling disease caused by *G. ultimum* when its oospores are applied as seed treatments on sugar beet seeds (Martin 1987). Likewise, *Py. periplocum* inhibits the ability of *Botrytis cinerea,* the primary pathogen for grey mold, to infect grapevines by penetrating the pathogen's hyphae and coagulating its cytoplasm (Paul 1999). We also isolated novel *Pythium* species belonging to clade E and was not pathogenic to cotton seeds. Further taxonomic efforts on undescribed species will aid in the understanding of oomycete's role in the cotton microbiome.

Among important results, *P. nicotianae* is a known pathogen for cotton boll rot and has been previously reported to cause cotton seedling disease (Erwin and Ribeiro 1996), this presents the first report of its association with cotton seedling disease in Alabama (Allen and West 1986; Guidroz 1970). It is unclear and intriguing, the link between *P. nicotianae* in cotton seedlings and boll rot, which may warrant further investigation. However, among all species identified, *G. irregulare* was the most frequently isolated, found in all soil types, and killed cotton seeds readily in the lab. The differences in virulence of isolated oomycete species support previous findings but could vary by plant hosts (Rossman et al. 2017; Rojas et al. 2016). For example, the pathogenicity of *G. spinosum* to seeds of various crops like dry bean and onion (French 1989; Rossman et al. 2017) and that of *G. irregulare* to alfalfa and soybean (Rojas et al. 2016; Berg et al. 2017) has been reported. This emphasizes the importance of conducting periodic studies like this to determine

pathogens of crops in a location rather than studies on different crops. The virulence of oomycete species may also vary by temperature. For example, *Py. torulosum* has been demonstrated to be pathogenic at lower temperatures about 13°C, but reduced in aggressiveness towards dry bean seeds as the temperature increased to 18°C and 23°C (Rossman et al. 2017). This supports the observations in this study, and in future studies, we can focus on screening these isolates for their aggressiveness to cotton seeds at different temperatures. Additionally, previous studies have demonstrated a difference among species that cause root rot versus seed rot or both. For example, Rojas et al. (2016) observed that *G. heterothallicum* was pathogenic to soybean seedlings but not to seeds. Our study focused on seed rot we demonstrated that *G. heterothallicum* was non-pathogenic to seeds, but root rotting has not been tested.

This study also shows the distribution of oomycete species by edaphic factors, particularly sand content, CEC and SOM. These edaphic factors largely follow the diverse soil regions across Alabama with a gradient of clay in the North to sandier soils in southern coastal regions. More oomycete species were associated with soils with higher CEC and the opposite was observed in fields with lower CEC. These findings are consistent with previous study by Rojas et al. (2016) on oomycetes associated with soybean seedlings in 11 states across North America. However, some species showed a clear preference or lack of preference in soil type. For example, *P. nicotianae* were isolated in high abundance in northern soils with higher clay content whereas *G. irregulare* was found in all soil types around the state. This may be due to several reasons such as preference of species for certain soil conditions (Chilvers et al. 2020a).

Various efforts have been made to manage oomycete seedling pathogens including the use of resistant varieties and seed treatment fungicides (Chilvers et al. 2020b). These seed treated fungicides, especially, metalaxyl, mefenoxam and ethaboxam are applied in crops including cotton and soybean (Belisle et al. 2019; Chilvers et al. 2020b; Isakeit 2016) and exhibit differential activity towards oomycete species/isolates (Noel et al. 2019; Weiland et al. 2014). This is because some species have developed resistance by mutation of target proteins as demonstrated towards ethaboxam by mutation of the amino acid, C239S in the target protein, β-tubulin (Noel et al. 2019). Thus, the combination of seed-treated fungicides for pathogenic oomycetes was recommended for effective management of oomycete species pathogenic to seedlings. As much as this multiple seed treatment fungicides are available for soybean seedling oomycetes, this is not well adapted in cotton (Scott et al. 2020; Isakeit 2016; Giesler and Miller 2017; Hu and Norton 2020). We did not

aim to associate oomycete diversity with seed treatment or cotton variety in this study and did not specifically control for those factors. Regardless, oomycetes were still isolated from cotton roots despite the seed treatment or variety and diversity patterns showed consistent patterns across years. Oomycetes have been previously isolated from soybean seedlings grown from treated seed (Noel et al. 2021). In the future, it will be insightful to screen the oomycete collection from this study for sensitivity to current seed treatment fungicides specific to oomycetes in cotton. Some cotton varieties including Prema, Maxxa, DeltaPine 6166 and 6100 have been reported to be highly resistant to *G. ultimum* (Wang and Davis 1997). However, little research into breeding efforts toward other oomycete species has been performed. Thus, future studies can screen varieties for resistance to the oomycete collection reported here for traits associated with resistance or tolerance to oomycetes.

Overall, the findings of this study demonstrate the possibility of control strategies tailored to fields based on soil properties and disease pressure history. It prioritizes certain oomycete species for further study based on abundance and virulence and serves as a baseline for future application of appropriate seed treatments and varieties for oomycetes in cotton. This study represents a major advancement in knowledge of oomycetes associated with cotton in Alabama and will facilitate sustainable control of cotton seedling disease caused by oomycetes.

Table 1: Metadata containing sampling locations and associated edaphic factors in 2021 and 2022

County	Location	Soil Type	Year	Field	Latitude	Longitude	%Sand	%Silt	%Clay	CEC[a]	SOM[b]	pH
Limestone	North	Limestone Valleys and Uplands	2021	1	34.69	-86.89	16	44	40	7.63	5.5	5.7
				2	34.69	-86.89	18	44	38	9.80	5.0	5.5
			2022	1	34.69	-86.88	11	56	34	7.72	3.0	4.9
				2	34.69	-86.89	12	46	42	6.44	2.4	4.9
Macon	Central	Blackland Prairie	2021	1	32.49	-85.89	63	18	19	3.70	1.2	6.3
				2	32.42	-85.89	76	7	18	2.44	1.1	6.3
			2022	1	32.49	-85.89	81	<1	19	3.19	1.1	5.1
				2	32.42	-85.89	81	<1	19	8.25	0.8	7.5
Autauga	Central	Coastal Plain	2021	1	32.43	-86.45	64	6	30	5.38	2.7	5.9
				2	32.42	-86.45	59	11	30	6.21	3.2	5.8
			2022	1	32.43	-86.44	58	14	28	7.27	2.8	5.4
				2	32.42	-86.45	62	9	29	5.75	2.1	5.7
Escambia	South	Coastal Plain	2021	1	31.15	-87.05	74	8	18	4.19	2.1	6.3
				2	31.15	-87.05	66	14	20	3.75	1.9	6.0
			2022	1	31.14	-87.05	82	<1	18	4.21	1.4	5.4
				2	31.14	-87.05	80	<1	20	3.30	1.7	5.2
Baldwin	South	Coastal Plain	2021	1	30.54	-87.88	47	28	26	9.73	5.4	5.9
				2	30.54	-87.88	33	32	35	10.97	7.1	5.9
			2022	1	30.54	-87.88	61	14	25	6.59	2.8	5.4
				2	30.54	-87.88	57	15	28	5.57	2.8	5.1
Lawrence	North	Appalachian Plateau	2021	1	34.68	-87.36	33	47	20	16.17	7.2	6.5
				2	34.68	-87.29	27	44	29	11.63	5.1	6.2
			2022	1	34.67	-87.23	15	52	34	16.83	3.5	5.6
				2	34.68	-87.27	22	40	38	8.99	2.7	6.1
Henry	South	Coastal Plain	2021	1	31.38	-85.31	82	1	18	1.43	1.3	6.1
			2022	1	31.38	-85.31	80	<1	20	3.28	1.2	5.3

[a]CEC = Cation Exchange Capacity (milliequivalents per 100g soil)

[b]SOM = Soil Organic Matter Content (%)

Table 2. Variety and seed treatment of collected cotton samples across Alabama in 2021 and 2022

County	Location	Year	Field	Seed Variety	Seed treatment
Limestone	North	2021	1	Not available	Not available
			2	DeltaPine 2038 B3XF	Acceleron[a]
		2022	1	DeltaPine 2038 B3XF	Acceleron[a]
			2	DeltaPine 2115 B3XF	Acceleron[a]
Macon	Central	2021	1	Not available	Not available
			2	Delta Pine 399	Acceleron[a]
		2022	1	DeltaPine 1646	Acceleron[a]
			2	DeltaPine 1646	Acceleron[a]
Autauga	Central	2021	1	DeltaPine 1646	Acceleron[a]
			2	DeltaPine 1646	Acceleron[a]
		2022	1	DeltaPine 1646	Acceleron[a]
			2	DeltaPine 1647	Acceleron[a]
Escambia	South	2021	1	Stoneville 4990 B3XF	Prime[c]
			2	Phytogen 400 W3FE	Trio[b]
		2022	1	DeltaPine 1646	Acceleron[a]
			2	DeltaPine 1646	Acceleron[a]
Baldwin	South	2021	1	DP2055	Acceleron[a]
			2	DP399	Acceleron[a]
		2022	1	DeltaPine 2038 B3XF	Acceleron[a]
			2	Phytogen 400 W3FE	Trio[b]
Lawrence	North	2021	1	Phytogen 443 W3FE	Trio[b]
			2	Phytogen 443 W3FE	Acceleron[a]
		2022	1	DeltaPine 1646	Acceleron[a]
			2	DeltaPine 1646	Acceleron[a]
Henry	South	2021	1	DP2038	Acceleron[a]
		2022	1	DeltaPine 2020	Acceleron[a]

Acceleron[a] = Active ingredients include Metalaxyl*, fluxapyroxad, myclobutanil, and pyraclostrobin

Trio[b] = Active ingredients include Metalaxyl*, Mefenoxam*, fludioxonil, myclobutanil, and azoxystrobin

Prime[c] = Active ingredients include Pyraclostrobin, Fluxapyroxad

Table 3. A list of identified oomycete species collected across different counties in North, Central and South Alabama in 2021 and 2022

Isolate Code	County Collected	Location	Region	Date Collected	Species
OEO_O26	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O27	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O28	Madison	TVREC1	North	2021	Globisporangium irregulare
OEO_O29	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O30	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O31	Madison	TVREC1	North	2021	Pythium longandrum
OEO_O32	Madison	TVREC1	North	2021	Phytophthora nicotianae
OEO_O33	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O34	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O35	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O36	Madison	TVREC1	North	2021	Pythium longandrum
OEO_O37	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O38	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O39	Madison	TVREC1	North	2021	Globisporangium rostratifingens
OEO_O40	Madison	TVREC1	North	2021	Globisporangium perplexum
OEO_O41	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O42	Shorter	EVS1	Central	2021	Globisporangium irregulare
OEO_O44	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O45	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O47	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O48	Shorter	EVS1	Central	2021	Globisporangium spinosum
OEO_O52	Shorter	EVS1	Central	2021	Globisporangium sylvaticum
OEO_O53	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O54	Madison	TVREC2	North	2021	Globisporangium heterothallicum
OEO_O55	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O56	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O57	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O58	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O59	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O60	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O61	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O62	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O63	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O64	Madison	TVREC2	North	2021	Globisporangium rostratifingens

OEO_O65	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O66	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O67	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O68	Madison	TVREC2	North	2021	Globisporangium sylvaticum
OEO_O70	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O71	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O72	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O73	Madison	TVREC2	North	2021	Globisporangium sylvaticum
OEO_O74	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O75	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O77	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O78	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O81	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O82	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O83	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O84	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O85	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O86	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O87	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O88	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O89	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O90	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O91	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O93	Madison	TVREC2	North	2021	Pythium dissotocum
OEO_O94	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O95	Madison	TVREC2	North	2021	Phytopythium helicoides
OEO_O98	Madison	TVREC2	North	2021	Globisporangium rostratifingens
OEO_O99	Madison	TVREC2	North	2021	Phytophthora nicotianae
OEO_O103	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O105	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O106	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O109	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O111	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O116	Autauga	Prattville2	Central	2021	Phytophthora cactorum
OEO_O126	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O128	Shorter	EVS2	Central	2021	Globisporangium perplexum
OEO_O129	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O130	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O131	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O132	Shorter	EVS2	Central	2021	Pythium torulosum

OEO_O133	Shorter	EVS2	Central	2021	Globisporangium irregulare
OEO_O135	Escambia	Brewton1	South	2021	Pythium inflatum
OEO_O137	Escambia	Brewton1	South	2021	Lagenidium sp.
OEO_O138	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O139	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O140	Escambia	Brewton1	South	2021	Globisporangium irregulare
OEO_O143	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O144	Escambia	Brewton2	South	2021	Pythium aristosporum
OEO_O145	Escambia	Brewton2	South	2021	Phytopythium cucurbitacearum
OEO_O146	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O147	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O148	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O149	Escambia	Brewton2	South	2021	Pythium torulosum
OEO_O150	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O151	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O153	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O155	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O157	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O158	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O159	Escambia	Brewton2	South	2021	Phytopythium helicoides
OEO_O160	Escambia	Brewton2	South	2021	Globisporangium acanthophoron
OEO_O161	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O163	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O164	Escambia	Brewton2	South	2021	Phytopythium cucurbitacearum
OEO_O165	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O168	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O170	Escambia	Brewton2	South	2021	Globisporangium spinosum
OEO_O172	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O173	Escambia	Brewton2	South	2021	Globisporangium irregulare
OEO_O176	Autauga	Prattville1	Central	2021	Globisporangium spinosum
OEO_O177	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O178	Autauga	Prattville1	Central	2021	Globisporangium spinosum
OEO_O180	Autauga	Prattville1	Central	2021	Globisporangium rostratifingens
OEO_O181	Autauga	Prattville1	Central	2021	Globisporangium irregulare
OEO_O183	Autauga	Prattville2	Central	2021	Pythium torulosum
OEO_O184	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O186	Autauga	Prattville2	Central	2021	Globisporangium irregulare
OEO_O188	Shorter	EVS2	Central	2021	Pythium longandrum
OEO_O194	Shorter	EVS2	Central	2021	Globisporangium spinosum
OEO_O195	Shorter	EVS2	Central	2021	Globisporangium irregulare

