

EVALUATING COTTON SEED TREATMENT EFFICACY, EFFECTS ON SEEDLING
DISEASES AND MICROBIAL DIVERSITY IN ARKANSAS

By

Mariana Araujo Alves Gomes de Souza

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ABSTRACT

Cotton is one of the most significant crops primarily grown worldwide for fiber, feed, and oil production. In the United States, it is primarily cultivated in the ‘Cotton Belt’, a region spanning from Virginia to California and covering approximately 10 million acres. In Arkansas, where cotton is typically grown from late April to October, the crop is susceptible to various fungal diseases that can reduce both lint quality and yield. Among the main diseases of economic importance, the seedling disease complex is a significant global issue affecting the establishment and production of cotton stands. It refers to a range of diseases, primarily caused by *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola* (*Berkeleyomyces basicola*), that compromise cottonseed germination and seedlings' emergence, survival, and development. Fungicide seed treatments are a key tool in managing cotton seedling diseases, offering critical protection against soilborne and seedborne pathogens. However, their effectiveness depends on the composition and prevalence of pathogen populations, which vary annually and regionally, as well as environmental conditions. The objectives of this study were to evaluate the effectiveness of four standard fungicide seed treatments in improving seedling emergence and survival across multiple years and locations in cotton fields in Arkansas. Additionally, we aimed to characterize the soil- and root-associated microbial communities in cotton, investigating how microbial composition varies by location, year, and seed treatment. For that, a field trial was conducted in Judd Hill (2019 – 2023) and Marianna (2021 – 2023), Arkansas. Four treatments containing a base insecticide (imidacloprid) were evaluated. Treatments consisted of no fungicide (T1), metalaxyl (T2), penflufen (T3), and a mix of prothioconazole, myclobutanil, penflufen, metalaxyl (T4). Our results suggest that the use of seed treatments is effective in controlling seedling disease complex, but their efficacy depends on environmental conditions and surrounding microbes.

This thesis is dedicated to Mom and Dad.
Your support has given me the confidence to face any challenge.
Thank you for everything.

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LITERATURE REVIEW

1. Cotton Production and Importance

Cotton is one of the most significant crops primarily grown worldwide for fiber, feed, and oil production (Campbell et al., 2011). It belongs to the family *Malvaceae* and the genus *Gossypium*. Its four cultivated species are *Gossypium herbaceum* L. (Asiatic cotton), *Gossypium arboreum* L. (Asiatic cotton), *Gossypium hirsutum* L., and *Gossypium barbadense* L. (Egyptian cotton), where the first two species are diploids ($2n = 26$), and the latter are allotetraploids ($2n = 52$). However, among those cultivars, *Gossypium hirsutum*, also called upland cotton, is the most broadly planted, making up 90% of global fiber production, with an additional 5–8% produced by *G. barbadense* (Tiwari and Wilkins, 1995; Ji et al., 2002; Aslam et al., 2020).

Cotton is cultivated globally in more than 70 countries, covering over 32 million hectares of land under various environmental circumstances, contributing significantly to the economies of many countries (Saranga et al., 2001; Bange et al., 2016; USDA 2018b; FAO 2018; Jabran et al., 2019). China is the largest cotton-producing country in the world, with an annual production of approximately 6 million tons, which accounts for one-quarter of the global total, followed by India with 5.33 million tons and the United States with 3.82 million tons. Altogether, the three countries accounted for 65% of the world's cotton in 2022 (Meyer and Dew, 2022). Countries such as Pakistan, Brazil, Australia, Uzbekistan, Turkey, Turkmenistan, Burkina Faso, Mali, Greece, and Myanmar also contribute significantly to global cotton production (Tokel et al., 2022).

The top cotton export country in the world is the United States, which exports 3.22 million tons (FAOSTAT, 2023; USDA, 2022; Statista, 2022). The average cotton lint yield and area harvested in the U.S. over the last decade were 946 kg ha⁻¹ and 3.8 million hectares, respectively, whereas the corresponding averages for the world were 775 kg ha⁻¹ and 32.2 million hectares

(USDA–NASS, 2022).

In the United States, cotton is primarily cultivated in 17 southern states, known as the "Cotton Belt", a region spanning from Virginia to California and covering approximately 10 million acres (NCCA, 2022). Texas leads U.S. cotton production, contributing about 40% of the total, followed by Georgia and Arkansas (Statista, 2022; Meyer, 2022a). The U.S. Department of Agriculture (USDA) categorizes cotton-producing states into four regions: the Southwest (Kansas, Oklahoma, and Texas) is the top producer of Upland cotton, followed by the Midsouth or Delta region (Arkansas, Louisiana, Mississippi, Missouri, and Tennessee). The Southeast region (Alabama, Florida, Georgia, North Carolina, South Carolina, and Virginia) ranks third, while the West region (Arizona, California, and New Mexico) contributes the smallest share (Mumma & Hudson, 1999; AgMRC, 2022). Arkansas is one of the major producers of cotton in the United States, ranking third nationally in 2022, with a production of 1.55 million bales (NASS-USDA, 2022). Cotton is one of the most important row crops grown in the state, with most of its planted acreage concentrated in eastern Arkansas, within the fertile Lower Mississippi River Valley region (NASS-USDA, 2022).

2. Environmental and Climatic Factors

Cotton is a significant crop cultivated across diverse soil types and climatic regions (Wang et al., 2011; Shah et al., 2017). Although it originally comes from tropical and subtropical regions where it grows as a perennial plant, cotton is typically cultivated as an annual crop (Constable and Bange, 2015). Its early growth, including germination and seedling development, is influenced by soil physicochemical properties and environmental factors (Bradow and Bauer, 2010). The plant taproot system (McMichael, 1986) plays a crucial role in its ability to access water and nutrients, which are key to healthy growth (Min et al., 2014). However, climate change has brought more

frequent and severe abiotic stresses, such as drought, waterlogging, and temperature extremes, negatively impacting cotton productivity. Drought stress, for example, negatively impacts photosynthesis, boll formation, and both the yield and quality of cotton fiber (Lokhande and Reddy, 2014). Extreme temperatures—whether too high or too low—negatively impact fiber quality (Zheng et al., 2012; Qian et al., 2017).

Cotton development is closely influenced by air temperature during the growing season, with significant development occurring only when temperatures exceed a critical threshold (Roussopoulos et al., 1998; Munro, 1987; McMahon & Low, 1972). For cotton, this threshold temperature is 60°F (15.6°C), below which little to no growth takes place. To quantify growth, degree days (DD60's) are calculated by averaging the daily maximum and minimum air temperatures and subtracting 60. This measurement of accumulated DD60s is a mean development of a valuable tool for tracking cotton growth stages (Kerby et al., 1987; Landivar and Benedict, 1996; Oosterhuis, 1990). The formula for calculating DD60s is as follows:

$$DD60 = \frac{^{\circ}F \text{ maximum} + ^{\circ}F \text{ minimum}}{2} - 60$$

Cotton, being a tropical plant, is highly sensitive to cold temperatures (Hake et al., 1990). For optimal germination, soil temperatures at planting should exceed 60°F (16°C) to ensure uniform germination, while temperatures closer to 30°C promote faster germination, uniform stands, and higher germination percentages (Shumate et al., 2024; Bradow & Bauer, 2010; Krzyzanowski & Delouche, 2011; Tharp, 1960). Wanjura et al. (1967) demonstrated that in the Southern High Plains of Texas, a minimum soil temperature between 60°F and 68°F (15.6°C – 20°C) is necessary to support successful seedling emergence. Soil temperatures below 50°F (10°C) can cause chilling injury to cotton. If this happens, particularly during the most critical stage when the seed is absorbing water, this stress may lead to seed death after the root tip, or radicle emerges about half

an inch or to the failure of a normal taproot development. Chilling within the first five days of planting often results in weak plants, delayed maturity, and lower yields. While dry seeds are highly tolerant to chilling, they become sensitive once placed in moist soil and begin absorbing water. Because shallow-planted cotton seeds experience significant temperature fluctuations, planting should be avoided if temperatures are expected to drop below 50°F within the first few days (Hake et al., 1990). Planting cotton in soils cooler than 55°F (12.8°C) increases the risk of stand loss, seedling diseases, and cold stress, all of which can contribute to reduced yields (Sansone et al., 2002). Cold weather hinders cotton growth and increases its susceptibility to fungal pathogens, which thrive at temperatures around 65°F (Hake et al., 1990).

3. Cotton Seedling Diseases

Cotton diseases significantly impact both yield and quality, posing a major threat to the economic sustainability of farmers (Chi et al., 2021). Estimates of total cotton disease losses range from 6% to 12% of yield annually (Lawrence et al., 2022). In 2023 alone, diseases reduced U.S. cotton yields by 7.4%, equivalent to a loss of 1.4 million bales (Faske and Sisson, 2024). The leading yield-reducing disease in 2023 was root-knot nematode, followed by reniform nematode, seedling diseases, *Stemphylium* leaf spot, and boll rots (Faske and Sisson, 2024).

Among the main diseases of economic importance for the cotton crop, the seedling disease complex is a significant global issue affecting the establishment and production of cotton stands (Howell, 2001; Wang and Davis, 1997). It refers to a range of diseases that compromise cottonseed germination and seedlings' emergence, survival, and development. In 2023, seedling diseases caused an estimated yield loss of 140,745 bales of cotton (480 lb. bales) (Faske and Sisson, 2024). In the field, cotton seedling diseases often appear as gaps or skips in the planting rows, caused by seed rot or preemergence damping-off, where seedlings die before emerging from the soil.

Additionally, seedlings can die from seedling diseases, such as postemergence damping-off, within the first one to four weeks after planting. These diseases not only cause plant losses but also delay early-season crop development, leading to growth setbacks and additional management challenges. In severe cases, the damage may be extensive enough to require replanting (Rothrock and Buchanan, 2017).

Numerous studies in the United States, along with extension publications, have identified cotton seedling pathogens, including true fungi such as *Fusarium* spp., *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* [A.B. Frank]), and *Thielaviopsis basicola* (Berk. & Broome) Ferraris, as well as various oomycetes species belonging to the genus *Pythium* (Hu and Norton 2020; Rothrock and Buchanan 2017; Rothrock et al. 2012; The Cotton Foundation 2007; Wrather et al. 2002; DeVay, 2001). These soilborne pathogens can exist independently or together, maintaining high inoculum levels in fields for years (Watkins, 1981). These pathogens can cause various symptoms on seeds, roots, and hypocotyls under favorable environmental conditions, including seed decay before germination, seedling decay before emergence, girdling of emerged seedlings at or near the soil surface, and rotting of root tips (Blasingame, 1993). When seeds rot or stand failure occurs due to damping-off, it can have moderate to severe impacts on the crop (Rothrock and Buchanan, 2017).

Most soilborne pathogens persist in the soil as dormant propagules, requiring a specific trigger from a plant to break dormancy or germinate before interaction occurs (Huisman, 1988). When plant structures such as seeds, roots, or hypocotyls stimulate these propagules under favorable conditions, the pathogen—or a combination of pathogens—initiates infection and colonization, leading to the development of disease symptoms (Wilson, 2017).

3.1. Cotton Seedling Diseases Caused by Oomycetes

3.1.1. *Pythium* spp.