OEO_O201	Madison	TVREC1	North	2021	Globisporangium spinosum
OEO_O202	Shorter	EVS1	Central	2021	Globisporangium rostratifingens
OEO_O211	Madison	TVREC2	North	2021	Globisporangium irregulare
OEO_O212	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O213	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O214	Baldwin	Gulfcoast1	South	2021	Globisporangium spinosum
OEO_O215	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O217	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O220	Baldwin	Gulfcoast1	South	2021	Globisporangium perplexum
OEO_O221	Baldwin	Gulfcoast1	South	2021	Pythium acanthicum
OEO_O222	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O223	Baldwin	Gulfcoast1	South	2021	Pythium acanthicum
OEO_O224	Baldwin	Gulfcoast1	South	2021	Phytopythium helicoides
OEO_O226	Baldwin	Gulfcoast1	South	2021	Globisporangium irregulare
OEO_O227	Baldwin	Gulfcoast1	South	2021	Pythium torulosum
OEO_O229	Baldwin	Gulfcoast1	South	2021	Pythium vanterpoolii
OEO_O231	Baldwin	Gulfcoast2	South	2021	Globisporangium acanthophoron
OEO_O232	Baldwin	Gulfcoast2	South	2021	Globisporangium irregulare
OEO_O233	Baldwin	Gulfcoast2	South	2021	Globisporangium acanthophoron
OEO_O235	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O238	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O239	Baldwin	Gulfcoast2	South	2021	Pythium inflatum
OEO_O242	Baldwin	Gulfcoast2	South	2021	Phytopythium cucurbitacearum
OEO_O243	Baldwin	Gulfcoast2	South	2021	Globisporangium rostratifingens
OEO_O244	Baldwin	Gulfcoast2	South	2021	Phytopythium cucurbitacearum
OEO_O246	Baldwin	Gulfcoast2	South	2021	Globisporangium irregulare
OEO_O249	Baldwin	Gulfcoast1	South	2021	Globisporangium rostratifingens
OEO_O254	Baldwin	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O255	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O257	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O259	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O262	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O264	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O265	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O266	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O267	Lawrence	Lawrence1	North	2021	Globisporangium perplexum
OEO_O268	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O269	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O272	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O274	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae

OEO_O275	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O276	Lawrence	Lawrence1	North	2021	Phytophthora nicotianae
OEO_O279	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O280	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O281	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O284	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O286	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O287	Lawrence	Lawrence1	North	2021	Globisporangium acanthophoron
OEO_O288	Lawrence	Lawrence1	North	2021	Pythium sp.
OEO_O290	Lawrence	Lawrence1	North	2021	Pythium dissotocum
OEO_O291	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O292	Lawrence	Lawrence1	North	2021	Globisporangium attrantheridium
OEO_O293	Lawrence	Lawrence2	North	2021	Pythium periplocum
OEO_O294	Lawrence	Lawrence2	North	2021	Pythium dissotocum
OEO_O295	Lawrence	Lawrence2	North	2021	Globisporangium irregulare
OEO_O297	Lawrence	Lawrence2	North	2021	Pythium dissotocum
OEO_O298	Lawrence	Lawrence2	North	2021	Pythium inflatum
OEO_O303	Lawrence	Lawrence2	North	2021	Globisporangium sylvaticum
OEO_O306	Lawrence	Lawrence2	North	2021	Pythium oligandrum
OEO_O308	Lawrence	Lawrence2	North	2021	Globisporangium sylvaticum
OEO_O309	Lawrence	Lawrence2	North	2021	Globisporangium orthogonon
OEO_O310	Lawrence	Lawrence2	North	2021	Globisporangium perplexum
OEO_O311	Lawrence	Lawrence2	North	2021	Pythium inflatum
OEO_O314	Lawrence	Lawrence2	North	2021	Pythium sp.
OEO_O316	Lawrence	Lawrence2	North	2021	Globisporangium heterothallicum
OEO_O319	Lawrence	Lawrence2	North	2021	Globisporangium acanthophoron
OEO_O320	Henry	Wiregrass	South	2021	Pythium acanthicum
OEO_O321	Henry	Wiregrass	South	2021	Pythium acanthicum
OEO_O326	Henry	Wiregrass	South	2022	Globisporangium orthogonon
OEO_O327	Henry	Wiregrass	South	2022	Pythium torulosum
OEO_O332	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O333	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O334	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O335	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O337	Baldwin	GulfCoast1	South	2022	Pythium deliense
OEO_O338	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O339	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O340	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O341	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O342	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron

OEO_O343	Baldwin	GulfCoast1	South	2022	Pythium deliense
OEO_O344	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O345	Baldwin	GulfCoast1	South	2022	Phytopythium helicoides
OEO_O346	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O347	Baldwin	GulfCoast1	South	2022	Phytopythium cucurbitacearum
OEO_O348	Baldwin	GulfCoast1	South	2022	Globisporangium spinosum
OEO_O349	Baldwin	GulfCoast1	South	2022	Globisporangium acanthophoron
OEO_O350	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O351	Baldwin	GulfCoast1	South	2022	Globisporangium irregulare
OEO_O352	Baldwin	GulfCoast1	South	2022	Phytophthora nicotianae
OEO_O356	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O357	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O358	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O359	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O360	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O361	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O362	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O363	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O364	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O366	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O367	Baldwin	GulfCoast2	South	2022	Phytophthora nicotianae
OEO_O368	Escambia	Brewton1	South	2022	Globisporangium irregulare
OEO_O370	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O371	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O372	Escambia	Brewton1	South	2022	Pythium oligandrum
OEO_O373	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O376	Escambia	Brewton1	South	2022	Globisporangium irregulare
OEO_O381	Escambia	Brewton1	South	2022	Pythium oligandrum
OEO_O382	Escambia	Brewton1	South	2022	Pythium deliense
OEO_O391	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O392	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O394	Escambia	Brewton2	South	2022	Pythium oligandrum
OEO_O395	Escambia	Brewton1	South	2022	Pythium acanthicum
OEO_O396	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O397	Escambia	Brewton2	South	2022	Globisporangium irregulare
OEO_O398	Lawrence	Lawrence1	North	2022	Globisporangium nunn
OEO_O399	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O400	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O401	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O402	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae

OEO_O403	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O404	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O405	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O406	Lawrence	Lawrence1	North	2022	Globisporangium pleroticum
OEO_O407	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O408	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O409	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O410	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O411	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O412	Lawrence	Lawrence1	North	2022	Pythium dissotocum
OEO_O413	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O415	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O416	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O418	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O419	Lawrence	Lawrence1	North	2022	Phytophthora nicotianae
OEO_O420	Lawrence	Lawrence1	North	2022	Globisporangium sylvaticum
OEO_O421	Lawrence	Lawrence1	North	2022	Globisporangium acanthophoron
OEO_O422	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O424	Lawrence	Lawrence1	North	2022	Globisporangium heterothallicum
OEO_O426	Lawrence	Lawrence1	North	2022	Phytopythium cucurbitacearum
OEO_O427	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O428	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O430	Lawrence	Lawrence1	North	2022	Globisporangium acanthophoron
OEO_O431	Lawrence	Lawrence1	North	2022	Globisporangium perplexum
OEO_O432	Lawrence	Lawrence1	North	2022	Globisporangium rostratifingens
OEO_O433	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O434	Lawrence	Lawrence2	North	2022	Globisporangium nunn
OEO_O435	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O436	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O437	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O438	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O439	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O440	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O441	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O442	Lawrence	Lawrence2	North	2022	Globisporangium sylvaticum
OEO_O443	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O444	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O447	Lawrence	Lawrence2	North	2022	Globisporangium rostratifingens
OEO_O448	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O449	Lawrence	Lawrence2	North	2022	Globisporangium perplexum

OEO_O450	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O451	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O452	Lawrence	Lawrence2	North	2022	Globisporangium acanthophoron
OEO_O453	Lawrence	Lawrence2	North	2022	Globisporangium irregulare
OEO_O454	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O455	Lawrence	Lawrence2	North	2022	Globisporangium sylvaticum
OEO_O456	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O457	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O458	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O459	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O460	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O461	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O462	Lawrence	Lawrence2	North	2022	Globisporangium perplexum
OEO_O463	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O464	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O465	Lawrence	Lawrence2	North	2022	Phytophthora nicotianae
OEO_O466	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O467	Madison	TVREC1	North	2022	Phytophthora nicotianae
OEO_O468	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O469	Madison	TVREC1	North	2022	Pythium sp.
OEO_O472	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O473	Madison	TVREC1	North	2022	Pythium dissotocum
OEO_O474	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O475	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O476	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O477	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O479	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O480	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O482	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O483	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O484	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O485	Madison	TVREC1	North	2022	Pythium oopapillum
OEO_O486	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O487	Madison	TVREC1	North	2022	Globisporangium rostratifingens
OEO_O488	Madison	TVREC1	North	2022	Pythium dissotocum
OEO_O489	Madison	TVREC1	North	2022	Globisporangium irregulare
OEO_O490	Madison	TVREC1	North	2022	Globisporangium sylvaticum
OEO_O491	Madison	TVREC1	North	2022	Globisporangium acanthophoron
OEO_O492	Madison	TVREC1	North	2022	Phytopythium cucurbitacearum
OEO_O494	Madison	TVREC2	North	2022	Globisporangium rostratifingens

OEO_O495	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O496	Madison	TVREC2	North	2022	Globisporangium pleroticum
OEO_O497	Madison	TVREC2	North	2022	Globisporangium irregulare
OEO_O498	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O499	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O501	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O504	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O505	Madison	TVREC2	North	2022	Globisporangium acanthophoron
OEO_O507	Madison	TVREC2	North	2022	Pythium periplocum
OEO_O508	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O510	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O512	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O513	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O514	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O515	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O516	Madison	TVREC2	North	2022	Globisporangium rostratifingens
OEO_O517	Madison	TVREC2	North	2022	Globisporangium orthogonon
OEO_O518	Madison	TVREC2	North	2022	Phytophthora nicotianae
OEO_O540	Shorter	EVS2	Central	2022	Phytopythium cucurbitacearum
OEO_O547	Shorter	EVS2	Central	2022	Globisporangium orthogonon
OEO_O552	Shorter	EVS2	Central	2022	Globisporangium orthogonon
OEO_O554	Autauga	Prattville1	Central	2022	Globisporangium irregulare
OEO_O555	Autauga	Prattville1	Central	2022	Pythium acanthicum
OEO_O557	Autauga	Prattville2	Central	2022	Globisporangium irregulare
OEO_O560	Escambia	Brewton1	South	2022	Globisporangium orthogonon

Table 4. Disease severity rating matrix of cotton seeds in seed virulence assay

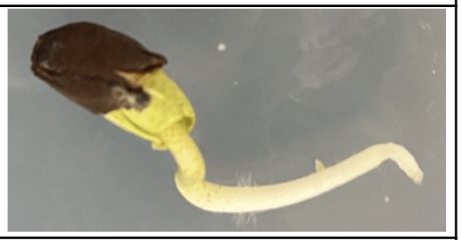
Rating	Image
0 (Germinated healthy seed)	
1 (Delayed development with minimal or no discoloration)	
2 (Germination with isolated lesions)	
3 (Germination with coalesced lesions)	
4 (No germination and/or seed fully colonized)	

Table 5. Virulence rating of species based on difference in mean disease severity index from the controls. Represented species include those identified in 2021 and 2022 with isolation frequency above 2% in any of the two years.

Species	Isolates[a]	Mean[b] ± SE[c]	*P* value[d]	Virulence_Rating[e]
Globisporangium acanthophoron	1	70.833 ± 5.833	0	Virulent
Globisporangium irregulare	4	89.583 ± 1.3	0	Virulent
Globisporangium sylvaticum	1	100 ± 0	0	Virulent
Phytophthora nicotianae	4	100 ± 0	0	Virulent
Phytopythium helicoides	1	100 ± 0	0	Virulent
Pythium acanthicum	1	81.667 ± 3.632	0	Virulent
Globisporangium spinosum	3	69.444 ± 12.103	0	Mildly Virulent
Phytopythium cucurbitacearum	1	65 ± 2.5	0	Mildly Virulent
Pythium dissotocum	1	39.167 ± 4.41	0.003	Mildly Virulent
Pythium torulosum	1	33.333 ± 1.667	0.039	Mildly Virulent
Globisporangium heterothallicum	3	14.583 ± 4.372	1	Non-Virulent
Globisporangium orthogonon	1	26.667 ± 4.167	0.499	Non-Virulent
Globisporangium perplexum	1	17.5 ± 0	1	Non-Virulent
Globisporangium rostratifingens	1	12.5 ± 2.5	1	Non-Virulent
Pythium sp. Clade E	2	10.833 ± 2.205	1	Non-Virulent
Positive Control[f]		99.444 ± 0.556	-	
Negative Control[g]		10.833 ± 1.153	-	

Isolates[a] = Number of Isolates tested in seed virulence assay for each species

Mean[b] = Mean of average disease severity index (across two assay repetitions) for replicates of all isolates tested for each species

SE[c] = Standard Error

***P* value[d]** = Probability values obtained from Dunnett's test. $P \leq 0.05$ indicates that the species mean is significantly different from the negative control. $P \geq 0.05$ indicates species mean is not significantly different from negative control

Virulence_Rating[e] = Classification of species into Non-Virulent, Mildly Virulent and Virulent. Virulent species have means that are significantly different from the negative control but not fron the positive control, non-virulent species have means not significantly different from the negative control but different from the positive control and mildly virulent species have means that are significantly different from both positive and negative control

Positive Control[f] = Plate included *Pythium irregulare* OEO-O28 which consistently had disease severity ratings between 95% and 100% throughout the experiment

Negative Control[g] = Plate without any oomycete present

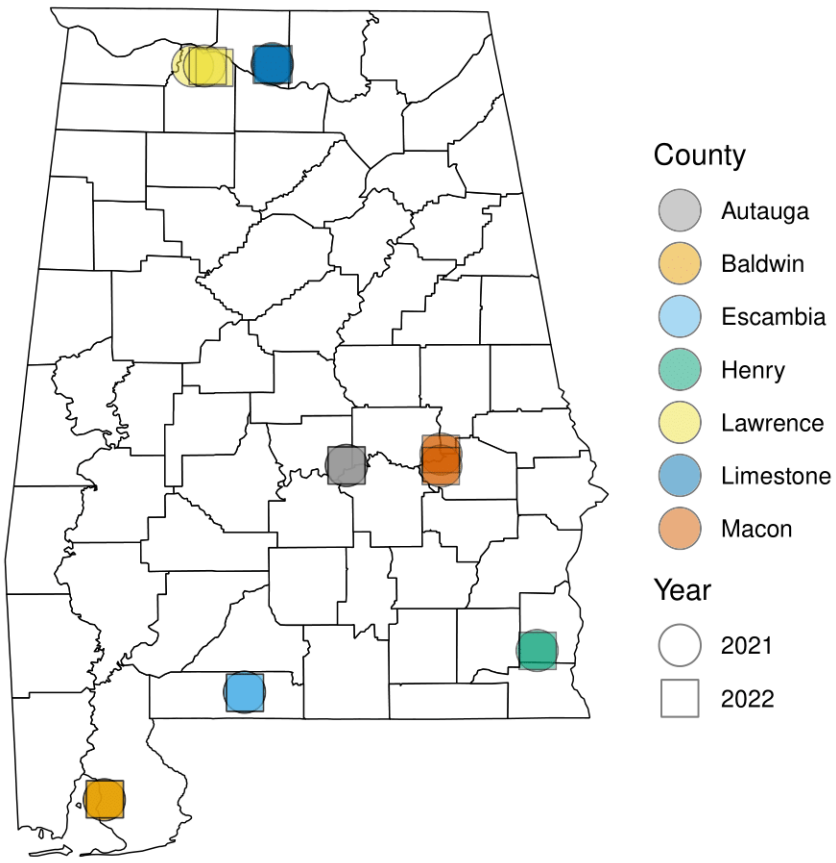


Figure 1. Map of fields sampled in 2021 and 2022 across Alabama counties. Thirty symptomatic cotton seedlings were collected two to four weeks after planting.