Pythium is a genus comprising many significant species, several of which are plant pathogens known to cause diseases in various host plants, resulting in substantial economic losses (Rossman et al., 2017; Schroeder et al., 2013; Hendrix and Campbell, 1973). Taxonomically, *Pythium* is classified under the domain Eukarya, kingdom Chromista/Stramenopila, phylum Oomycota, class Oomycetes, order Pythiales, and family *Pythiaceae* (Lévesque et al., 2010).

Pythium species can significantly impact cotton crops, causing various symptoms such as seed rot and preemergence damping-off, which directly contribute to poor stand establishment and yield losses (Arndt, 1943; Howell, 2001; Howell, 2002; Spencer and Cooper, 1967; DeVay et al., 1982). Younger seedlings (6 days old) are particularly susceptible to infection, as compared to older seedlings (12 days old), emphasizing the critical timing of *Pythium* infection and the need for effective management strategies during early plant development (Arndt, 1943; Spencer and Cooper, 1967). In addition, *Pythium* spp. cause root rot and hypocotyl lesions, further compromising plant health (Arndt, 1943; Howell, 2001). Severe stand losses in cotton have been attributed to *Pythium* spp. (DeVay et al., 1982; Fulton and Bollenbacher, 1959; Howell, 2001; Johnson et al., 1978; Ogle et al., 1993). Notably, a significant negative correlation has been observed between the percentage of seedlings from which *Pythium* spp. were isolated and the percent emergence, highlighting their role in seedling establishment issues (Johnson and Doyle, 1986). The importance of *Pythium* spp. in the seedling disease complex was further underscored by the use of Metalaxyl, a fungicide with selective activity against these pathogens, in the National Cottonseed Treatment Trials. Of 119 trials with a fungicide response, Metalaxyl significantly improved stands in 40 trials, demonstrating the widespread impact of *Pythium* spp. on cotton stand

establishment (Rothrock et al., 2012).

Environmental conditions, particularly soil temperature and moisture, play a critical role in the development and severity of diseases caused by *Pythium* species in cotton. Losses from *Pythium* spp. have been shown to increase as soil temperatures decrease, with the greatest losses observed at temperatures between 12°C and 24°C, and with increasing rainfall during the first three days after planting (Rothrock et al., 2012). These findings align with earlier research in Tennessee, where field studies over seven years demonstrated a direct relationship between soil moisture and temperature at planting and the isolation frequency of *Pythium*. Specifically, isolation frequency was negatively correlated with minimal soil temperature and positively correlated with soil moisture content (Johnson et al., 1969).

Controlled studies further substantiate the impact of temperature on disease development. For instance, the optimal temperature for root rot caused by *Pythium irregulare* was 15°C, within a broader range of 15 to 31°C (Roncadori and McCarter, 1972). Similarly, *Pythium ultimum* caused the most damage at temperatures of 18 to 21°C (Arndt, 1943), while germinated seeds exposed to low temperatures (10°C for 3–5 days) in infested soils exhibited significantly reduced emergence (McCarter and Roncadori, 1971). Temperature preferences also vary among *Pythium* species; for example, *P. ultimum* thrives at low to moderate temperatures, while *P. aphanidermatum* has an optimal temperature of 37°C (Howell, 2002).

Soil moisture and texture also influence the activity and severity of *Pythium* infections. Increased soil water content has been shown to enhance *Pythium* growth, creating conditions favorable for disease development (Griffin, 1963; Hillocks, 1992). Additionally, soil texture influences the severity of infections, with clay soils exhibiting a higher susceptibility to *Pythium* compared to sandy soils (Johnson and Doyle, 1986).

3.2. Cotton Seedling Diseases Caused by Fungi

3.2.1. *Rhizoctonia solani*

Rhizoctonia solani Kühn, teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk is one of the most significant soilborne fungal pathogens in the cotton seedling disease complex. Within this species, isolates are classified into anastomosis groups (AGs) and intraspecific groups (ISGs) (Ogoshi, 1987). *Rhizoctonia* isolates that can fuse hyphae (anastomose) are genetically related. These AGs are critical for characterizing and identifying *R. solani* because the species exhibits diverse biotypes with varying pathogenic capabilities and lacks easily distinguishable morphological features. Early research by Parmeter et al. (1969) identified four primary AGs from a study of 138 isolates. Subsequent studies have expanded this classification to include additional AGs (Carling et al., 1994; Carling et al., 2002). In cotton, the primary seedling disease-causing group of *R. solani* is AG-4 (Rothrock and Buchanan, 2017). AG 4, associated with soils historically used for cotton production, has been identified as the most prevalent group affecting cotton in Arkansas (Weinhold, 1977; Rothrock et al., 1995). However, other AGs have been reported to cause symptoms under controlled conditions (Carling et al., 1994; Carling et al., 2002; Wrather et al., 2002).

Rhizoctonia solani is prevalent in most agricultural soils and can reproduce and exist primarily as vegetative mycelium and/or sclerotia (Adams, 1988; Shearwood, 1970). It is a facultative parasite capable of saprophytic growth on soil or organic matter. This pathogen primarily affects subterranean parts of cotton plants, leading to seed rot, pre-emergence death, and post-emergence damping-off, which can severely impact stand establishment and yield (Rothrock, 1996). Among these symptoms, post-emergence damping-off is the most frequently observed, characterized by brown to reddish-brown lesions on the hypocotyl near or below the soil line.

These lesions, often referred to as "sore shin" are sunken and can girdle the hypocotyl, ultimately killing the seedling (Garber and Leach, 1971; Rothrock, 1996; Rothrock, 2001; Rude, 1984).

Some studies have shown that cotton seedlings become increasingly resistant to *R. solani* with age (Hunter et al., 1978; Neal, 1942), suggesting that younger plants are more vulnerable to infection. Research highlights the widespread importance of *R. solani* in stand establishment. For example, it was reported as the most significant pathogen associated with diseased cotton seedlings in Mississippi (Davis, 1975; Ranney, 1962) and Oklahoma (Ray and McLaughlin, 1942). The use of PCNB, a fungicide selective for *R. solani*, in the National Cottonseed Treatment Trials significantly increased plant stands in 44 of 119 trials where a fungicide response was observed, underscoring the pathogen's impact (Rothrock et al., 2012).

The development of diseases caused by *Rhizoctonia solani* is typically more pronounced in sandy, acidic soils, particularly under cool, wet conditions (Collins et al., 2015; Rothrock, 1996). Low soil temperatures (10°C) have been linked to reduced emergence in germinated seeds exposed to *R. solani* for 3–5 days (McCarter and Roncadori, 1971; Shao and Christianson, 1982). While soil moisture showed little effect on plant colonization (Huisman, 1988; Johnson et al., 1969), there was a negative correlation between temperature and exudate production, while exudate levels showed a positive correlation with the growth of *Rhizoctonia solani* (Hayman, 1969). Despite these findings, data from the National Cottonseed Treatment Trials indicated limited differences in fungicide response across soil temperatures ranging from 12 to 24°C and varying rainfall conditions (Rothrock et al., 2012). This suggests that factors beyond temperature and moisture may also play a role in the success of seed treatments against *Rhizoctonia solani*.

3.2.2. *Thielaviopsis basicola* – *Berkeleyomyces*

Thielaviopsis basicola (Berk. & Broome) Ferraris, now reclassified as *Berkeleyomyces*

basicola by taxonomists, is a globally distributed root pathogen broad host range, including economically important crops like cotton (Geldenhuis et al., 2006; Coumans et al., 2011). This fungus is prevalent in both agricultural and non-agricultural soils, underscoring its widespread adaptability and impact (Stover, 1950; Yarwood, 1974). Initially believed to be a saprophyte (Massee, 1912; Gayed, 1972), *T. basicola* is now recognized as a hemibiotrophic plant pathogen, exhibiting characteristics of an obligate parasite during its ecological interactions with host plants (Hood and Shew, 1997; Nan et al., 1992; Bateman, 1963).

The survival and persistence of *T. basicola* are largely attributed to its production of chlamydospores, which serve as crucial survival structures during unfavorable environmental conditions, such as periods of drought or cold (Tsao & Bricker, 1966; Meyer et al., 1994; Hood and Shew, 1997). These chlamydospores ensure the pathogen persists in the soil between growing seasons, remaining viable for over 3 years (Allen, 2001). In addition to chlamydospores, *T. basicola* produces endoconidia, which are implicated in secondary infection cycles, facilitating its spread within host populations (Mathre & Ravenscroft, 1966; Papavizas & Lewis, 1971; Meyer et al., 1994). The endoconidia also contributes to the pathogen's survival outside the host, transitioning into a secondary state known as the secondary chlamydospore. This form is highly durable and capable of surviving in the soil for more than 15 months, further complicating efforts to manage the disease (Stover, 1950; Schippers, 1970).

The widespread presence of *Thielaviopsis basicola* poses a significant challenge for cotton growers across key production areas in the United States. In Mississippi, the pathogen was found in half of the surveyed locations (18 of 36), with some fields experiencing 100% infection in cotton seedlings (Roy & Bourland, 1982). Similar trends were observed in Arkansas and Texas, where *T. basicola* was detected in over 70% of cotton fields, highlighting the scale of its impact on cotton

farming (Rothrock, 1997; Wheeler et al., 2000). The effects of *T. basicola* extend beyond its prevalence, as its presence directly harms plant health. In Mississippi, higher levels of the pathogen were linked to reduced cotton stand establishment and increased root and hypocotyl disease severity (Roy & Bourland, 1982). These symptoms not only weaken seedlings but also contribute to long-term yield losses. Research in controlled field studies demonstrated that even moderate levels of *T. basicola* in the soil (100 chlamydospores per gram of soil) could reduce seed cotton yields by 15% to 21% over two out of three years (Jaraba et al., 2014). These findings underscore the real-world challenges faced by cotton producers dealing with *T. basicola* and emphasize the importance of developing and implementing effective disease management strategies to safeguard cotton yields.

Cotton plants infected with *Thielaviopsis basicola* are characterized by distinct symptoms that include blackened roots and belowground portions of the hypocotyls, accompanied by chlorotic and stunted plant growth (Allen, 2001; Melero-Vara & Jimenez-Diaz, 1990; Rothrock, 1992). Fortunately, the damage is usually limited to the outer layers of the root, sparing the endodermis and vascular cylinder, which are essential for water and nutrient transport (Allen, 2001; Mathre et al., 1966; Mauk & Hine, 1988; Walker et al., 1999). However, in more severe cases, the development of lateral roots is significantly inhibited or completely suppressed, further weakening the plant's ability to grow and thrive (Allen, 2001).

The severity of black root rot in cotton, caused by *Thielaviopsis basicola*, is strongly influenced by environmental factors and soil conditions. Cool temperatures, particularly below 24°C, are conducive to disease development, making early season conditions after planting especially favorable for the pathogen (Allen, 2001; Blank et al., 1953; Maier, 1966; Rothrock, 1992). Additionally, wet or poorly drained soils exacerbate disease severity compared to well-

drained soils (King & Presley, 1942). Soil moisture and texture also play critical roles in how the disease develops. For instance, at 24°C, soils with higher moisture levels (-10 J/kg matric potential) had more root colonization (32%) compared to drier soils (-30 J/kg matric potential), which saw only 12% colonization (Rothrock, 1992). Similarly, soil texture influences the pathogen's behavior, with clay soils often leading to more severe symptoms compared to sandy soils (Hillocks, 1992). Studies found that soils with high sand content (87%) had reduced root colonization, and less pathogen reproduction compared to soils with moderate sand content (48–74%) (Jaraba et al., 2014). These insights highlight the importance of managing soil and environmental conditions to minimize the impact of black root rot, underscoring the need for targeted strategies that account for local climate and soil characteristics.

3.2.3. *Fusarium* species

The genus *Fusarium* includes numerous plant pathogenic species of significant economic importance (Nelson, 1992). Among these, *Fusarium oxysporum* Schlechtend and *Fusarium solani* (Mart.) Sacc. have garnered considerable attention due to their roles as major plant pathogens, causing substantial economic losses across diverse agricultural production systems worldwide (Sanogo & Zhang, 2016; Abd-Elsalam et al., 2006; Colyer, 1988; Colyer, 2001; Melero-Vara and Jimenez-Diaz, 1990; Roy and Bourland, 1982). In cotton production, *Fusarium* species are among the most isolated fungi from diseased seedlings and frequently represent the predominant fungal genus recovered (Colyer, 1988; Davis, 1975; Fulton and Bollenbacher, 1959; Johnson et al., 1978; Johnson and Doyle, 1986; Melero-Vara and Jimenez-Diaz, 1990; Ray and McLaughlin, 1942; Roy and Bourland, 1982).

Symptoms of *Fusarium* infection in cotton seedlings are often confused with those caused by other pathogens, such as *Pythium* spp., *Rhizoctonia solani*, and *Thielaviopsis basicola*. Infected

seedlings display various symptoms, including seed decay, brown or black lesions on the hypocotyls and roots, stunting, chlorosis, reduced root systems, and seedling death (Davis et al., 2006; Hillock, 1992; Sanogo and Zhang, 2016). Wilted cotyledons are commonly observed, and when seedling death occurs, uneven stands may develop in the field, which can serve as an indicator of pathogen presence. Examining the vascular tissue reveals browning within the hypocotyl helps differentiate *Fusarium* spp. from other cotton seedling diseases (Davis et al., 2006; Colyer, 2001).

Fusarium spp. can persist indefinitely in infested fields, thriving in a wide range of soil types that provide optimal conditions for cotton production (Bennett et al., 2008; Elliott, 1923; S.N. Smith et al., 1970; S.N. Smith & Snyder, 1975). This pathogen exhibits considerable adaptability, colonizing organic matter as a saprophyte and even parasitizing non-host plants, such as weeds (Colyer, 2001). Furthermore, *Fusarium* spp. can survive in the soil for over a decade, even in the absence of cotton as a host (S.N. Smith et al., 2001). Their persistence is facilitated by the production of three types of asexual spores: microconidia, macroconidia, and chlamydospores. Chlamydospores, in particular, play a pivotal role in their long-term survival due to their resistance to unfavorable environmental conditions. These spores are essential for the fungus's dissemination and survival in diverse soil environments (DeVay et al., 1997).

Fusarium wilt (FW) is favored by elevated temperatures, with symptoms typically emerging during the seedling stage when temperatures exceed 23°C. Plants become increasingly susceptible at flowering as temperatures reach the optimal range of 28–32°C (Hillocks et al., 1992; Abdel-Raheem & Bird, 1968). Warm, moist soil conditions further promote root infection by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV), while the application of high levels of nitrogen fertilizers has been shown to increase the incidence of FW in cotton (Abdel-Raheem & Bird, 1967).

Soil type and pH also influence disease prevalence: races 1 and 2 of FOV are more prevalent in sandy soils with neutral to acidic pH (5.0–6.5), whereas races 3 and 4 are more commonly associated with clay soils (Hillocks, 1992). The prevalence, diverse pathogenic nature, and survival tactics of *Fusarium* species make them a major concern in cotton seedling diseases. Their ability to persist and adapt highlights the need for effective management strategies to protect stand establishment and maximize yield.

3.3. Cotton Seedling Management

Effective control of cotton seedling diseases involves a combination of cultural practices aimed at minimizing environmental stress and pathogen exposure. Key strategies include delaying planting until soil temperatures are optimal for cotton germination and growth, accompanied by a favorable weather forecast (DeVay and Rothrock, 2001; Hillocks, 1992; Kerby et al., 1989; Minton and Garber, 1983; Rude, 1984). In the United States, cotton is planted as early as March and as late as June and harvested from August to December (Meyer, 2022b). Additionally, using high-quality seed, ensuring proper planting depth, and planting on well-prepared, raised seedbeds in well-drained soils are critical measures to promote healthy stand establishment and reduce disease risk (Allen, 2001; Rothrock, 1992; King & Presley, 1942; Maier, 1966; Wheeler et al., 2000).

3.4. Chemical Seed Treatments

Fungicide seed treatments are crucial in modern agriculture, offering broad-spectrum protection against seed-borne and soilborne pathogens (Lamichhane et al., 2020). The active ingredients (a.i.) in these treatments typically target an entire genus or multiple species, making them an effective first line of defense in disease management strategies. When applied correctly, fungicide seed treatments provide significant agronomic benefits, including improved seedling emergence, enhanced plant height and vigor, and increased plant and root biomass. These

outcomes are attributed to the suppression of pathogens during critical early growth stages (Anderson and Buzzell, 1982; da Silva et al., 2017; Dorrance and McClure, 2001; Guy et al., 1989).

The introduction of systemic fungicides in the early 1970s revolutionized seed treatment practices by offering effective control over both seed-borne and soilborne pathogens. These fungicides quickly became the preferred choice for seed treatment due to their dual-action capabilities (Ayesha et al., 2021). Today, systemic fungicide treatments are a good strategy for disease management for a wide range of field and vegetable crops globally (Bhushan et al., 2013; Lamichhane et al., 2020).

In the United States, cottonseed is routinely treated with fungicides before sale, reflecting both the widespread occurrence of seedling diseases and the effectiveness of these treatments (Rothrock et al., 2007). The primary goal of chemically treating seeds is to eliminate existing pathogens and/or protect them from soil-borne pathogens, particularly during the germination process. Additionally, growers can enhance disease management by applying supplementary fungicides directly to the seed before planting or through in-furrow applications during planting. These practices offer improved protection for emerging seedlings and have been shown to effectively control seedling diseases (Chambers, 1995; Colyer and Vernon, 2005; Minton and Garber, 1983; Minton et al., 1982).

The National Cottonseed Treatment Program, under the Cotton Disease Council, has systematically assessed cotton seedling survival across a range of environmental conditions throughout the Cotton Belt to evaluate the performance of commercial fungicide seed treatment combinations. Since its establishment in 1993, researchers evaluated the impact of seedling diseases and fungicide seed treatments on cotton by assessing stand improvements from industry-standard seed treatments and experimental compounds compared to untreated seeds (Rothrock et

al., 2012). They also investigated the role of specific pathogens by testing selective fungicide treatments against untreated seeds, using metalaxyl to target *Pythium* and PCNB for *Rhizoctonia solani*, among others. This study also investigated how environmental factors influenced cotton stand establishment by recording soil temperature, moisture levels, and rainfall data for each trial site. Results showed that fungicide seed treatments significantly enhanced stand establishment in most trials, emphasizing the importance of seedling diseases. Both selective treatments, metalaxyl and PCNB, effectively improved stands, highlighting the widespread presence of *Pythium* and *R. solani*, respectively. Additionally, combination seed treatments consistently outperformed untreated seeds under all environmental conditions. However, environmental factors played a crucial role in fungicide effectiveness (Rothrock et al., 2012). This study provided important insights into the complex interactions between cotton seedling diseases and their environment, helping to develop better strategies for healthier crops and improved yields.

Fungicide seed treatments are categorized by the Fungicide Resistance Action Committee (FRAC) based on their mode of action and resistance risk (Frac List 2024). Commonly used seed treatment fungicides fall under FRAC codes 3, 4, 7, and 11. FRAC Code 3 includes demethylation inhibitors (DMIs), which disrupt fungal growth by blocking ergosterol biosynthesis—a key component of the plasma membrane essential for certain fungi (Wyenandt, 2021). Triazole fungicides, such as myclobutanil and triadimenol, have demonstrated efficacy against black root rot (*Thielaviopsis basicola*) (Toksoz et al., 2009). Phenylamide fungicides (PA) (FRAC Group 4) are a potent class of fungicides specifically targeting oomycete pathogens, including *Phytophthora* and *Pythium* spp. These fungicides are highly effective against several significant plant pathogens. However, like other fungicide classes, they carry a high risk of resistance development. Phenylamides inhibit ribosomal RNA (rRNA) biosynthesis in oomycetes, disrupting multiple life

stages such as hyphal growth, haustorium development, and sporangia formation (Wyenandt, 2020a).

Penflufen, a fungicide, is a key active ingredient in seed treatment formulations used to manage seedling diseases in soybeans caused by *R. solani* (Ajayi-Oyetunde et al., 2017). Cross and Druce (2012) identified penflufen as a newly developed succinate dehydrogenase inhibitor fungicide, effective as a seed treatment against various seed- and soil-borne fungal diseases, including smuts, bunts, and Rhizoctonia root rot in cereals. FRAC code 7 includes succinate dehydrogenase inhibitors (SDHIs), which inhibit complex II of fungal mitochondrial respiration by binding to succinate dehydrogenase (SDH) and blocking electron transfer from succinate to ubiquinone. Due to their highly specific modes of action, this group is susceptible to resistance development (Wyenandt, 2020b). Strobilurin fungicides, also known as QoI fungicides (FRAC code 11), are highly effective in managing a wide range of common vegetable pathogens. These fungicides work by disrupting fungal respiration, specifically by binding to the cytochrome b complex III at the Qo site in the mitochondria (Wyenandt, 2013). In simple terms, they inhibit the fungi's ability to carry out normal respiration, ultimately preventing growth and infection. While strobilurins offer strong disease control, their precise mode of action makes them particularly vulnerable to resistance development in certain fungal populations.

Despite extensive research on cotton seedling diseases and the use of fungicide seed treatments, several gaps remain. While fungicide seed treatments are widely used, their effectiveness can be inconsistent due to regional and annual variations in pathogen populations and environmental conditions. Soil temperature and moisture are critical factors influencing seedling disease severity, with studies showing that cooler temperatures and increased rainfall shortly after planting can exacerbate seedling losses due to pathogens (Colyer et al., 1991; Johnson

et al., 1969). In multi-year field studies, *Pythium* isolation frequency was negatively correlated with soil temperature and positively correlated with soil moisture, highlighting the importance of environmental conditions in shaping disease dynamics (Davis et al., 1997). Additionally, fungicide efficacy may vary depending on the dominant pathogen at a given location. For example, seed treatments targeting *Pythium* spp. were found to have greater stand protection under wet, cool conditions, whereas *R. solani* caused stand losses over a broader range of temperatures and soil moisture levels (Walker, 1928; Hunter et al., 1960). More research is needed to understand the factors influencing this variability to refine seed treatment strategies that account for site-specific environmental conditions and pathogen pressures.

While previous studies have primarily focused on the direct impact of fungicides on target pathogens, there is limited understanding of how these treatments affect the broader soil- and root-associated fungal communities. Given that non-pathogenic microbes can play beneficial roles in plant health and disease suppression, alterations in microbial composition due to fungicide use may have unintended consequences. Understanding these interactions is critical for optimizing disease management strategies that balance pathogen control with maintaining beneficial microbial diversity.