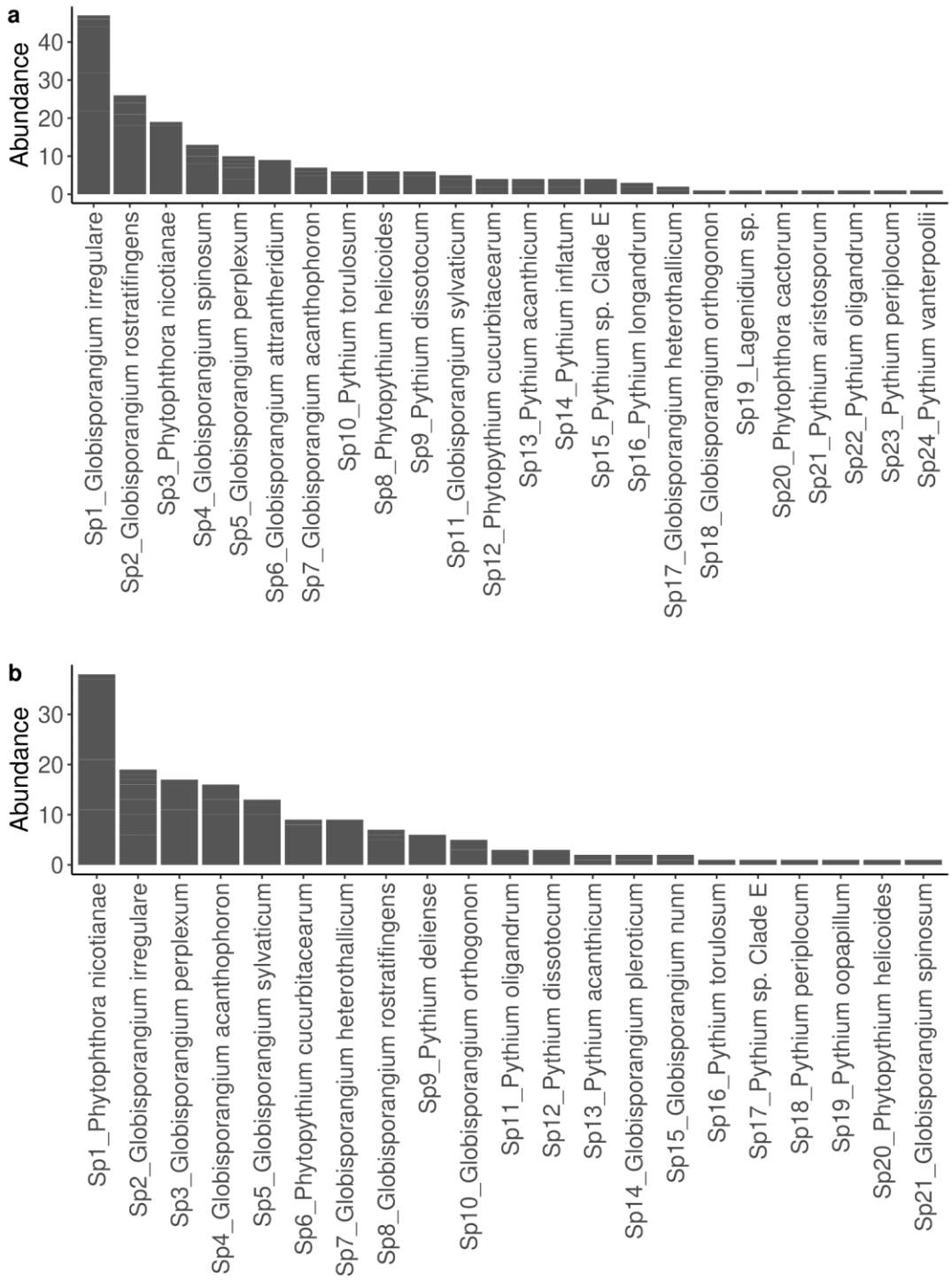


Figure 2. Abundance of oomycetes isolated from symptomatic cotton seedlings on a semi-selective medium (CMA-PARPB) in Alabama, USA. (a) 23 species isolated in 2021 and (b) 22 species isolated in 2022

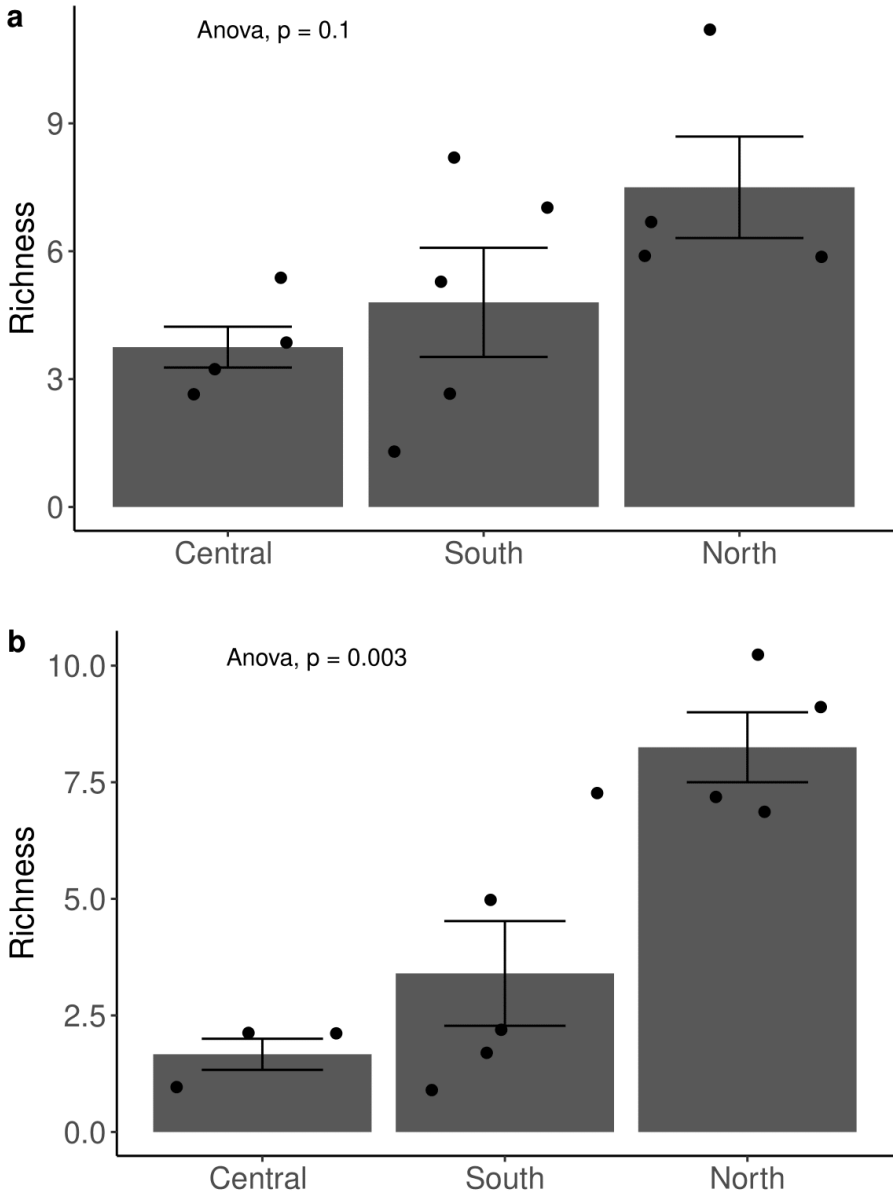


Figure 3. Oomycete richness highest in the north and lowest in central Alabama. (a) 2021 (b) 2022. Oomycete richness differs by location in 2022

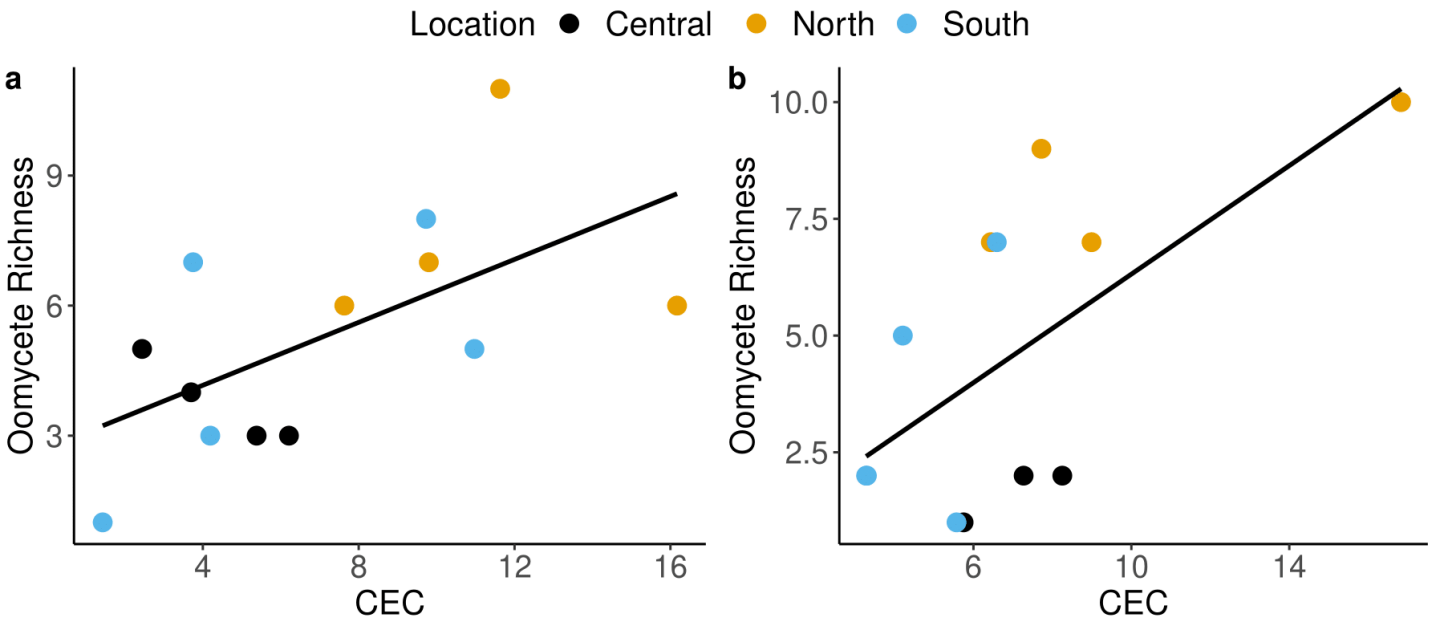


Figure 4. Oomycete richness positively correlates with Cation Exchange Capacity of field soils in (a) 2021 and (b) 2022

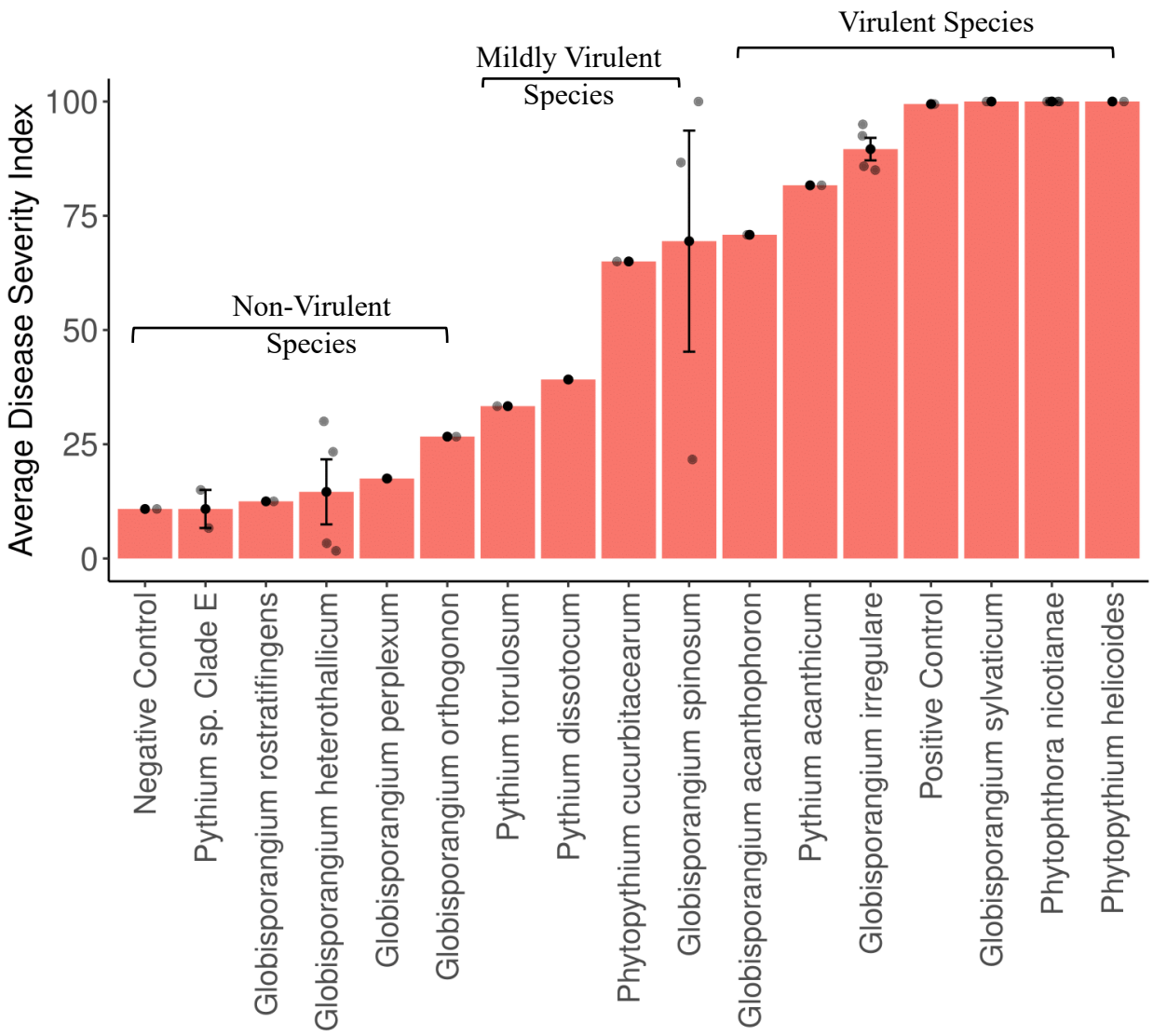


Figure 5. Inter-specific variation of oomycete species in seed virulence. Non-Virulent species have mean of average disease severity that is not significantly different from the negative control but different from positive control. Mildly virulent species have mean of average disease severity that is significantly different both negative and positive controls. Virulent species have mean of average disease severity that is not significantly different from the positive control but different from negative control. Species indicated were identified in 2021 and 2022 and have frequency above 2% in any of the two years.