Furthermore, most research has examined short-term impacts on seedling emergence and survival. Still, fewer studies have explored how repeated fungicide use over the years can influence microbial diversity and composition over multiple growing seasons. Long-term shifts in microbial communities could have lasting effects on soil health, pathogen dynamics, and plant resilience. Additionally, while seedling disease management typically targets a complex of pathogens, including *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*, there is limited information on how individual pathogens respond to different active ingredients under

varying environmental conditions. A more detailed understanding of these pathogen-specific responses could improve treatment recommendations and lead to more targeted disease management strategies.

This study aims to address these gaps by evaluating the effectiveness of fungicide seed treatments across multiple years and locations while characterizing microbial community shifts associated with different seed treatments. By integrating field-based agronomic evaluations with microbial and ecological analyses, this research contributes to a more comprehensive understanding of seed treatment efficacy and its broader implications for cotton production. These insights will help refine disease management strategies, ensuring more effective and sustainable approaches to cotton production under varying environmental conditions.

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CHAPTER 1: EVALUATION OF FUNGICIDE SEED TREATMENTS UNDER FIELD CONDITIONS FOR COTTON SEEDLING DISEASE CONTROL

ABSTRACT

Cotton (*Gossypium hirsutum* L.) is one of the world's most important natural fiber crops, playing a crucial role in the global economy and everyday life. Among the main diseases that impact cotton economically, the seedling disease complex is a significant global issue that affects the establishment and overall production of cotton. It refers to a range of diseases that compromise cottonseed germination and seedlings' emergence, survival, and development. Fungicide seed treatments are an effective strategy for controlling soilborne pathogens. However, their effectiveness can vary depending on environmental conditions, pathogen presence, and the specific active ingredients used. Understanding how different fungicide treatments perform under various field conditions is essential for optimizing disease control strategies. The objective of this study was to evaluate the effectiveness of four standard fungicide seed treatments in improving seedling emergence and survival in cotton fields in Arkansas and their performance over the years. A field trial was conducted in Judd Hill and Marianna, Arkansas. Four treatments containing a base insecticide (imidacloprid) were evaluated. Treatments consisted of no fungicide (T1), metalaxyl (T2), penflufen (T3), and a mix of prothioconazole, myclobutanil, penflufen, metalaxyl (T4). Plant stands were recorded from 2019 to 2023 for Judd Hill and 2021 to 2023 for Marianna. Across the years, there were no statistical differences between the treatments at Judd Hill, while T2 and T4 had the highest stand counts at Marianna. In 2021, T2 and T4 had higher yields at Judd Hill, but the differences were not significant. Similarly, no significant differences were found at Marianna. Our results suggest that the use of seed treatments is effective in controlling seedling disease complex, but their efficacy depends on temperature and surrounding microbes.

1. INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the world's most important natural fiber crops, playing a crucial role in the global economy and everyday life. In 2023, cotton generated approximately \$5 billion in revenue in the United States, with Arkansas contributing over \$504 million, making it the third-largest producer in the country (USDA, 2023). Cotton is cultivated in subtropical to tropical regions across various latitudes. Although naturally a perennial plant, it is often managed as an annual crop, a practice that, along with frequent stress conditions, can intensify disease issues (Rothrock et al., 2015). In Arkansas, where cotton is typically grown from late April to October, the crop is susceptible to various fungal and bacterial diseases that can reduce both lint quality and yield. In 2023, such diseases reduced U.S. cotton yields by 7.4%, amounting to a loss of 1.4 million bales (Faske & Sisson, 2024).

Among the most economically significant diseases affecting cotton is the seedling disease complex. This complex compromises seed germination and impairs the emergence, survival, and development of seedlings (DeVay, 2001; Ogle et al., 1993; Hillocks, 1992; Melero-Vara and Jimenez-Diaz, 1990). Its economic impact is substantial, with estimated annual production losses exceeding \$40 million, excluding the additional replanting costs (Blasingame, 2006; USDA, 2021; Lawrence et al., 2021). Moreover, cotton seeds represent the second-highest operating cost for farmers, totaling approximately \$1 billion annually (McCowen, 2022). Therefore, protecting seeds from seedling diseases is critical (Rothrock et al., 2012). The primary pathogens associated with this complex are *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*. These pathogens can operate individually or in combination, affecting roots and hypocotyls (DeVay, 2001; Roy and Bourland, 1982; Johnson et al., 1978; Fulton and Bollenbacher, 1959).

Symptoms of cotton seedling diseases include seed rot, preemergence, and postemergence

damping-off, which can lead to stand losses, hypocotyl lesions, and root rot (Rothrock et al., 2007). In the field, these diseases often manifest as the absence of plants or skips in the planting row due to rotted seeds or seedlings that die before or shortly after emergence, typically within the first one to four weeks after planting. These conditions weaken plants, delay early-season growth, and result in poor stand uniformity, which negatively affects yield. Additionally, seedling diseases introduce further management challenges, such as improper timing of herbicide, insecticide, or fertilizer applications. In severe cases, replanting may become necessary (Rothrock et al., 2007; Rothrock et al., 2017).

The soil environment plays a critical role in developing seedling diseases, with soil temperatures and moisture levels during the first few weeks after planting being particularly influential. These factors affect both the host plants and the pathogens (Johnson et al., 1969; Minton et al., 1982; Riley et al., 1969). Optimal conditions for rapid cotton seed germination and robust seedling development include soil temperatures of 65°F or higher, along with well-prepared beds that ensure proper water infiltration and drainage. However, to extend the growing season and reduce competition from weeds—which can outcompete cotton plants for water and harbor insect pests—growers often plant early. This early planting can expose seeds to cool, moist soils, which favor the growth of pathogens and increase the risk of seedling diseases.

Seed treatments are universally used for managing cotton seedling diseases, involving the application of various fungicides to cottonseed before sale to protect the crop from a range of pathogens (Kelly et al., 2018; Davis et al., 1997; Hillocks, 1992; Minton and Garber, 1983). In response to the need for effective disease management across diverse environmental conditions throughout the U.S. Cotton Belt, the National Cotton Seed Treatment Program was established in 1993. For over 20 years, the National Cotton Seed Treatment program has analyzed soilborne

pathogen populations known to cause cotton seedling diseases across the U.S. Cotton Belt. Conducted at the University of Arkansas from 1995 to 2017, the program transitioned to the University of Tennessee in 2018, where investigators have continued all established protocols. Initially established to assess cotton seedling survival under various fungicide seed treatment combinations nominated by industry representatives across diverse environmental conditions and pathogen populations, the program has provided valuable insights for cotton growers nationwide (Guyer et al., 2019). The National Cotton Seed Treatment Program also conducts disease ratings, pathogen isolations from seedlings, and assessments of soilborne pathogen populations by collecting seedlings and soil samples from non-treated control plots at each location.

Building upon the work of the National Cotton Seed Treatment Program, this study further evaluates the effectiveness of fungicide seed treatments in managing cotton seedling diseases in Arkansas, specifically focusing on their impact on seedling emergence and survival under local environmental conditions. The program has provided valuable insights into seedling survival and pathogen populations across the U.S. Cotton Belt, particularly through the analysis of soilborne pathogens and their interactions with different fungicide treatments.

The standard treatments evaluated in this study included metalaxyl (treatment 2), penflufen (treatment 3), and a combination of metalaxyl, penflufen, prothioconazole, and myclobutanil (treatment 4). Treating cotton seeds with metalaxyl is a common practice to protect seeds from *Pythium* spp. (Thomson, 1991). Penflufen targets *Rhizoctonia solani*, another major seedling disease pathogen. Seeds treated with this active ingredient have demonstrated antifungal activity, helping to reduce infection and improve seedling survival (Di et al., 2021). The combination of different active ingredients (metalaxyl + penflufen + prothioconazole + myclobutanil) is a widely used standard seed treatment among cotton growers. It provides broad-spectrum protection against

multiple soilborne pathogens, including *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*, and has been shown to improve seedling establishment under field conditions (Kelly et al., 2023).

There are a limited number of studies looking at seed treatment effects on seed germination; while the studies available indicated interactions with weather; these studies were overdone over ten years ago. We aim to look at more recent interactions with current standard chemistries over the years to determine if seed treatments still contribute to disease control if conditions are conducive. Therefore, the objective of this study was to evaluate the effectiveness of four standard fungicide seed treatments in improving seedling emergence and survival in Arkansas cotton fields and their performance over multiple years. We hypothesize that treatment four, which combines four different active ingredients, will significantly enhance seedling survival compared to others. Field trials were conducted to compare these treatments under varying environmental conditions, offering valuable insights for cotton growers in Arkansas and similar regions.

2. MATERIALS AND METHODS

2.1. Field Trials

Field trial experiments were conducted at two research stations in Arkansas: Judd Hill in Poinsett County (Northeast Arkansas), and Marianna in Lee County (Central East Arkansas). The trials took place at Judd Hill from 2019 to 2023. Marianna's location was included in the study from 2021 to 2023 to account for different environmental conditions and disease pressure. The experiments were planted following a randomized complete block design, with replications between locations ranging from four to five. The experimental plots were composed of four-row plots 30 feet long and 38-inch row spacing, with a planting rate of 5 seeds per foot.

2.2. Fungicide Seed Treatments

Every year, the National Cottonseed Treatment Program (NCST) evaluates cotton seedling survival for several fungicide seed treatment combinations under diverse environmental conditions and populations of cotton seedling pathogens. The program comprises four standard treatments and eleven fungicide seed treatments nominated by chemical industry representatives. These treatments were used on common commercial cultivars. In 2019, the cotton cultivar DP 1522 B2XF was used at Judd Hill. From 2020 to 2023, the cultivar DP 1646 was used for both locations, Judd Hill and Marianna (Table 1.1).

Over the five years, the four standard treatments were tested across Judd Hill and Marianna. The treatments and their respective application rates were as follows: 1 = Nontreated check (Imidacloprid only), 2 = Metalaxyl, 3 = Penflufen, and 4 = Metalaxyl + Penflufen + Myclobutanil + Prothioconazole. Fungicides were mixed with water to achieve a total slurry rate of 30 fl. oz/cwt, and Imidacloprid was applied at 12.8 fl. oz/cwt to all seeds, including those in treatment 1, which did not receive a fungicide treatment. Including Imidacloprid in all treatments was intended to reduce potential insect damage during the trials

2.3. Seed Germination

The seed germination rate for each treatment was evaluated under controlled conditions using a potting mix in the greenhouse. Ten seeds were placed in plastic trays, and emergence was recorded 14 days after planting. Seed germination rates were determined by dividing the number of seeds that germinated in each treatment by the total number of seeds planted in that treatment, then multiplying the result by 100 to express it as a percentage, following the equation:

$$\text{Seed Germination Rate} = \frac{\text{Seeds germinated}}{\text{Total Seeds}} \times 100$$

Where *Seeds germinated* = the number of seeds germinating in each treatment, and

Total Seeds = the total number of seeds planted in that treatment.

2.4. Stand Counts and Yield

Stand count data for each treatment within a location was converted to percent emergence with the following equation:

$$\text{Percent Emergence} = \frac{\text{Counts}}{\text{Total Planted Seeds}} \times 100$$

Where *Counts* = the number of emerged plants per treatment per replicate, and *Total Planted Seeds* = the total number of seeds planted for the plot length. For each trial, stand counts were taken 30 days after planting for each treatment across all replicates within a location. Data were then analyzed with R Studio version 4.3.

For yield, each row of each replicate was harvested. Yield from the two central rows was averaged and converted to seed cotton pounds per acre (lb/ac).

2.5. Data Analyses

Seed treatment efficacy was analyzed using a linear mixed-effects model using the “lmer” function from the “lme4” package. The fixed effects in the model were location, year, treatment, and their interactions, and random effects were associated with different combinations of replicates, years, and locations. A Tukey HSD means separation test to determine significant differences between treatments was also performed in R Studio. An alpha level of 0.05 was used to determine significance when evaluating treatment effects.

3. RESULTS

3.1. Effect of Seed Treatments on Cotton Stands at Judd Hill (2019-2023) and Marianna (2021-2023)

Before the field season, germination in controlled conditions was examined to determine potential issues with seed quality. Percent emergence before planting was evaluated using a potting

mix in the greenhouse, and all treatments had germination higher than 80%.

From 2019 to 2023 at Judd Hill and from 2021 to 2023 at Marianna, field trials were conducted to assess the efficacy of four standard fungicide treatments in managing cotton seedling diseases. Field emergence was determined as stand counts and was recorded 30 days after planting (Table 1.2). The treatments included Treatment 1 (control with Imidacloprid only), Treatment 2 (Metalaxyl + Imidacloprid), Treatment 3 (Penflufen + Imidacloprid), and Treatment 4 (a broad-spectrum combination of Prothioconazole, Myclobutanil, Penflufen, Metalaxyl, and Imidacloprid). The stand count analysis across the years and locations showed that location ($P < 0.001$), year ($P < 0.001$), treatment ($P = 0.024$), and the interaction location x year ($P < 0.001$) significantly affected stand (Table 1.3). Thus, the significant interaction indicates that the response varied by year, depending on environmental conditions and/or pathogen pressure at each specific location, as expected. Overall, at Judd Hill, no significant differences in stand counts between treatments were observed within the years, suggesting that the treatments had comparable effects on stand establishment. At Marianna, treatment effects were more pronounced, especially in 2022 and 2023.

At Judd Hill, over the years, there was no significant difference in field emergence between the treatments within each year (Figure 1.1). In 2019, the percentage ranged from 74.2% to 81.3%. Treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) had the highest stand count, and the lowest was treatment 3 (Penflufen), suggesting *Rhizoctonia* was less important in this field this year. In 2020, the percentage ranged from 77.4% to 86.8%, with no significant differences between treatments. Treatment 2 (Metalaxyl) had the highest stand count, and the lowest was treatment 1 (Imidacloprid only). In 2021, the percentage ranged from 63.9% to 68.8% (Table 1), with no significant differences between treatments. Treatment 4 (Prothioconazole +

Myclobutanil + Penflufen + Metalaxyl) had the highest stand count, and the lowest was treatment 2 (Metalaxyl). Stand counts at Judd Hill in 2022 were notably high, ranging from 85.4% to 91.0%, with no significant differences among treatments. Treatment 3 (Penflufen) had the highest stand count, and the lowest was treatment 2 (Metalaxyl). In 2023, Judd Hill stand counts ranged from 88.2% to 96.2%, with no significant differences between treatments. Treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) had the highest stand count, and the lowest was treatment 1 (Imidacloprid only).

At Marianna in 2021, the percentage ranged from 83.5% to 88.2%, with no significant differences among treatments (Figure 1.1). Treatment 2 (Metalaxyl) had the highest stand count, and treatment 1 (Imidacloprid only) had the lowest. In 2022, the percentage ranged from 96.3% to 100%, with a significant difference between the treatments. Treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) had the highest stand count, and treatment 2 (Metalaxyl) had the lowest. In 2023, the percentage ranged from 73.4% to 88.8%, and significant differences were also observed. Treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) had the highest stand count, and treatment 1 (Imidacloprid only) had the lowest.

In most of the years, Treatment 4 (broad-spectrum) showed numerically higher stand counts at Judd Hill and demonstrated significantly improved stands at Marianna in 2022 and 2023. In addition, Treatment 2 (Metalaxyl) showed significantly higher stand counts in 2023 at Marianna compared to the control and Penflufen (Treatment 3). However, Treatment containing only Metalaxyl resulted in numerically lower stand counts in 2021 and 2022 at Judd Hill, with a significant reduction in 2022 at Marianna.

3.2. Effect of Seed Treatments on Cotton Yield at Judd Hill (2019-2023) and Marianna (2021-2023)

The yield analysis across the years and locations showed that location ($P < 0.001$), year ($P < 0.001$), treatment ($P < 0.001$), and interaction location x year ($P < 0.001$) all significantly affected yield. The significant interaction suggests that year response was dependent on the environment and/or pathogen pressure for a particular location.

Average yields across fungicide seed treatments and control plots fluctuated by location and year, with only 1 out of 8 trials showing a statistically significant yield increase from fungicide treatments compared to the control (Figure 1.2). In the other seven trials, fungicide treatments did not significantly improve yields over the control, suggesting similar efficacy of the treatments in that environment. At Judd Hill, there were no significant differences in yield among treatments within years, except in 2021, where Treatments 2 (Metalaxyl) and 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) produced significantly higher yields compared to the control (Treatment 1), while Treatment 3 (Penflufen) had the lowest yield, showing a statistically significant reduction. All differences were considered significant at $p \leq 0.05$. At Marianna, yields did not differ significantly between treatments within any year, suggesting similar efficacy of the treatments in that environment.

4. DISCUSSION

Seedling diseases are a significant barrier to maximizing cotton production potential. In the United States, cottonseed is routinely treated with various fungicide combinations before sale. This practice aims to protect the crop against a range of seedling disease pathogens (Davis et al., 1997; Hillocks, 1992; Milton and Garber, 1983). Control measures have proven essential in cool soil conditions that favor seedling disease development (Brown and McCarter, 1976; Colyer et al.,

1991; Roncadori and McCarter, 1972). However, the advantages of cotton seed fungicides are less evident in fields with low pathogen inoculum densities or soils that support quick seedling emergence and growth (Davis et al., 1997). The effect of fungicide seed treatments on stand establishment, along with the role of the cotton seedling disease complex in reducing stand counts, were evaluated by comparing stand counts across four different fungicide treatments over multiple years and locations. To contextualize these findings, we incorporated pathogen pressure data from the National Cottonseed Treatment Committee (NCST) to help explain treatment efficacy and stand reductions.

Based on the efficacy results of the fungicide seed treatments, stand counts measured 30 days after planting showed variability across years and locations. This variability underscores the influence of environmental and site-specific factors on stand establishment. At Judd Hill, the broad-spectrum fungicide seed treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) consistently showed a numerical increase in stand establishment compared to both the non-treated seeds (treatment 1 – Imidacloprid only) and selective treatments (treatment 2 – Metalaxyl and treatment 3 – Penflufen) in 2019, 2021, and 2023. In 2020, all treatments showed numerically higher stands than the non-treated seed (treatment 1 – Imidacloprid only), with treatment 2 (Metalaxyl) numerically resulting in the highest stands among them, while in 2022, treatment 3 (Penflufen) numerically improved stands. At Marianna in 2021, all treatments showed numerically higher stands than the non-treated seed (treatment 1 – Imidacloprid only), with treatment 2 (Metalaxyl) numerically resulting in the highest stands among them. In 2022, the broad-spectrum fungicide seed treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) demonstrated a significant improvement in stands over the non-treated seeds (treatment 1 – Imidacloprid only) and selective treatments (treatment 2 – Metalaxyl and treatment 3 –

Penflufen). In 2023, both the broad-spectrum fungicide treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) and treatment 2 (Metalaxyl) significantly improved stands compared to the non-treated seeds and treatment 3 (Penflufen).

At Judd Hill in 2019, the broad-spectrum fungicide seed treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) showed a numerical increase in stand establishment compared to both the non-treated seeds (treatment 1 – Imidacloprid only) and selective treatments (treatment 2 – Metalaxyl and treatment 3 – Penflufen). This result suggests that multiple pathogens contributed to stand loss. The Cottonseed Treatment Committee's 2019 report noted the presence of *Thielaviopsis basicola* (35% isolation frequency), *Fusarium* spp. (67% isolation frequency), *Pythium* spp. (67% isolation frequency from selective media and 25% isolation frequency detected by ELISA), and *Rhizoctonia solani* (detected in soil at 27.4 propagules/100 cm³ and in seedlings at a low frequency of 5%) at Judd Hill. Despite the prevalence of *Fusarium* spp., most of these isolates were likely nonpathogenic, consistent with findings from the 2018 report, where high *Fusarium* levels did not correlate with stand loss (Kelly et al., 2019). Seeds are treated with Myclobutanil to protect against Black Root Rot, caused by *Thielaviopsis basicola*. We believe that *Thielaviopsis basicola* plays a role in reducing stand counts at this site, as treatments without Myclobutanil (Treatments 1, 2, and 3) resulted in consistently lower stand counts compared to Treatment 4, which includes Myclobutanil. This aligns with findings from other studies, such as Toksoz et al. (2009), who observed that Myclobutanil effectively controls Black Root Rot in naturally infested soils by reducing root and hypocotyl discoloration caused by *T. basicola*. Their work also showed that in some experiments, higher application rates (42 g a.i./100 kg seed) provided greater disease reduction than lower rates (21 g a.i./100 kg seed), supporting our observation of Myclobutanil's effectiveness in pathogen management. *Rhizoctonia* species

propagules can be estimated using the toothpick-baiting-method (Paulitz and Schroeder, 2005). In this method, the soil is added to fill a pot up to a marked fill line; then, the pot is placed in a water-filled tray to saturate the soil. After saturation, the pot is drained overnight. Flat toothpicks are then inserted vertically into the soil, left for 48 hours, and transferred to TSM selective medium (Spurlock et al., 2011) to promote the growth of *Rhizoctonia* species. Although *Rhizoctonia* was isolated from both soil and seedlings in 2019 at Judd Hill, its impact on stand establishment might have had a relatively minor effect, as Treatment 3 (Penflufen) showed the lowest numerical effectiveness in improving stand counts. *Pythium* spp. was also isolated from seedlings at Judd Hill in 2019. In contrast to *Rhizoctonia*, *Pythium* appears to impact stands, as Treatment 2 (Metalaxyl), which is selective against this pathogen, resulted in a numerical increase in stands, reinforcing its role in stand loss. Overall, these results suggest that the superior efficacy of Treatment 4 at Judd Hill in 2019 can be attributed to its broad-spectrum action, which likely managed a diverse range of pathogens, including *Thielaviopsis basicola* and *Pythium* spp., contributing to its numerically enhanced performance in improving stand establishment.