3. CHAPTER THREE

Microbial Assembly in the Spermosphere of Cotton or Soybean

Abstract

Seed exudates released by crops stimulate and select microbes that influence plant health. This plant environment called the spermosphere is the transient, immediate area of soil around a germinating/imbibing seed that is rich in microbial activity. It represents the first contact of plants and seed associated microbes with horizontally acquired soil microbes. Compared to other plant environments, the spermosphere is less studied and the associated microbial interactions are not well understood. This could be linked to the lack of an easy and efficient method to collect spermosphere samples. Thus, the objectives of this study were to develop a suitable method to collect the spermosphere for high throughput sequencing, investigate the assembly of microbes in cotton and soybean spermosphere compared to bulk soil and how microbial interactions change in the spermosphere over time. The sampling method we employed sufficiently collected the spermosphere as defined in space and time for high throughput sequencing. The spermosphere microbial communities of both crops differed from bulk soil as six hours after seeds are sown. The changes observed were more related to a reduction in taxa evenness time point 0 to 18 after seeds were sown. Soybean spermosphere had the greatest reduction in taxa evenness, followed by cotton and bulk soil which only had a slight change in taxa evenness over time. *Paenibacillus* and *Brevibacillus* were enriched in soybean spermosphere. Overall, these findings could be applied to improve PGPR bioactivity and plant growth.

This chapter is written for intended publication in Phytobiomes

Olofintila, O.E., Noel, Z.A., 2023. Microbial assembly in cotton or soybean spermosphere. *Phytobiomes.*

Introduction

Soybean (*Glycine max* (L.) Merr.) is a leguminous crop ranked among the top five important crops in the world as an oil and protein source, nitrogen fixation capabilities, and other industrial uses (Raza et al. 2017). Currently, the United States is the second largest producer of soybean with an average yearly production of about 4 billion bushels on over 90 million acres of land (USDA 2020) and production value of 46 billion USD (USDA 2022b). Cotton (*Gossypium hirsutum* L.) is the most important fiber crop in the world. The United States is the third largest producer of cotton in the world, which generates an annual revenue of about 21 billion USD (Meyer 2020) and has a yearly production of 17.62 million bales (USDA 2022a) planted on about 13.51 million acres of land (USDA 2021b). However, both soybean and cotton production are threatened at a young susceptible growth stage by soil-borne pathogens including fungi and oomycetes leading to reduced stands and yields (Bradley et al. 2021; Strayer-Scherer 2021). Pathogens and other beneficial microbes are members of complex microbial communities that influence plant growth (Shade et al. 2017). Many plant-associated habitats like the rhizosphere constitute the area of soil directly around roots, while the phyllosphere includes above-ground plant parts (Knief et al. 2012).

Less studied is the spermosphere that comprises the area of soil influenced by seeds and seed exudates. The spermosphere harbors microbes that affect life or death of the plant soon after sowing seeds (Aziz et al. 2021; Nelson 2004a; Li et al. 2022). For example, Nelson (1988) showed that *Pythium* can fully colonize seeds within the first 12 hours after cotton seeds are sown. The spermosphere is defined as "the short-lived, rapidly-changing and microbiologically dynamic five-to-ten-millimeter zone of soil around a germinating seed" (Nelson 2004a). It was first observed by Slykhuis (1947) whom observed the inhibition of a fungal pathogen, *Fusarium culmorum*, by three fungal species around a germinating seed but not in bulk soil. This environment was named, "The Spermatosphere" but in the late 1950s, it became well defined as a "zone of elevated microbial activity" influenced by seed exudates and termed "The Spermosphere" (Verona 1958). The moment seeds imbibe water, exudates are released that recruit microbes that may favor or inhibit its germination. For example, the release of long-chain unsaturated fatty acids in cotton seed exudates promoted the germination of sporangia of *Pythium ultimum* leading to complete colonization of the seed within the first 12 hours (Nelson 2004b). Competitors like *Enterobacter cloaceae* in the spermosphere of cotton and cucumber degraded the unsaturated fatty acids faster

than the rate of exudate release thereby, inhibiting sporangia germination of *Pythium* and disease (Windstam and Nelson 2008b; Kageyama and Nelson 2003). These interactions may be variable depending on the crop species since *Enterobacter cloacae* protects against preemergence damping-off caused by *Pythium ultimum* in cucumber, cotton, and rye, but not in corn, peas, lima or snap beans (Nelson et al. 1986; Kageyama and Nelson 2003). The difference in efficacy was associated with differences in exudate composition; cotton was reported to contain less simple sugar in its exudates while corn contained more sugar thus altering the rate of metabolism of *Pythium* stimulator molecules present in seed exudates. Bacteria and fungi have used this mechanism to increase plant growth or reduce disease severity (Brewster et al. 1997; Weller 2007; Nelson 1988; Newitt et al. 2019; Sopheareth et al. 2013). However, successful inclusion of microbial inoculants relies partially on the ability to colonize the plant quickly and compete with native microbes.

Consequently, the spermosphere represents the basis for horizontal acquisition of microbes from the soil (Shade et al. 2017; Nelson 2018), and the vertical inheritance of microbes originating in the seed (Barret et al. 2015; Shade et al. 2017). As such, the phyllosphere and root microbiome may also be influenced by the spermosphere through initial interactions (Johnston-Monje et al. 2016). Therefore, understanding the microbial dynamics in the spermosphere is critical for advancing knowledge on crop microbiome assembly and successful inclusion of sustainable management strategies.

Despite the importance of the spermosphere, it remains understudied compared to the soil, rhizosphere, and phyllosphere partially due to the rapid development and small size (Nelson 2018). The few studies that focused on the spermosphere are primarily culture-based, which is limited in the number of microbes that can be recovered and identified (Nelson 2004b). However, some studies that incorporated the culture-independent techniques either focused on the contribution of the seed microbiome to the spermosphere and used soilless or sterile growth media or used pre-germinated seeds and collect samples beyond the spatial and temporal properties of the spermosphere (Johnston-Monje et al. 2021; Moroenyane et al. 2021). One major challenge in the study of the spermosphere using high-throughput culture-independent techniques may be owing to the lack of a quick and reliable method of collecting spermosphere samples (Schiltz et al. 2015).

Here, we developed a simple method to sample the spermosphere of cotton and soybean by constraining the soil zone and sampling precisely one to three millimeters around an imbibing and germinating seed, which was easily amenable to high-throughput sequencing. As such, as a

proof-of-principle, we sequenced bacteria from the spermosphere to provide the most in-depth analysis of spermosphere microbiome assembly to date. We hypothesized that the spermosphere would develop rapidly closely following seed imbibition and would develop differently depending on crop species. Therefore, the objectives of this study were twofold: 1) characterize the bacterial microbial communities associated with cotton and soybean spermosphere compared to bulk soil and 2) determine how microbial communities change over time as a seed imbibes water and germinates.

Materials and Methods

Soil collection and preparation. Soil used in this study was collected from Prattville Agricultural Research Unit (32°42'45.5"N 86°44'53.92"W) in Prattville, Alabama, since this soil showed consistent germination and emergence of both cotton and soybean in preliminary experiments (*data not shown*). Approximately three liters of soil from the top 10 centimeters was collected and transported to the lab. Soil was sieved to eliminate stones and pebbles and air-dried for 24 hours to ensure homogeneity in water content. Soil was used immediately after air drying. Six to seven grams (6 ml) of soil was transferred to each well of the 12-well microtiter plates (VWR American cat no.:10861-556, USA), containing three one-millimeter holes in the bottom of all wells for drainage. Each well in the 12-well microtiter plates measured 6.8 ml. Each well containing soil was watered with 1.5 ml of sterile water, and the water was allowed to circulate for an hour before seeds were sown.

Untreated Williams-82 soybean or untreated Delta Pine 1646 B2XF cotton were used in this study and were sorted to discard discolored seeds, cracked seeds, or seeds with cracked seed coats (Nelson et al. 1986). Since seed weight is related to the amount of exudates, soybean seeds weighed between 170 and 250 mg and cotton seeds weighed between 60 and 110 mg. Seeds were surface-sterilized by soaking in 6% bleach solution for 10 minutes in a sterile petri-dish and washed three times with sterile distilled water. Seeds were surface sterilized to maximize the effect of seed exudates on the growth of microbes from the soil. Six replicate seeds were sown into individual wells at the center of the wells, halfway into the 15 mm depth of the well, using flamed forceps. Wells containing just soil without seeds, hereafter called bulk soil, were used as a control. The 12-well microtiter plates were placed in a planting tray which was covered with the

planting tray lid to keep soil from drying out. Planting trays containing 12-well microtiter plates were placed inside a growth chamber at 25°C.

Collection of spermosphere. Spermosphere samples were collected at 0, 6, 12, and 18 hours after sowing. Spermosphere samples were collected using an 11 mm cork borer cleaned with 70% ethanol and flame sterilized between samples (Figure 6). The 11 mm cork borer was specifically used since the spermosphere is defined as the first 5-10 mm around a germinating seed (Nelson 2004b) and allowed the collection of soil three to five millimeters above and below a soybean and five to seven millimeters above and below cotton seeds since the average size of soybeans were six millimeters and cotton was four millimeters. In preliminary experiments, microbial populations in spermosphere soils sampled with this method increased significantly about 1.15 log in soybean and about 0.8 log in cotton compared to bulk soil (Figure 7). Spermosphere soil containing the seed inside the core was transferred into sterile envelopes and 0.25 ml immediately transferred to 2 ml disruptor tubes (Omega Bio-tek E.Z.N.A Soil DNA; Norcross GA), then stored at -80ºC until DNA extraction. The remaining soil clinging to the seed was washed off, the seed was blotted dry, and the weight of the seed was recorded after sample collection and compared to the initial individual seed weight to determine the water imbibed.

DNA extraction, amplification, and sequencing. The total DNA was extracted from bulk and spermosphere soils following the manufacturer's recommendation. The ZymoBIOMICS microbial community DNA standard (Zymo Research, Irvine, CA) was used as a positive control mock community. Negative controls samples consisted of DNA extraction controls and PCR controls. Amplification and library construction of 16S rDNA was performed with a three-step Polymerase Chain Reaction (PCR) (Lundberg et al. 2013; Longley et al. 2020). Briefly, the 16S region of the ribosomal DNA (rDNA) were amplified using the forward and reverse primers 515F and 806R (Caporaso et al. 2011). Following the amplification of the respective rDNA regions, the amplicons were linked to respective variants of frameshift primers, and then a 10 bp barcode was added for sample identification. Table 7and Table 7 show the primers and cycling conditions used to construct amplicon libraries. DNA amplification was confirmed with gel electrophoresis and successfully amplified libraries were normalized using SequalPrep™ Normalization Plate Kit (Thermo Fisher, USA). Normalized amplicons were then pooled and concentrated 20:1 using the

50K Dalton Millipore filters (Sigma-Aldrich, USA) and the pooled library was cleaned using AMPure XP beads at a ratio of 0.7X (Beckman Coulter, USA). Cleaned amplicon pools were verified by gel electrophoresis, quantified using Qubit fluorometer (Thermo Fisher, USA), and sequenced on an Illumina MiSeq 2x300 bp using the v3 500 cycles kit.

Read processing. The quality of demultiplexed reads were assessed using the FastQC and primer sequences were removed using cutadapt 4.0 (Martin 2011). Reads were then filtered and trimmed using VSEARCH 2.21.1 with expected error thresholds of 1.0 (Rognes et al. 2016). Singletons were removed and reads clustered based on the traditional 97% identity using USEARCH v11.0.667. The OTU table was generated with VSEARCH global alignment search, and taxonomy was assigned using SINTAX algorithm, against the SILVA 138.1 database.

Data analysis. Data were primarily analyzed using phyloseq v. 1.34.0 (McMurdie and Holmes 2013) and vegan 2.5-7 (Oksanen et al. 2020) of the statistical software R v. 4.0.4 (R core team 2013). All plots were generated using the data visualization package 'ggplot2 v. 3.3.5' (Wickham 2016). The OTU table, taxonomy table and OTU sequences generated from the read processing were merged with the mapping file into a phyloseq object (McMurdie and Holmes 2013). Contaminant OTUs detected in the negative controls were removed with decontam 1.10.0 (Davis et al. 2018). The OTUs associated with the bacterial mock community used as a positive control and one sample that had reads below 10,000 was removed due to low sequence coverage. Pielou's evenness (Pielou 1966) and richness (Chao and Chiu 2016) was used to determine differences between bulk soil, cotton, and soybean spermosphere samples using Kruskal-Wallis one-way analysis of variance in R.

Read counts were then normalized using the cumulative sum scaling with metagenomeSeq 1.32.0 (Paulson et al. 2013) and subjected to principal coordinate analysis based on Bray-Curtis distances. This analysis was followed by a Permutational Analysis of Variance (PERMANOVA) implemented with the 'adonis2' function to determine the differences in centroids of prokaryote communities across time-points and crops or bulk soil. Differences in multivariate dispersion were also evaluated using the 'betadisper' function.