In 2020, all treatments resulted in numerically higher stand counts compared to the non-treated seed (treatment 1 – Imidacloprid only), with treatment 2 (Metalaxyl) numerically showing the highest stand counts among them. This suggests that *Pythium* spp. likely contributed to stand loss that year. The Cottonseed Treatment Committee's 2020 report identified *Pythium* spp. as a major pathogen at Judd Hill, with an isolation frequency of 81% and high pathogenicity in 2020. This supports our findings, where Metalaxyl, which targets *Pythium* spp., numerically increased stand establishment compared to the non-treated seeds, emphasizing the role of *Pythium* in seedling survival. Davis et al. (1997) conducted a 3-year study in the San Joaquin Valley and found that Metalaxyl alone had a positive impact on stands only in 1995 trials, with no significant effect in

1993 or 1994. Although *Thielaviopsis basicola* (4%) and *Fusarium* spp. (100%) were also isolated, these isolates were largely non-pathogenic. *Rhizoctonia solani* was detected in soil at 29.5 propagules/100 cm³ but was not found in seedlings, further supporting that *Pythium* spp. appeared to be the primary pathogen influencing stand establishment in 2020.

In 2021, stand counts at Judd Hill ranged from 63.9% to 68.8%, representing the lowest levels observed across all years and locations. The broad-spectrum fungicide seed treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) showed a numerical increase in stand establishment compared to both the non-treated seeds (treatment 1 – Imidacloprid only) and selective treatments (treatment 2 – Metalaxyl and treatment 3 – Penflufen). This result suggests that multiple pathogens contributed to stand loss. However, treatment 2 (Metalaxyl) numerically resulted in the lowest stand count, suggesting that *Pythium* spp. might have had a relatively minor impact on stand establishment. The Cottonseed Treatment Committee's 2021 report indicated low frequencies of *Thielaviopsis basicola* (1%) and *Fusarium* spp. (22%), with the latter being likely non-pathogenic. They initially isolated potential *Pythium* spp. from seedlings. However, further pathogenicity testing revealed that none of these isolates were accurately classified as members of the *Pythium* genus. Consequently, all *Pythium* isolation data were excluded due to concerns about the reliability of the identification. The report also showed that *Rhizoctonia solani* was isolated from seedlings (14%) but was not detected in soil samples, which may be due to the dry soil conditions and/or potential issues in the pathogen isolation process. Overall, the lowest stand counts and the lack of significant differences in stand counts, despite the presence of *R. solani*, suggest that pathogen levels were likely insufficient to cause substantial variation in seedling establishment. Additionally, the absence of reliable *Pythium* spp. data limits the assessment of Treatment 2 (Metalaxyl) efficacy.

In 2022, treatment 3 (Penflufen) numerically improved stands, suggesting that *Rhizoctonia solani* played a role in successful stand establishment. The Cottonseed Treatment Committee's 2022 report found *R. solani* in seedlings (28%) but not in soil. The absence of *R. solani* in soil samples may have been influenced by dry soil conditions or potential issues during pathogen isolation. Regarding other pathogens, the report noted an isolation frequency of *Thielaviopsis basicola* (44%) and *Fusarium* spp. (28%). Even though *Fusarium* spp. were present, it is important to note that not all isolates were likely pathogenic, reinforcing that the observed effects on stand establishment were more strongly influenced by *R. solani*. *Pythium* spp. were isolated (44%), and based on the pathogenicity assay, most of the isolates were pathogenic, but their impact on stand establishment was likely minimal.

In 2023, all treatments resulted in numerically higher stand counts compared to the non-treated seed (treatment 1 – Imidacloprid only), with treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) numerically showing the highest stand counts among them. This suggests that multiple pathogens contributed to stand loss that year. The Cottonseed Treatment Committee's 2023 report noted the presence of *Fusarium* spp. (80% isolation frequency). However, although it had a relatively high isolation frequency, not all isolates were likely pathogenic. *Pythium* spp. was also isolated (50% isolation frequency), but the pathogenicity assay indicated that most isolates were not pathogenic. Notably, *Thielaviopsis basicola* was absent in 2023, contrasting with previous years. *Rhizoctonia solani* was isolated from seedlings (50%) but not in soil samples. The absence of *R. solani* in soil samples may have been influenced by dry soil conditions or potential issues during the pathogen isolation process.

At Marianna in 2021, all treatments resulted in numerically higher stand counts compared to the non-treated seed (treatment 1 – Imidacloprid only), with treatment 2 (Metalaxyl) numerically

showing the highest stand counts among them. This suggests that *Pythium* spp. likely contributed to stand loss that year. As previously mentioned, the Cottonseed Treatment Committee's 2021 report initially isolated potential *Pythium* spp. from seedlings. However, subsequent pathogenicity testing revealed that these isolates could not be accurately classified as *Pythium* species. As a result, all data related to *Pythium* isolation were excluded due to concerns regarding the reliability of the identification. *Fusarium* spp. was isolated (24%), but results from the pathogenicity screening suggested that they may all have been non-pathogenic. Unlike at Judd Hill, *Thielaviopsis basicola* was absent across all years at Marianna. The report also showed that *Rhizoctonia solani* was isolated from seedlings (22%) and in soil samples (11.5 propagules/100 cm³).

In 2022, the broad-spectrum fungicide seed treatment (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) significantly improved stand establishment compared to both the non-treated seeds (treatment 1 – Imidacloprid only) and the selective treatments (treatment 2 – Metalaxyl and treatment 3 – Penflufen), suggesting that multiple pathogens contributed to stand loss. However, treatment 2 (Metalaxyl) significantly resulted in the lowest stand count, indicating that *Pythium* spp. may have had a relatively minor impact on stand establishment that year. The Cottonseed Treatment Committee's 2022 report revealed no presence of *Thielaviopsis basicola*, while *Fusarium* spp. exhibited a high isolation frequency (60%), though most isolates were likely non-pathogenic. *Pythium* spp. were isolated from seedlings (88%), and most of the isolates were pathogenic. *Rhizoctonia solani* was isolated from seedlings (24%), but it was not detected in soil samples, which could be attributed to dry soil conditions and/or potential issues in the pathogen isolation process.

In 2023, Marianna's stand counts were numerically lower than in 2022. Treatments 2 (Metalaxyl) and 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) significantly

outperformed others, while Treatment 1 (Imidacloprid only) was the least effective. These results suggest that *Pythium* spp. might have contributed to stand loss that year. The Cottonseed Treatment Committee's 2023 report revealed no presence of *Thielaviopsis basicola*, while *Fusarium* spp. exhibited a high isolation frequency (90%), but not all isolates were likely pathogenic. *Pythium* spp. were isolated from seedlings (100%), and most of the isolates were not pathogenic. *Rhizoctonia solani* was isolated from seedlings (30%), but it was not detected in soil samples, which could be attributed to dry soil conditions and/or potential issues in the pathogen isolation process.

Cotton seedling diseases pose a significant threat to crop yield, sometimes necessitating costly replanting efforts in severe cases (Minton and Garber, 1983). Fungicide seed treatments are a critical management strategy, protecting young cotton plants from early pathogen attacks (Chambers, 1995; Minton and Garber, 1983; Minton et al., 1982). The study results indicated a strong interaction between location and year ($P < 0.001$), suggesting that yield response is influenced by yearly variations in environmental conditions and pathogen pressures unique to each location. This significant location-by-year interaction underscores the dynamic impact of soil temperature, precipitation, and other site-specific environmental factors on cotton productivity. However, the non-significant interaction between location and treatment suggests that the relative effectiveness of fungicide treatments was consistent across locations, reinforcing the benefit of broad-spectrum treatments under diverse environmental conditions.

From 2019 to 2023, yield results varied across locations, with no significant differences among treatments overall. However, at Judd Hill in 2021, Treatments 2 (Metalaxyl) and 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) produced significantly higher yields compared to Treatments 1 (Control) and 3 (Penflufen), the latter of which showed significantly

the lowest yield. This lack of consistent yield differences among fungicide seed treatments, despite variations in stand counts, implies that other factors, such as environmental factors and disease pressure, may play a role in yield outcomes. Previous studies by Rothrock et al. (2012) demonstrated that environmental conditions—particularly soil temperature and rainfall shortly after planting—can significantly influence stand establishment and disease severity. Their research also showed that, at higher soil temperatures (25°C), seedling stands were relatively stable across a range of rainfall amounts, but as soil temperatures decreased, stands declined, especially with increased rainfall (Rothrock et al., 2012).

Across the study period, average yields were generally higher at Judd Hill than at Marianna. At Judd Hill, Treatment 4 (Prothioconazole + Myclobutanil + Penflufen + Metalaxyl) numerically produced the highest average yield, reaching 6,376.4 lb/a, likely reflecting its broad-spectrum activity against multiple pathogens. At Marianna, Treatment 2 (Metalaxyl) numerically resulted in the highest average yield over the years, with 4,605.7 lb/a, emphasizing the importance of targeting specific pathogens like *Pythium* spp., in this environment to optimize yield.

This study builds upon the foundation established by the National Cotton Seed Treatment Program by evaluating the effectiveness of four fungicide seed treatments in Arkansas, specifically assessing their impact on seedling emergence and survival under local environmental conditions. By analyzing how these treatments perform in the presence of region-specific pathogen pressures, this research contributes to refining disease management strategies and improving seed treatment recommendations for cotton growers.

TABLES AND FIGURES

Table 1.1. Summary of location, cultivar, year, and chemical products and their rates used to control cotton seedling pathogens from 2019 to 2023.

Location	Cultivar	Year	Treatment	A.I. (%)	Rate (oz/cwt)	Target pathogen
Judd Hill	DP 1522 B2XF	2019	1	Imidacloprid (47.8)	13	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.6	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.9	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.2	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.3	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.8	<i>Pythium</i> spp.
	DP 1646	2020	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.85	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
		2021	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.85	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
		2022	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.85	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
		2023	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (40)	1.2	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
Marianna	DP 1646	2021	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.85	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
		2022	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (22.37)	1.85	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.
		2023	1	Imidacloprid (47.8)	12.8	Control
			2	Metalaxyl (28.35)	1.5	<i>Pythium</i> spp.
			3	Penflufen (22.7)	0.64	<i>Rhizoctonia solani</i>
			4	Myclobutanil (40)	1.2	<i>Rhizoctonia solani</i> , <i>Thielaviopsis basicola</i>
				Prothioconazole (41.0)	0.16	<i>Rhizoctonia solani</i> , <i>Fusarium</i>
				Penflufen (22.7)	0.32	<i>Rhizoctonia solani</i>
				Metalaxyl (28.35)	0.75	<i>Pythium</i> spp.

*- All treatments included Imidacloprid (48.7%), 16 oz/cwt.

Table 1.2. Planting, sampling, and stand count dates over the years and location.

Location	Year	Planting Date	Sampling Date	Stand Counts	Number of Seeds Planted
Judd Hill	2019	5/29/19	7/1/19	7/1/19	250 seeds
Judd Hill	2020	6/3/20	7/6/20	7/6/20	125 seeds
Judd Hill	2021	5/24/21	6/28/21	6/28/21	150 seeds
Marianna	2021	5/14/21	6/14/21	6/14/21	120 seeds
Judd Hill	2022	5/18/22	6/14/22	6/14/22	177 seeds
Marianna	2022	5/12/22	6/13/22	6/13/22	177 seeds
Judd Hill	2023	5/2/23	6/5/23	6/5/23	150 seeds
Marianna	2023	5/3/23	6/6/23	6/6/23	150 seeds

Table 1.3. ANOVA results of linear mixed models.