Indicator species analysis was used to determine the taxa that were significantly associated with soybean spermosphere, cotton spermosphere, or bulk soil using the indicspecies 1.7.12 (De

Cáceres et al. 2010). We determined the core microbiome of the spermosphere microbiota by determining the taxa that contributed the last 2% increase in Bray-Curtis distance (Shade and Stopnisek 2019). Briefly, this method involves rarefying the dataset to the lowest sequencing depth, calculating occupancy and mean relative abundance of each OTU, ranking OTUs based on occupancy and abundance, then calculating the Bray-Curtis dissimilarity iteratively adding the next ranked OTUs. The log mean relative abundance and occupancy of each OTU was then fit to a neutral model using the tyRa 0.1.0 package (Sprockett et al. 2020). The neutral model can represent a null expectation of community assembly, and OTUs that deviate from that null expectation are hypothesized to assemble non-randomly (Burns et al. 2016; Shade and Stopnisek 2019), which may warrant further investigation. The Data files and scripts used for this analysis are available on GitHub (https://github.com/OLUWAKEMI-SOLA/SpermosphereMicrobiomeofCottonandSoybean).

Results

Sequencing outputs. All expected mock OTUs were recovered and identified in the positive controls (mock community samples) and contributed to 99% of the composition of the positive controls. Nine prokaryotic OTUs were filtered after detection in negative control samples resulting in a total of 2,090,814 reads of 8760 OTUs across 70 samples. The median read depth was 29,237.5 reads per sample and rarefaction curves were starting to plateau at this read depth indicating that much of the diversity in samples were adequately captured (Figure 8).

Water imbibition increased exponentially in the first six hours. The individual measurement of seed weight for both soybean and cotton seeds before planting and after spermosphere sample collection indicate that water was imbibed by both seeds from the surrounding soil. Overall, soybean seeds imbibed more water than cotton seeds and both seeds increased in seed weight exponentially within the first six hours after planting indicating water imbibition within this timeframe (Figure 9). However, water imbibition plateaus after six hours with no significant increase in seed weight.

Temporal change in prokaryote community in the cotton and soybean spermosphere. Principal coordinate analysis indicated distinct prokaryote communities within soybean and cotton spermosphere compared to bulk soil with time (Figure 10). Prokaryote communities were driven by crop ($P < 0.001$), time ($P < 0.001$), and an interaction of both factors ($P < 0.001$) indicated in Table 9. This prompted a closer look into the differences observed between crops by splitting the data by time-point (Table 10). At 0 hours, no significant difference in prokaryote communities existed between bulk soil compared to soybean and cotton spermosphere, as expected ($P = 0.452$). However, as early as 6 hours we observed significant difference between both crop spermosphere and bulk soil with the soybean spermosphere having a distinct community ($P < 0.001$). This was extended through 12 hours ($P < 0.001$) and 18 hours ($P < 0.001$) where the spermosphere formed unique communities depending on crop species (Figure 11).

Elevated microbial dominance in the spermosphere. We determined the changes in microbial communities observed by quantifying richness and evenness. Overall, we observed a reduction in evenness over time. Particularly, the reduction in evenness was higher in soybean spermosphere and lowest in bulk soil prokaryote communities, indicating a distinct effect of the crop on taxa dominance (Figure 13). Particularly, at time-point 0, there was no significant difference in evenness of prokaryote taxa ($P > 0.05$) but at 6 hours, we observe a reduction in evenness of prokaryote taxa in soybean and cotton spermosphere, compared to bulk soil ($P < 0.05$). At 12 and 18 hours, both cotton and soybean spermosphere samples had reduced evenness considerably compared to bulk soil ($P < 0.001$). However, crop did not alter richness (Figure 12).

Enrichment of Bacilli in the spermosphere of soybean. Taxa associated with the spermosphere samples of both crops and bulk soil was determined using the indicator species analysis. Bulk soil composed 62 indicator taxa, soybean spermosphere 52 taxa and cotton spermosphere had the lowest frequency of indicator taxa, 26 (Figure 14). The spermosphere of both cotton and soybean differed in the composition of indicator taxa. There was a clear dominance of Bacilli and Gammaproteobacteria in the soybean spermosphere. Bacilli (51.92% OTUs), Gammaproteobacteria (28.85%), Actinobacteria (11.54%) and four other classes that included 7.69% OTUs were taxa associated with soybean spermosphere. Cotton spermosphere constituted Planctomycetes (19.23%), Gemmatimonadetes (15.34%), Actinobacteria (15.34%) and eleven

other classes that contained the remaining 50.09% OTUs. In contrast, bulk soil, 20.63% of the indicator OTUs belonged to Alphaproteobacteria, Gemmatimonadetes (12.7%), Blastoclatellia (7.94) and Thermoleophilia (6.35%). Eighteen other classes contained the remaining 52.38% OTUs. The cotton spermosphere was enriched in Planctomycetes compared to bulk soil, which had lower Planctomycetes and soybean with no Planctomycetes at all.

The core microbiome of soybean and cotton spermosphere. Several microbes were identified as core members of the spermospere microbiome and bulk soil, all having an occupancy of one indicating that they are found in every sample. Several also fell outside the 95% confidence interval of the neutral model, indicating they had less abundance than expected and were selected by the environments (Figure 15). The core microbiota of bulk soil, soybean and cotton spermoshere appeared similar in composition based on bacteria class. In soybean, these taxa predominantly belong to Alphaproteobacteria with 22.32% abundance, Thermoleophilia with 15.18% and Actinobacteria with 11.61% (Figure 15a). In the cotton spermosphere (Figure 15b), Alphaproteobacteria (22.22%), Thermoleophilia (15.56%) and Actinobacteria (10.67%) were the most abundant core taxa. Bulk soil core microbiome (Figure 15c) constituted Alphaproteobacteria (22.93%), Thermoleophilia (15.79) and Actinobacteria (11.48). However, the abundance of some members was different in the spermospheres and bulk soil. For example, Rhizobiales (8.93%) constituted the most abundant order in soybean spermosphere while Solirubrobacterales (8.00% and 8.61%) was in cotton and bulk soil respectively, both orders of bacteria belonging to Alphaproteobacteria. Rhizobiales was also observed in core cotton spermosphere and bulk soil microbiota, but, in lower abundance (7.56% and 8.13%).

In contrast, the taxa that were not core but had higher abundance than expected (i.e., enriched) were distinct by crop. In soybean spermosphere, these taxa consisted of Bacteroidia (27.5%), Bacilli (20%), Gammaproteobacteria (17.5%), four other classes that made up 5% each and another six constituting 2.5% each. Notably, the genera *Flavobacterium*, *Sphingobacterium*, *Tumebacillus*, *Paenibacillus,* and *Bacillus* made up a quarter of the enriched taxa. The composition and relative abundance of non-core taxa in the cotton spermosphere was different from the soybean spermosphere, particularly, Bacteroidia (25%), Gammaproteobacteria (21.43%), Actinobacteria (14.29%), Polyangia (10.71%), Clostridium (7.14%) and six other classes that made up 3.57% each. Many of these were unidentified genera (32.14%) and *Flavobacterium* (7.14%).

Planctomycetes constituted 10.74% of non-core taxa occuring in lower abundance. Non-core microbes enriched in bulk soil were primarily Gammaproteobacteria (31.81%), Actinobacteria (13.63%), Bacteroidia (13.63%), four classes that made up 36.36% and Myxococcia (4.55%). Unidentified genera, Tumebacillus and Flavobacterium made up 31.82% of these classes.

Discussion

The objectives of this study were to develop a quick and efficient method to collect spermosphere samples for high throughput sequencing, characterize microbial communities associated with the spermosphere and determine the changes in the spermosphere soil over time. Sequencing results indicate that the method employed sufficiently sampled the spermosphere soil as defined in space, time, and microbial activity by Nelson (2004b), Stanghellini and Hancock (1971) and Verona (1958). We observed an initial exponential increase in water imbibition followed by an equilibrium which follows the pattern of water imbibition reported by Nelson (2004b). These two events are characteristic of the process of seed germination (Nelson 2004a). Thus, validating our collection of spermosphere samples, that is, around a germinating seed. The spermosphere microbiome was different from bulk soil by exhibiting greater change in diversity of prokaryote communities which developed as early as twelve hours after a seed is sown. The diversity observed within each group was increased taxa dominance over time which was greater in soybean spermosphere than cotton spermosphere. Major dominating taxa enriched in soybean spermosphere belong to genera with long history of being beneficial or plant growth promoting, demonstrating that beneficial associations with crops may occur as soon as 12 hours after sowing seeds.

In this study, we further define the development of the spermosphere of cotton and soybean at twelve hours after sowing which aligns with previous report of increased spore germination and full colonization of cotton seeds by *G. ultimum* twelve hours after sowing (Nelson 1988). Interestingly, this development of the spermosphere corresponds with water imbibition. We observed an exponential increase in water imbibed by both cotton and soybean seeds in the first 6 hours after which it plateaued, which is consistent with previous reports that documented the highest increase in water imbibition and exudation within the first few hours after planting (Ashraf

and Nisar 1972; Simon and Harun 1972). Since microbial activity in the spermosphere is primarily driven by seed exudates released by imbibing seeds, it may be expected that microbial communities change rapidly over short time frames when seed exudates are released, as observed in this study. This study therefore fills the knowledge gap and validates our sampling technique of microbial responses to seed exudates in the spermosphere at these early time-points.

Particularly, we observed the initial increase in water absorbed by seeds termed the Phase I hydration or actual imbibition (Nelson 2004b) which was consistent across both crops within the first 6 hours after sowing. However, we noticed that this initial increase was greater in soybean compared to cotton seeds. This could be a result of various seed properties such as seed size and exudate composition (Nelson 2004b; Vančura and Hanzlíková 1972). As soon as imbibition reaches a peak at 6 hours, it ceased, representing Phase II of germination which indicates the saturation of nutrient reserves and synthesis of products required for the extending radicle and marks the last phase of germination (Nelson 2004b).

We observed greater imbibition in soybean than cotton which could be linked to the greater change in prokaryote diversity observed in soybean spermosphere compared to cotton spermosphere. As mentioned briefly in the previous paragraph, this may be due to physical characteristics of the seed (Soldan et al. 2021). Vančura and Hanzlíková (1972) demonstrated increased quantities of seed exudates as seed size increased with highest and lowest exudation in bean and cucumber seeds respectively. Additionally, different varieties of common bean has been shown to differ in quantity of seed components exuded (Kato et al. 1997). The variety with largest seeds, Hokkaikintoki, released the highest quantities of sucrose, leucine, nitrogen, and potassium in its seed exudates while the small seeds of Kurodanekinugasa released the lowest amounts of exudates. Thus, we speculate that the greater change in microbial communities of the soybean spermosphere compared to cotton may be due to the larger size of soybean seeds that allows for increased imbibition, exudation and ultimately increased microbial activity.

We observed the dominance of certain taxa in the spermosphere microbiome over time. This suggests the selection of certain taxa in the spermosphere, supported by the reports of Ota et al. (1991) where specific nitrogen-fixing bacteria had increased dominance in the spermosphere of cocklebur seeds but not in bulk soil. Soybean and cotton spermosphere samples were different in indicator taxa. Particularly, Bacilli and Gammaproteobacteria were enriched in the soybean spermosphere and Planctomycetes were enriched in cotton spermosphere. This could

be a result of the difference in seed size and exudate composition. In contrast to Buyer et al. (1999), we observed varying spermosphere bacterial communities in different plant species. The contrast may be a result of the collection of spermosphere samples at the initial stages of seed germination and imbibition, which constitute the spermosphere rather than at later hours after radicle emergence (96 hours after planting).

We did not observe a major difference in the core microbiome of bulk soil, soybean and cotton spermospheres but observed differences in the enriched species associated with these environments. The bulk of core microbes identified belonged to Alphaproteobacteria and is consistent with the detection of Alphaproteobacteria as the most abundant member of core microbiome isolated from soil collected from the arctic region and six continents around the world (Malard et al. 2019; Delgado-Baquerizo et al. 2018). Consistent with Lauber et al. (2009) and Jones (2015), Rhizobiales constitute a major member of the core microbiota of bulk soil and spermospheres. Their consistent detection in the soil and spermosphere may be associated with their ability to form close associations with diverse leguminous crops and metabolize complex compounds such as phenolic compounds, hydrocarbons and heavy metals (Teng et al. 2015). However, since we only conducted this experiment with a single soil (i.e., a single microbial pool), a future study of the spermosphere across multiple soil types is warranted to confirm that these microbes are consistent members of the spermosphere core microbiome.

Importantly, Bacilli appears to be enriched in soybean spermosphere. Since these *Bacilli* including *Tumebacillus*, *Paenibacillus* and *Bacillus* have historically been associated with plant growth promotion and disease protection, it was notable that they were enriched in the spermosphere of soybean but not core members of the microbiota. It indicates their ability to utilize soybean exudate quickly for growth, but not consistently across all seeds. In support of this statement, seed exudates have been reported to induce chemotaxis and biofilm formation of *B. amyloliquefaciens* (*velezensis*) and seed colonization by enhancing active cell division (Yaryura et al. 2008). *Paenibacillus polymyxa* isolated from wheat and peanut rhizosphere increased the survival of *Arabidopsis thaliana* in the presence of the oomycete pathogen *Pythium aphanidermatum* when applied as root treatment. Regardless, the development of strategies to translate this knowledge into biologically based solutions for disease control on a wide geographic scale is still lacking.

The findings of this study such as the method developed for spermosphere sample collection could be applied to other sequencing methods like metagenomics or metatranscriptomics for functional characterization of spermosphere microbiomes. It can also be adapted to study interactions between seedling pathogens and chemical seed treatments, biocontrol, or bioactive metabolites in the spermosphere. Thereby, improving knowledge of microbial interactions in a spermosphere, which may lead to the development of novel or improved management strategies for seed and seedling diseases.

Table 6. Thermal Cycling conditions for the three-step polymerase chain reaction of prokaryote and fungal library preparation (adapted from Noel et al. (2022))

Prokaryote								
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	

Table 7. Primers used for the preparation of prokaryote and fungal library (Noel et al. 2022)

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

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

Table 8. Three-step amplicon library preparation for prokaryotes including all reagents used (Noel et al. 2022)

Reagent	Volume per reaction (uL)
Step 1	
2X Platinum Green Taq Master Mix(Thermo Fisher,USA)	6.25
10 uM 515F Primer (IDT, USA)	0.375
10 uM 806R Primer (IDT, USA)	0.375
Bovine Serum Albumin (BSA, 3%)	0.64
GC Enhancer (Thermo Fisher,USA)	2
H2O	0.36
Extracted DNA	2
Step 2	
2X Platinum Green Taq Master Mix(Thermo Fisher,USA)	6.25
10 uM 515F Primer Frameshift (IDT, USA)	0.375
10 uM 806R Primer Frameshift (IDT, USA)	0.375
Bovine Serum Albumin (BSA, 3%)	0.64
H2O	0.36
GC Enhancer (Thermo Fisher,USA)	2
Step 1 Product	2
Step 3	
2X Platinum Green Taq Master Mix (Thermo Fisher,USA)	8
Barcode Forward Primer	0.5
Water	1
GC Enhancer (Thermo Fisher,USA)	0.5
Unique 10 Nucleotide Barcode	1
Step 2 Product	4

Table 9. Permutational Analysis of Variance (PERMANOVA) shows that prokaryote communities in the spermosphere are influenced by crop, time and an interaction of crop and time

	[a]R^2	[b]F	[c]Pr(>F)
Crop	0.14012	7.8809	**0.001**
Time Point	0.22724	8.5204	**0.001**
Crop:Time Point	0.11703	2.194	**0.001**
Residual	0.51562		
Total	1		

[a]R^2 = Coefficient of determination

[b]F = Value on the F distribution

[c]Pr(>F) = Probability value

Table 10. Spermosphere Prokaryote communities differs between crops as early as time-point 6 (six hours after sowing)

	PERMANOVA			BETA-DISPERSION	
	[a]R^2	[b]F	[c]Pr(>F)	[b]F	[c]Pr(>F)
Time-Point 0					
Crop	0.11335	0.9588	0.437	0.9369	0.409
Time-Point 6					
Crop	0.27288	2.8146	**0.001**	0.5389	0.59
Time-Point 12					
Crop	0.42384	5.5172	**0.001**	0.8281	0.544
Time-Point 18					
Crop	0.41696	4.6484	**0.001**	0.0918	0.911

[a]R^2 = Coefficient of determination

[b]F = Value on the F distribution

[c]Pr(>F) = Probability value

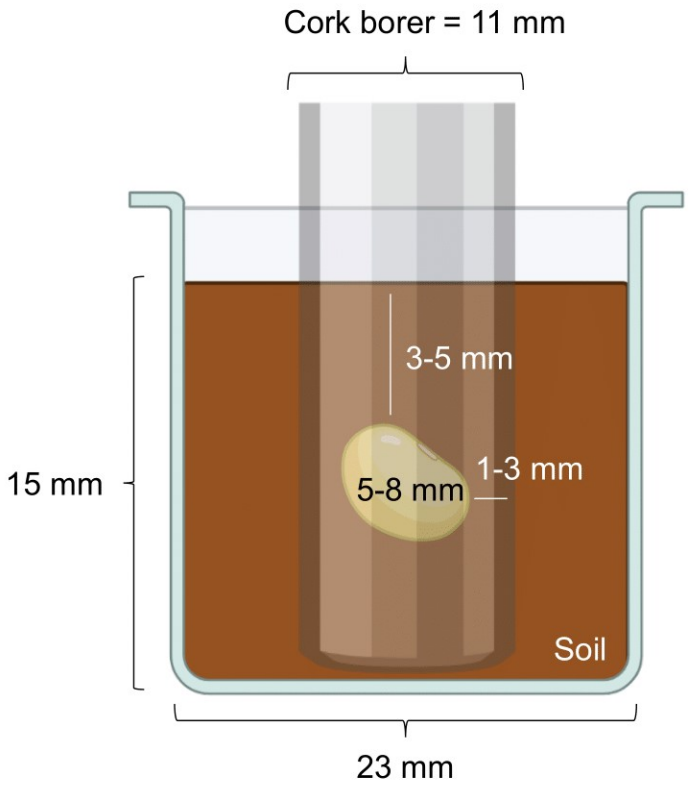


Figure 6. Illustration showing how spermosphere samples were collected

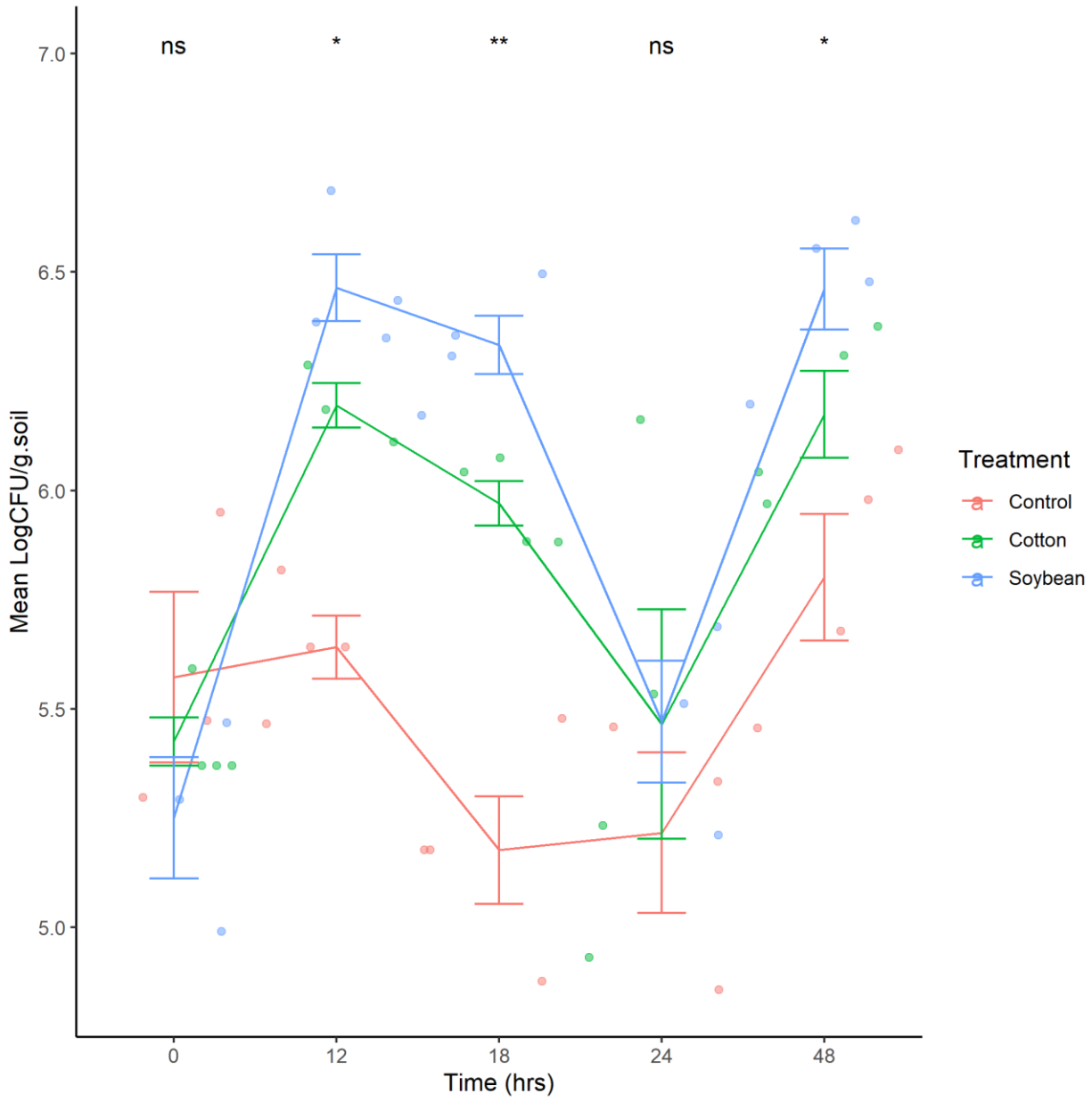


Figure 7. Preliminary Experiment- Increased bacterial populations in the spermosphere within the first 12 hours after seeds are sown. Soil used in this study was collected from EV. Smith Research Center, Shorter, Alabama. Asterisks denote the significance level of differences in means of three environments. The control represents bulk soil, cotton spermosphere and soybean spermosphere. The plots with 'ns' denot that the difference in means is insignificant, '*' when P-value (P) is less than 0.05 but greater then 0.01 and '**' when $P < 0.01$

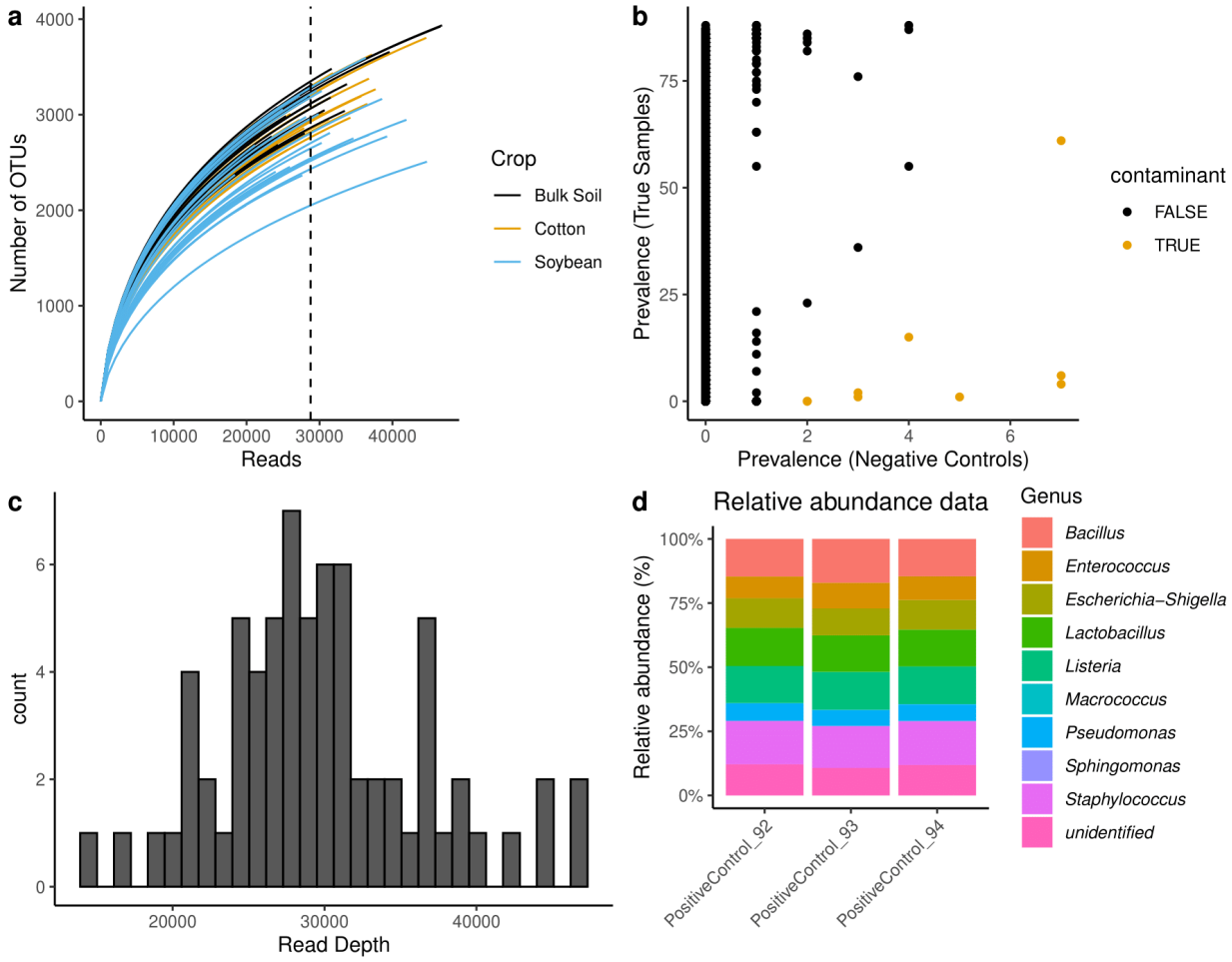


Figure 8. Sequencing results for prokaryote communities. (a) Rarefaction analysis showing a median read depth of 29,237.5 reads per sample. (b) Plot showing the filtered contaminants from negative control samples. (c) Histogram showing read-depth distribution. (d) Relative abundance plot for positive control samples known to contain the listed genera from the ZymoBiomics kit

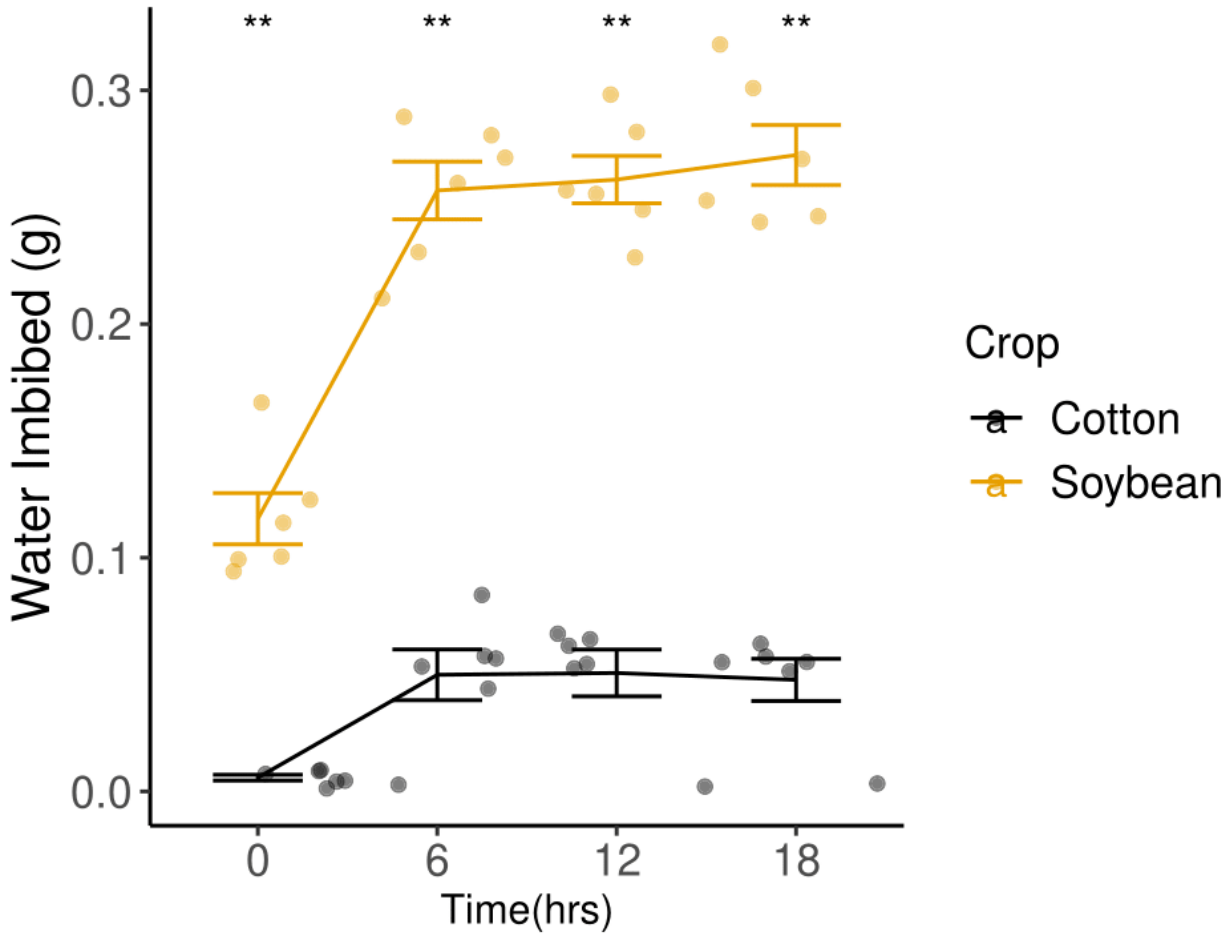


Figure 9. Kruskal-wallis one-way anova was used to test the differences in means of water imbibed by cotton and soybean seeds at the different time-points. Asterisks were used to denote the significance level on the plots with '**' when $P < 0.01$.