	F	Df	Df.res	Pr(>F)
ANOVA response: stand count				
Location	239.380	1	25.943	< 0.001 ***
Year	55.287	4	25.864	< 0.001 ***
Treatment	3.318	3	77.238	0.024 *
Location : Year	46.430	2	25.926	< 0.001 ***
Location : Treatment	0.080	3	77.304	0.970
Year : Treatment	0.722	12	77.228	0.725
Location : Year : Treatment	0.702	6	77.299	0.649
ANOVA response: yield				
Location	45.136	1	25.943	< 0.001 ***
Year	48.015	4	26	< 0.001 ***
Treatment	7.1038	3	78	< 0.001 ***
Location : Year	62.8228	2	26	< 0.001 ***
Location : Treatment	2.1397	3	78	0.102
Year : Treatment	1.6996	12	78	0.082
Location : Year : Treatment	0.661	6	77.299	0.682

* Significance $P < 0.05$ *** Significance $P < 0.001$ *** Significance $P < 0.001$

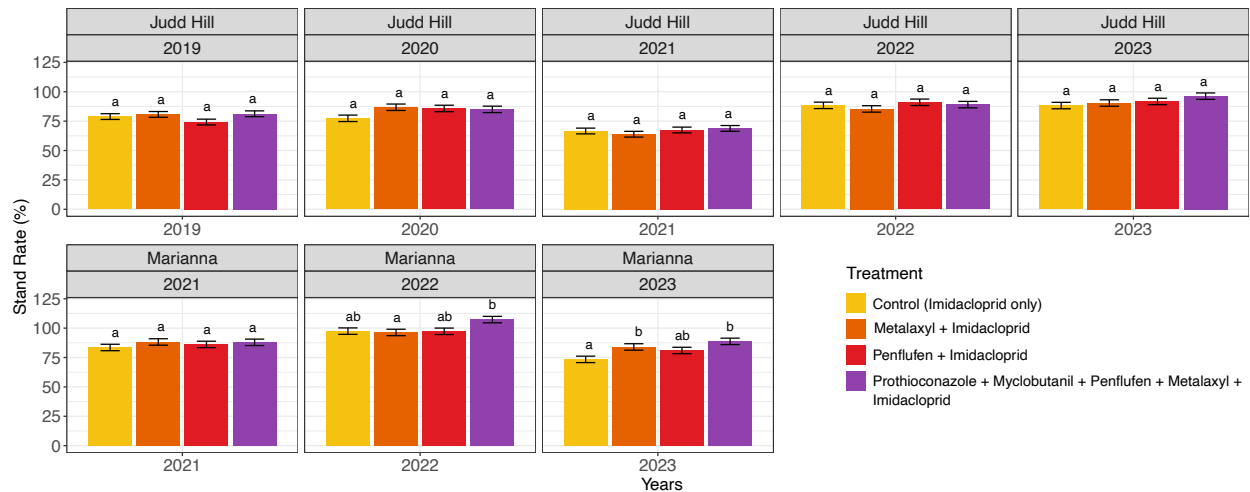


Figure 1.1. Stand counts of cotton seedlings of Judd Hill and Marianna (Arkansas) over the years, using 4 different fungicide seed treatments. Means with the same letters are not significantly different (post-hoc test with Bonferroni correction, ($P < 0.05$)).

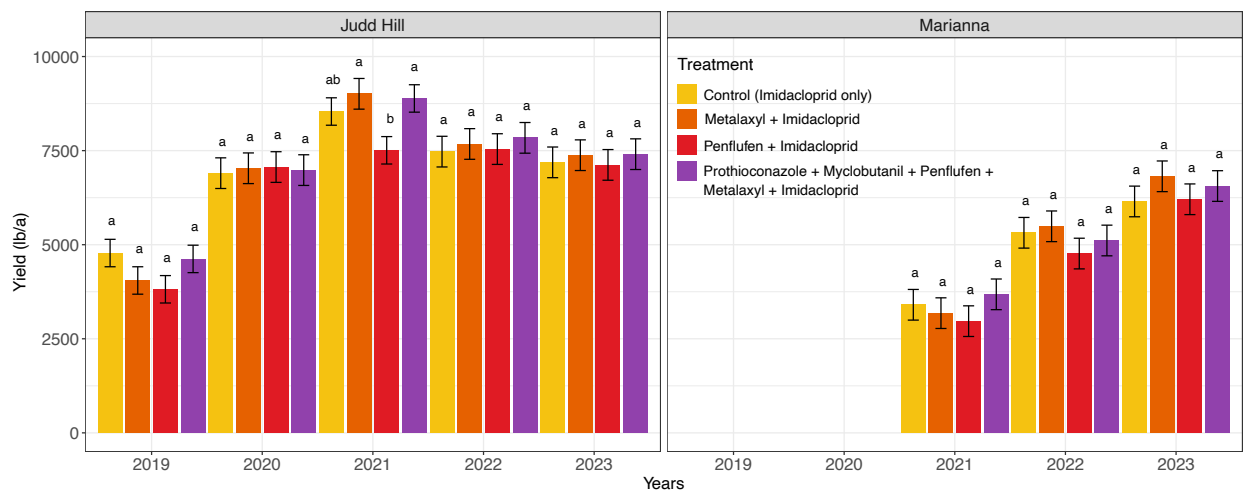


Figure 1.2. Yield of cotton seedlings over the years of Judd Hill and Marianna (Arkansas), using 4 different seed treatments. Means with the same letters are not significantly different (post-hoc test with Bonferroni correction, ($P < 0.05$)).

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CHAPTER 2: DETERMINE THE INFLUENCE OF ACTIVE INGREDIENTS ON COTTON SEED AND ROOT-ASSOCIATED COMMUNITIES

ABSTRACT

Fungicide seed treatments are a key tool in managing cotton seedling diseases, offering critical protection against soilborne and seedborne pathogens. Their success, however, depends on the composition and prevalence of pathogen populations, which vary annually and regionally, and environmental conditions. In this study, we aimed to characterize the soil- and root-associated microbial communities in cotton using the Illumina MiSeq platform and examine the differences in microbial composition between two locations across different years and treatments. Field trials were conducted over 5 years at Judd Hill and 3 years at Marianna, and treatments consisted of no fungicide (T1), metalaxyl (T2), penflufen (T3), and a mix of prothioconazole, myclobutanil, penflufen, metalaxyl (T4). All the treatments contained insecticide (imidacloprid) to mitigate the effect of insects during the trials. Soil and root samples were collected 30 days after plating for both locations. Total DNA was extracted from both sample types and used for Illumina sequencing. Our study demonstrates that fungicide seed treatments significantly influence microbial communities, with effects varying by active ingredient, sampling year, and environmental conditions. Metalaxyl and penflufen had an impact on their targeted pathogens but also altered fungal populations.

1. INTRODUCTION

Cotton (*Gossypium hirsutum* L.), commonly known as upland cotton, is a globally important cash crop, with the United States ranking as the third-largest producer, generating \$21 billion annually. In 2023, U.S. cotton production reached 12.43 million bales (USDA, 2023a), covering 10.23 million acres (USDA, 2023b). Arkansas, as the third-largest producer, contributed 1.41

million bales, trailing behind Texas and Georgia (USDA, 2023c). Despite its economic importance, cotton production faces numerous challenges, including pests and diseases, which can adversely affect the crop's yield and quality, potentially leading to significant economic losses for growers (Khan et al., 2020). Among these, soil-borne pathogens, such as fungi, are particularly harmful during the early growth stages, often leading to reduced stands and yields (Bradley et al., 2021; Strayer-Scherer, 2021).

Cotton seedling diseases, primarily caused by *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*, pose a significant threat to seed germination and early seedling development. These diseases have become a major concern due to their potential to drastically reduce yield and fiber quality (Hancock et al., 2004). Characterized by the death or decay of seeds and seedlings before or after emergence, known as pre- and post-emergence damping-off, these diseases often present with necrotic lesions along the hypocotyl and roots of seedlings. Even when infection doesn't lead to death, affected seedlings can become stunted and chlorotic, preventing them from reaching their full yield potential (Rothrock and Buchanan, 2017).

Effective management of cotton seedling diseases involves several cultural practices, including delaying planting until soil conditions are optimal for germination and growth, using high-quality seeds, planting at the appropriate depth, and establishing well-drained, raised seedbeds (DeVay & Rothrock, 2001; Hillocks, 1992; Kerby et al., 1989; Minton & Garber, 1983; Rude, 1984). Despite these measures, fungicide seed treatments remain the most essential tool for protecting cotton seedlings from these diseases (Afzal et al., 2020; Lamichhane et al., 2017). These treatments are routinely applied to cottonseed before commercialization, primarily to mitigate damage from biotic stresses such as pest predation and pathogen infection (Rothrock et al., 2012; Davis et al., 1997; Hillocks, 1992; Minton & Garber, 1983).

While fungicides are effective in controlling targeted soil and seed-borne pathogens, and provide considerable benefits, they are not species-specific and can unintentionally affect non-target beneficial fungi (Karlsson et al., 2014; Prior et al., 2017) and the potential for pesticide accumulation in the soil, which can impact soil biodiversity (Vasanthakumari et al., 2019; Fort et al., 2019; You et al., 2020; Nettles et al., 2016).

Fungicide seed treatments with a systemic nature may have unintended negative effects on endophytic fungi, which are widely recognized for their role in promoting plant growth, strengthening defense mechanisms, and mitigating both biotic and abiotic stresses (Khan et al., 2015; Matsumoto et al., 2021; Pal et al., 2022; Samreen et al., 2021). However, the negative impact on beneficial microorganisms could reduce the overall effectiveness of fungicides. Nettles et al. (2016) demonstrated that treating seeds with systemic fungicides, such as mefenoxam and sedaxane, significantly altered the soybean leaf endophyte fungal community. This suggests that fungicide applications aimed at controlling one pathogen may unintentionally disrupt beneficial microorganisms. Understanding the effects of fungicides on the beneficial roles of endophytes is crucial for evaluating the risks associated with fungicide use in agriculture and for improving fungicide application strategies.

On the other hand, the root-associated microbiome (including mycorrhizal, saprotrophic, and pathogenic) plays a crucial role in safeguarding plants against various stresses, including pathogenic infections (Finkel et al., 2017). Soil microorganisms are vital indicators of soil ecological health (Du et al., 2018; Zhang et al., 2014), and preserving a diverse microbial community is crucial for promoting sustainable agricultural practices (Wei et al., 2019). However, many chemicals used in seed coatings are delivered directly into the rhizosphere - the soil zone surrounding seedling roots (Thompson, 2010). These treatments effectively target soil-borne

pathogens and herbivores (Baird et al., 1994). However, they often lack species specificity and have broader impacts on non-target microbial communities, including beneficial fungi (Karlsson et al., 2014; Prior et al., 2017; Nettles et al., 2016).

In this study, our objectives were to (i) characterize the soil- and root-associated microbial communities in cotton using the Illumina MiSeq platform, (ii) assess the impact of various fungicide treatments on these communities, and (iii) examine the differences in microbial composition between two locations (Judd Hill and Marianna) across different years and treatments.