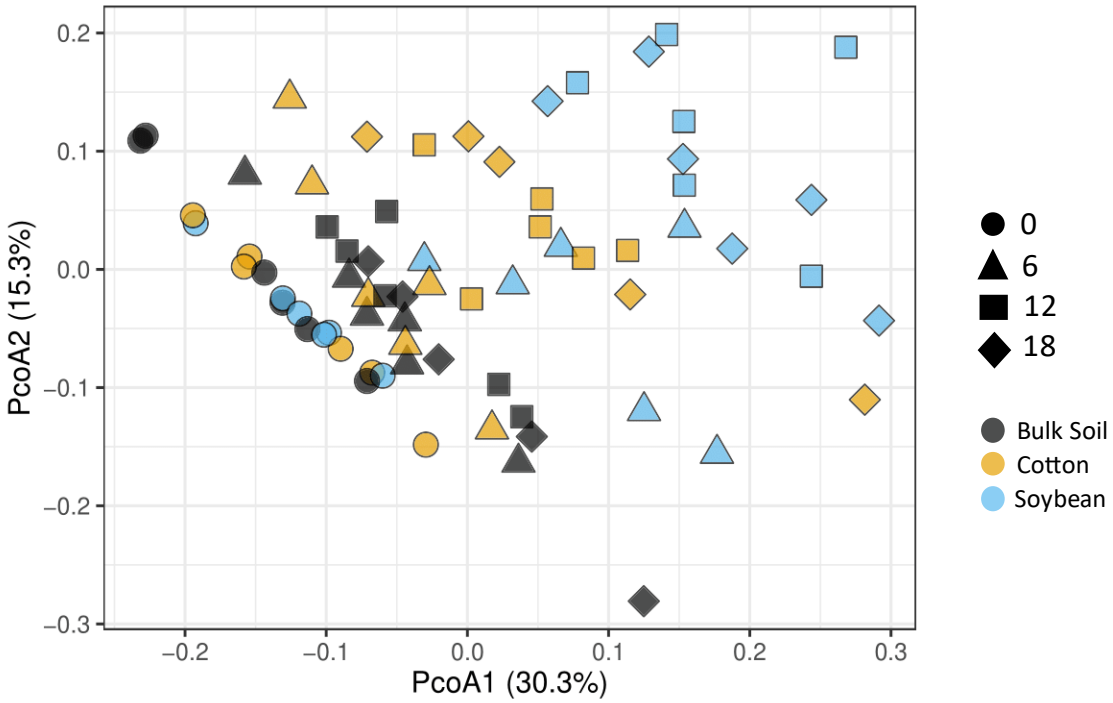


Figure 10. Spermosphere prokaryotic communities change over time. Each point represents a prokaryote community and the different colors represent the different plant environments. ● represents soybean spermosphere communities, ● represents cotton spermosphere communities and ● represents communities associated with bulk soil. The shapes represent the various time-points the environments were sampled. ● for associated communities at 0 hours after sowing, ▲ for associated communities at 6 hours after sowing, ■ for associated communities at 12 hours after sowing and ◆ for communities associated with samples collected 18 hours after sowing.

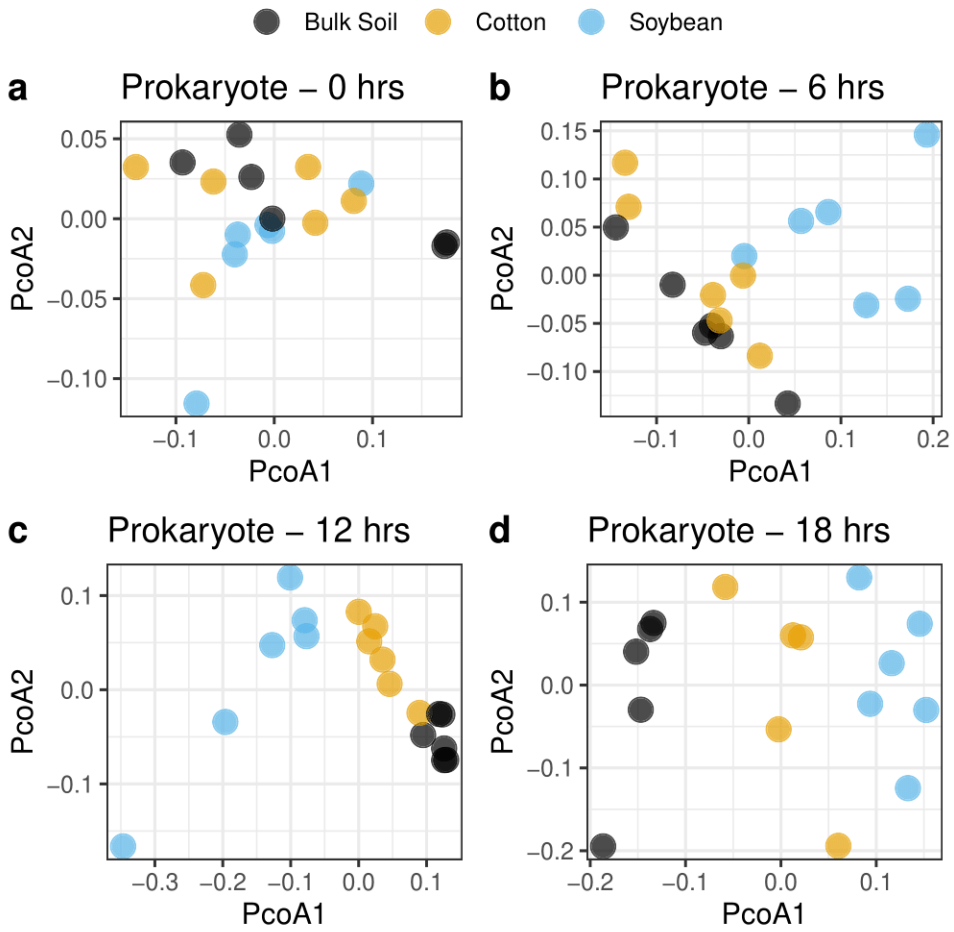


Figure 11. Prokaryotic communities of soybean and cotton changes as early as 6 and 12 hours after sowing. Each point represents a prokaryote community and the different colors represent the different plant environments. ● represents soybean spermosphere communities, ● represents cotton spermosphere communities and ● represents communities associated with bulk soil.

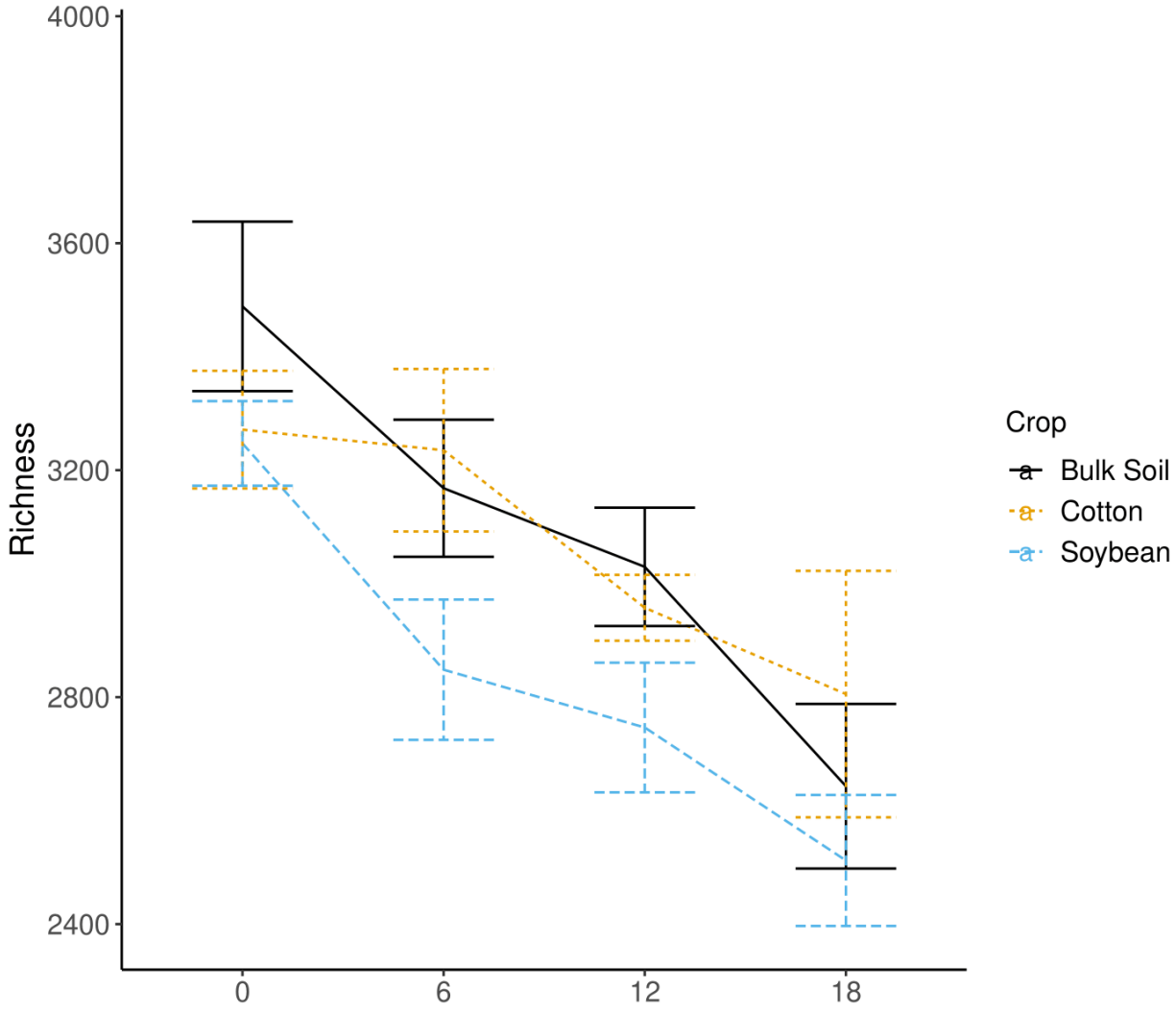


Figure 12. No significant difference in prokaryote richness between spermospheres and bulk soil

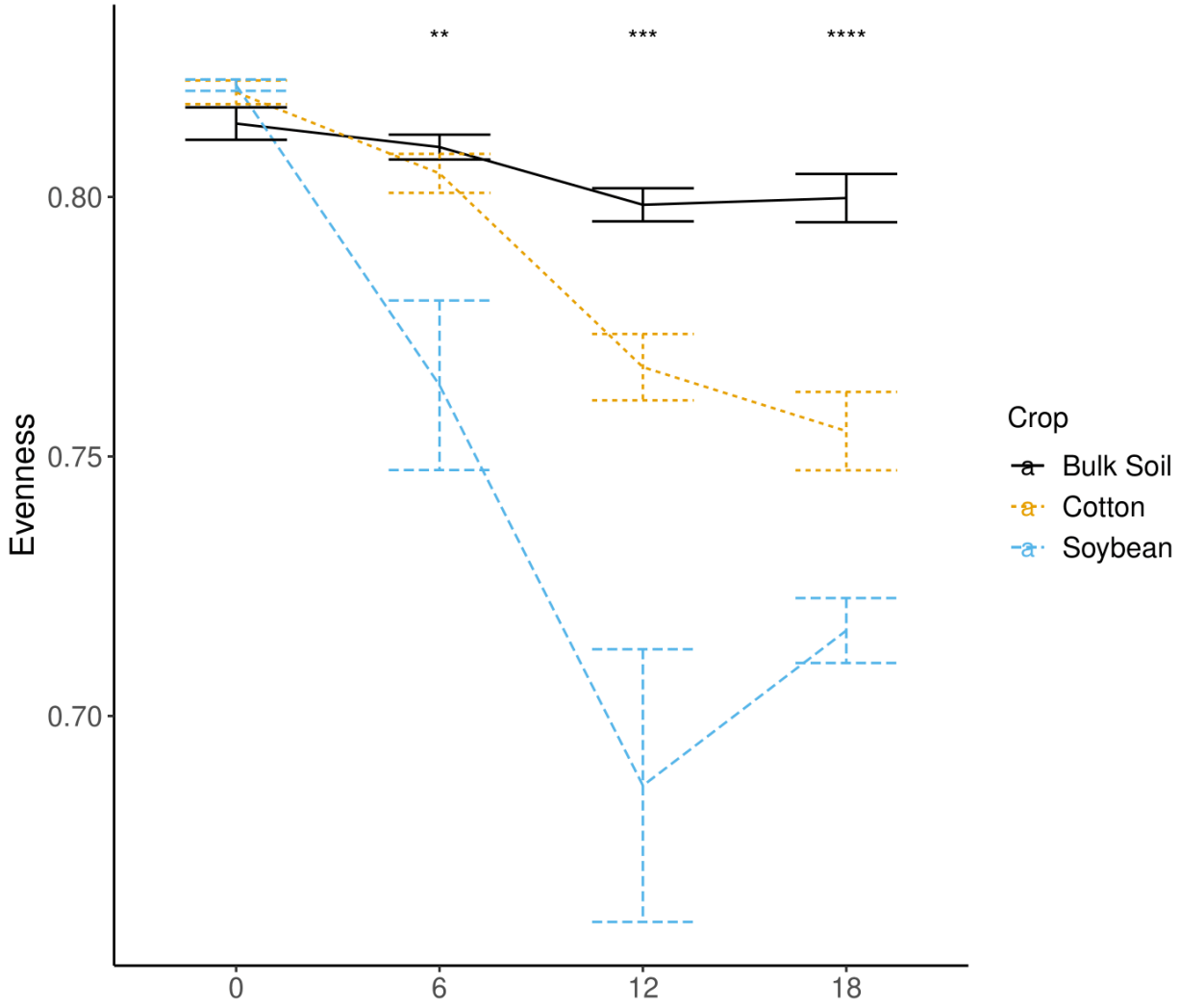


Figure 13. Increased taxa dominance in soybean and cotton spermospheres. Asterisks were used to denote the significance level on the plots with no asterisks when the difference in means is insignificant, '*' when P-value (P) is less than 0.05 but greater then 0.01, '**' when $P < 0.01$ and '***' when $P < 0.001$

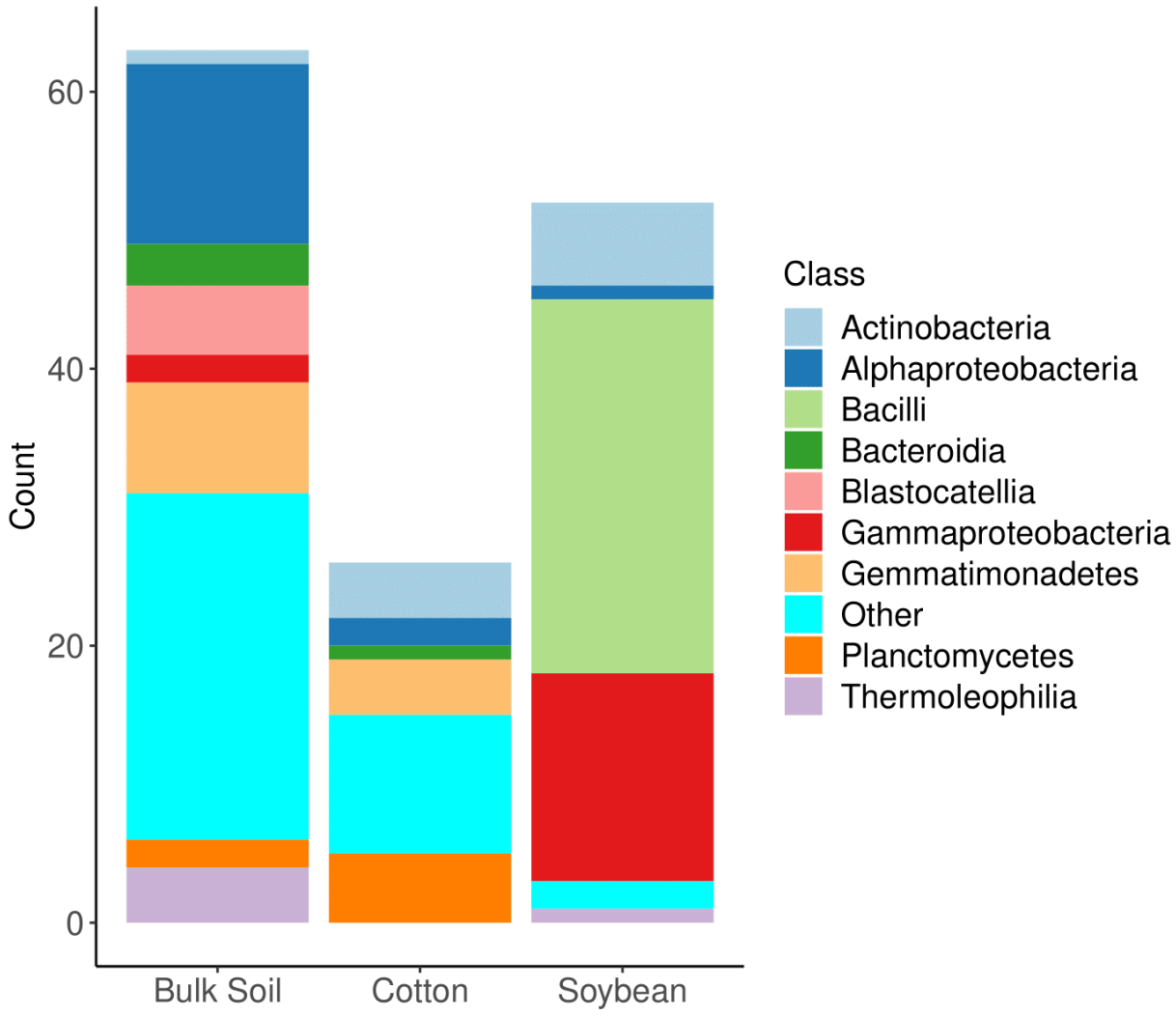


Figure 14. Soybean spermosphere enriched in Bacilli, Gammaproteobacteria and Actinomycetes selected by indicator species analysis (n = 18) (P <0.01). Colors represent bacterial class. Classes with indicator taxa below three were grouped as “Other”.

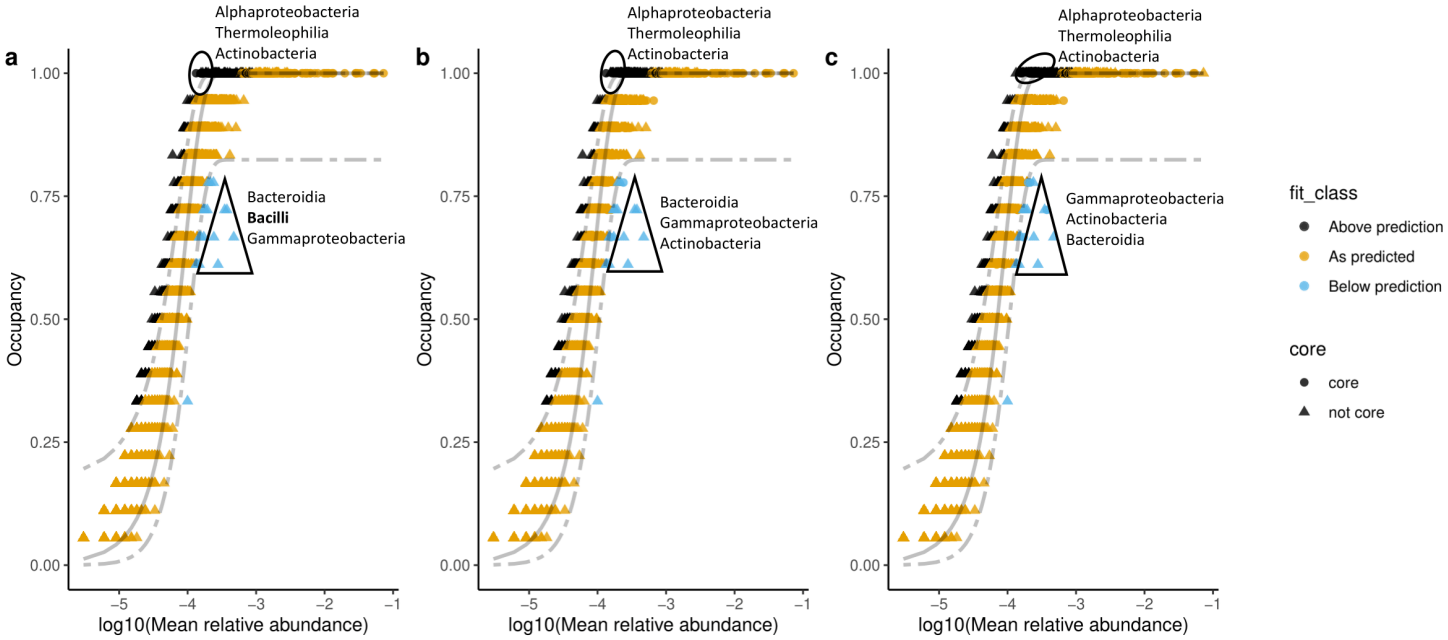


Figure 15. Abundance occupancy distribution for the (a) soybean (b) cotton spermosphere and (c) bulk soil prokaryote community across time points. Grey lines represent the neutral model fit plus or minus the 95% confidence interval. The core microbiome consisted of OTUs that contributed to the last 2% increase in Bray-Curtis distances and are indicated by black circles, whereas triangles are non-core members. Colors of points represent how individual OTUs fit within a neutral model. Black colored points had higher occupancy than expected given their abundance, whereas blue points had higher abundance than expected given their occupancy. Yellow colored points fell within the limits of expectation according to the neutral model.

4. CHAPTER FOUR

Conclusions and Impacts

Conclusions

Oomycetes are major pathogens responsible for seedling damping-off of important field crops including cotton and soybean. Chapter 2 focused on understanding the diversity of species associated with cotton in Alabama which represents one of the seventeen cotton belt states.

We identified 28 different species in Alabama of which the most important and pathogenic species were *Globisporangium irregulare,* and *Phytophthora nicotianae.* Thus, we conclude that management efforts towards cotton seedling diseases caused by oomycetes in Alabama should focus on these species, while recognizing the other species present in less abundance. Additionally, we report the close association of *Phytophthora nicotianae* with seed damping off in heavier soils. Seed treatments highly active towards *Phytophthora nicotianae* should be considered. This finding also informs the prospect of prescriptive management methods based on field properties.

In chapter 3, a novel and precise sampling method was used to collect spermosphere samples, we recovered different prokaryote communities in cotton and soybean spermosphere, which forms 6-12 hours after planting depending on crop. This informs the application of major management strategies for seed and seedling pathogens before or by this time-point. Thus, leading to the precision and optimization of employed strategies. The information from this study could also be applied in understanding seed exudate chemistry and breeding crops with exudates that assemble beneficial microbes and repel major seed pathogens. Overall, these data are important to improve stand establishment and yield.

Impacts

These data have been presented at the Plant Health meetings by the American Phytopathological Society (APS) held in 2021 and 2022 and at the 2022 APS Southern Division meeting. It was also presented at the College of Agriculture student research symposium. Additionally, it was presented as a seminar to faculty and students at the Department of Entomology and Plant Pathology in Auburn University. The death of cotton and soybean seedlings by some of the pathogenic oomycetes isolated from this study has been demonstrated to high school students during the E.A.G.L.E camps at Auburn.

List of Intended Publications

Refereed publications

1. Anticipated Journal: *Plant Disease*
   Olofintila, O.E., Lawrence, K.S., Noel, Z.A., 2023. Diversity of oomycetes associated with cotton seedlings in Alabama. *Plant Disease. Manuscript in preparation.*

2. Anticipated Journal: *Phytobiomes*
   Olofintila, O.E., Noel, Z.A., 2023. Microbial assembly in cotton or soybean spermosphere. *Phytobiomes. Manuscript in preparation.*

3. Anticipated Journal: *Applied and Environmental Microbiology*
   Jahangir Alam, **Oluwakemisola E. Olofintila**, Francesco S. Moen, Zachary A. Noel, Mark R. Liles, and Douglas C. Goodwin
   Broad antibiosis activity of *Bacillus velezensis* and *Bacillus subtilis* accounted for by a conserved capacity for lipopeptide biosynthesis. *Manuscript under review*.

4. Anticipated Publication with Frank S. Moen

5. Anticipated Journal: *The **Auburn** University Journal of Undergraduate Scholarship (**AUJUS**)*
   Charis Harrison, **Oluwakemisola Olofintila**, Laura Rodriguez, Zachary Noel
   Potential of *Chromobacterium* and *Janthinobacterium* ssp. as biocontrols against *Pythium ultimum*

6. Anticipated Journal: *To be determined*
   Alan Yocca, Mary Akinyuwa, Brannan Cliver, Harrison Estes, Abigail Guillemette, Omar Hasannin, Risheek Khanna, Jie Zhang, Qiong Zhang, **Oluwakemisola Olofintila**, Jinesh Patel, Lauren Lopes, Kadi Keita, Nicholas Bailey, Jennifer Hutchison, Madelene Loftin, Wren Jenkins, Erika Moore, Ishveen Kaur, Parbati Thapa, Martin Waldinger, Leslie Goertzen, Huiting Zhang, Stephen Ficklin, Loren Honaas, Alex Harkess
   A chromosome-scale assembly for D'Anjou pear

Non-refereed publications

1. Olofintila, O.E., Moen, F.S., Liles, M., **Noel, Z.A.**, 2021. The diversity and virulence of oomycetes associated with cotton seedlings in Alabama and options for biocontrol. (Abstr.) Phytopathology 111: S2.1.
2. Olofintila, O.E., Moen, F.S., Liles, M., **Noel, Z.A.**, 2022. The diversity and virulence of oomycetes associated with cotton seedlings in Alabama and the reduction of their disease severity using Bacillus biocontrol. (Abstr.) Phytopathology 112:S2.1. https://doi.org/10.1094/PHYTO-112-8-S2.1
3. Olofintila, O.E., Moen, F.S., Liles, M., **Noel, Z.A.**, 2022. The diversity and virulence of oomycetes associated with cotton seedlings in Alabama and the reduction of their disease severity using Bacillus biocontrol. (Abstr.) Phytopathology 112: S2.1

Presentations

4. **Olofintila, O.E**., Moen, F.S., Liles, M.R., Noel, Z.A**.**, 2021. The diversity and virulence of oomycetes associated with cotton seedlings in Alabama and options for biocontrol. Auburn University 2021 Research Symposium. ***Poster Presentation***

5. Harrison, C., **Olofintila, O.E**., Noel, Z.A**.**, 2021. Comparison of soybean spermosphere microbiomes in long-term irrigated versus non-irrigated soil form the Old Rotation. Spring 2021 Undergraduate Research Symposium. ***Poster Presentation***

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