2. MATERIAL AND METHODS

2.1. Soil

2.1.1. Sample Collection

Soil samples were collected at two research stations in Arkansas, Judd Hill in Poinsett County (Northeast Arkansas) from 2019 to 2023 and Marianna in Lee County from 2021 to 2023, to evaluate the effects of different active ingredients on seed- and root-associated fungal communities. The standard fungicide seed treatments were as follows: Treatment 1 = Nontreated check, Treatment 2 = Metalaxyl (1.5 fl. oz/cwt), Treatment 3 = Penflufen (0.64 fl. oz/cwt), and Treatment 4 = Metalaxyl (0.75 fl. oz/cwt), Penflufen (0.32 fl. oz/cwt), Myclobutanil (1.85 fl. oz/cwt), and Prothioconazole (0.16 fl. oz/cwt).

Soil samples were collected at a depth of 20 cm around the roots of cotton plants using a completely randomized sampling approach (Zig-Zag transect across the plots) for most of the years. In 2023, each soil sample was a composite of subsamples taken from ten random points within each plot, ensuring a representative mixture. This approach resulted in sixteen composite samples per location (four treatments replicated four times each). The soil samples were placed into labeled plastic bags for each plot and location and transported in a cooler to the laboratory. A

portion of each sample was stored at -20 °C for subsequent DNA extraction, while the remaining part was air-dried in the greenhouse for physicochemical analysis.

2.1.2. Environmental Factors

The Agricultural Diagnostic Laboratory of the University of Arkansas analyzed soil samples to measure available N, P, K, Cu, Zn, Fe, S, B, and Mn, as well as pH, organic matter (%LOI), exchangeable Ca, Mg, and Na. Climate data for each season were obtained from the Southern Regional Climate Center, including monthly average temperature (initial and final) and monthly total precipitation (initial and final).

2.1.3. Soil DNA Extraction

Total DNA was extracted from approximately 1.0 g of soil of each sample, using the E.Z.N.A.® Soil DNA kit (Omega Bio-tek, Norcross, Georgia, US), according to the manufacturer's instructions. The integrity of the DNA extracted from the soil samples was confirmed by electrophoresis in 1.0% agarose gel stained with EZ-Vision Blue light DNA Dye 10,000X in 1 x TBE buffer and visualized under UV light. All the DNA samples were stored at – 20°C until subsequent processing.

2.2. Plants

2.2.1. Plant Samples Collection

Root samples were randomly collected from Judd Hill (2019-2023) and Marianna (2021-2023), Arkansas, from all the plots 30 days after planting. Ten seedlings from each plot of each replicate were dug using a spade, and the above-ground portion was cut from all of them, leaving the remaining hypocotyl and roots. The roots were vigorously shaken to remove adhering soil particles loosely, then combined as a single composite sample in a plastic bag, transported in a cooler to the laboratory, and then stored at –20 °C. When frozen, they were placed in a freeze-

dryer (Labconco 77530-00 G FreeZone 6 Freeze Dryer System) at -80°C until the samples were completely dry. After that, the roots were stored in a 50 mL falcon tube until subsequent processing.

2.2.2. Root Total DNA Extraction

Total DNA was extracted from 50 mg of ground roots using the Omega Mag-Bind Plant DNA DS 96 kit (M1130, Omega BioTek, Norcross, GA) following the manufacturer's instructions. The integrity of the DNA extracted from the root samples was confirmed by electrophoresis in 1.0% agarose gel stained with EZ-Vision Blue light DNA Dye 10,000X in 1 x TBE buffer and visualized under UV light. All the DNA samples were stored at – 20°C until subsequent processing.

2.3. Soil and Roots Amplicon Library Preparation

Genomic DNA from soil and root samples, previously extracted as described above, was used. For the fungal community analysis, the internal transcribed spacer gene (ITS1 region) was amplified using the primer sets ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Gardes & Bruns, 1993; White et al., 1990). The PCR amplicon was performed in three steps, with a total reaction volume of 25 µL for each sample/step. The first step reaction mix included 7 µL of H₂O, 12.5 µL of 1 × PCR RepliQa Hifi Mix, 4.0 µL of genomic DNA, 0.75 µL of primer ITS1F (10 µM), and 0.75 µL of primer ITS2 (10 µM). After initial denaturation at 98 °C for 10 seconds, amplification was performed by 20 cycles of incubations for 5 seconds at 58 °C and 5 seconds at 68 °C. The second step reaction included 8.5 µL of H₂O, 12.5 µL of 1 × PCR RepliQa Hifi Mix, 2.5 µL of amplified products from step 1, 0.75 µL of frameshift primer ITS1F (1-6) (10 µM), and 0.75 µL of frameshift primers ITS2 (10 µM) as described in Lundberg et al. (2013). Amplification was performed by 15 cycles of incubations for

10 seconds at 98 °C and 5 seconds at 68 °C. PCR products from step 2 were purified using the ExoAP PCR cleanup protocol, where 6 µL of ExoAP Master Mix (40 µL of AP [5U/µL], 20 µL of Exo I [10U/ µL], and 940 µL of Molecular Biology Grade Water) were pipetted into PCR tubes containing approximately 20 µL of PCR product each. The tubes were incubated in a thermal cycler for 40 minutes at 37°C, 10 minutes at 80°C, and a hold at 8°C. For the third step, the mix included 1 µL of H₂O, 12.5 µL of 1 × PCR RepliQa Hifi Mix, 10 µL of purified products from step 2, 0.75 µL of primer Illumina (5'-AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG-3') (10 µM), and 0.75 µL of a unique barcode was added into each sample. After an initial denaturation at 98 °C for 10 seconds, amplification was performed by 15 cycles of incubations for 5 seconds at 63 °C and 5 seconds at 68 °C. PCR products from Step 3 were run alongside those from Step 2 to ensure the efficiency of the barcodes, using a 1.5% agarose gel electrophoresis. After that, step 3 products were purified using the ExoAP PCR cleanup protocol. After that, 2 µL of each sample was quantified using a Qubit FlexTM Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA), using the Qubit dsDNA Assay Kit as the manufacturer's instruction, and based on the quantification, the DNA was pooled to library construction. The library was then purified using magnetic beads under the manufacturer's instruction (Mag – Bind® TotalPure NGS, Omega BIO-TEK, Norcross, Georgia), and the final concentration was verified using qPCR assay (CFX Opus 96, BIO-RAD), nanodrop (2000C, Thermo Scientific), and Qubit FlexTM Fluorometer.

2.4. Data Processing and Taxonomic Annotation

Demultiplexing was performed as if the reads were single-end by importing FASTQ files into QIIME 2 (Bolyen et al., 2019) as 'CasavaOneEightSingleLanePerSampleDirFmt' sequences, then using the demux function for demultiplexing. The function demux summarize was then utilized to

generate a visualization file (.qzv) to observe the raw sequence counts for each sample. The quality of raw reads was assessed using FastQC (version 0.12.1), with results compiled into an HTML report via MultiQC (version 1.27.1). Cutadapt was utilized to trim adapters and primers from sequences and remove reads that had a low average quality score (Martin, 2011). The minimum read length after trimming was set to 50 base pairs. Sequences were then trimmed to 250 bp during the denoising step to ensure high-quality reads were used for downstream analyses. Low-quality sequences were also filtered out by setting a maximum expected error threshold of 2 (max-ee), a truncation quality score cutoff of 20 (trunc-q), and chimeras were removed. Reads were assigned taxonomy using the “feature-classifier classify-sklearn” command, with the 99% identity threshold for taxonomy assignment with the UNITE database version 9 (Abarenkov et al., 2023). The ecological functions of each ASV were determined using FungalTraits (Pöhlme et al., 2020). Fungal plant pathogens were separated from other functional groups and used in this study.

2.5. Statistical Analysis

The data processing was mainly completed with RStudio Server (2023.09.1), using vegan, phyloseq, and microeco packages. For alpha diversity analysis, the Observed and Shannon diversity index were used to calculate the richness using the “estimate_richness” function from the phyloseq package. To assess normality, the Shapiro-Wilk test (shapiro.test) was performed.

To assess differences in fungal communities across sample types within each group (year, treatment, and location), we conducted a Principal Coordinate Analysis (PCoA). The visual patterns observed in the PCoA were statistically validated using a permutational multivariate analysis of variance (PERMANOVA; adonis test) (Anderson, 2001), with 999 permutations and a Bray-Curtis distance matrix. Significant effects identified by the PERMANOVA were further investigated through pairwise PERMANOVA comparisons.

Redundancy analysis (RDA) was performed to examine the relationship between fungal community structure and environmental factors. The significance of each environmental variable in RDA was assessed using the ‘terms’ function, while the explanatory axis significance was tested using the ‘axis’ function. Additionally, a Mantel test was conducted to evaluate correlations between community structure and environmental factors. Pearson correlation was applied to soil samples, while Spearman correlation was used for root samples (Mantel test, 999 permutations).

3. RESULTS

3.1. Alpha Diversity of Fungal Communities

After quality filtering, 1,817,656 sequences were obtained across 209 samples, with 529 unique features identified. The number of sequences per sample ranged from 47 to 63,111, with a median of 6,262 and a mean of 8,696.9. The first and third quartiles of sequence distribution were 3,536 and 10,708.0, respectively. Diversity analysis for data from 2019-2020 was analyzed separately from 2021–2023 due to the availability of only one location (Judd Hill) in the earlier years. Data were also separated into two types (soil and roots) to study the effect of treatments on the fungal communities over the years separately. For soil samples, since the data was normally distributed for both 2019-2020 ($W = 0.96$, $p\text{-value} = 0.70$) and 2021-2023 ($W = 0.97$, $p\text{-value} = 0.24$), an ANOVA test (aov) was performed to compare Shannon index values across groups. For root samples, since the data was not normally distributed for both 2019-2020 ($W = 0.94$, $p\text{-value} = 0.03$) nor 2021-2023 ($W = 0.94$, $p\text{-value} < 0.001$), a Kruskal-Wallis test (Kruskal.test) was used to compare Shannon index values across groups.

For soil samples from 2019 to 2020, the values for the Shannon diversity index ranged from 0.99 to 2.17, which suggests some variation in the diversity of the soil fungal communities across different samples. Additionally, the number of observed species ranges from 3 to 13 across

samples. It appears that in some cases, there was a higher species richness in the samples, while others showed lower richness. However, neither the year, the treatment, nor their interaction significantly altered the Shannon diversity index (Table 2.1). These results suggest that the diversity of soil fungal communities, as measured by the Shannon index, was relatively stable across the treatments and years at Judd Hill between 2019 and 2020.

From 2021 to 2023, the values for the Shannon diversity index ranged from 0 to 1.94, indicating a variation in fungal diversity. Additionally, the number of observed species ranged from 1 to 11 across samples, with a few samples showing only 1 or 2 species, while others showed more species richness. The ANOVA results for soil samples showed that year (p -value = 0.019) and location (p -value = 0.007) significantly influenced diversity (Table 2.2, Figure 2.1). Neither treatment nor the interactions between year and treatment, year and location, treatment and location, nor year, treatment, and location significantly affected alpha diversity. These results suggest that fungal diversity in soil could have varied significantly between years and locations but not as much due to the treatment.

For root samples from 2019 to 2020, the values for the Shannon diversity index ranged from 0 to 1.77, indicating a variation in fungal diversity across root samples. Additionally, the number of observed species ranged from 1 to 7 across samples, with some samples showing low species richness while others showed more species. The Kruskal-Wallis results showed that year (p -value < 0.001) and the interaction between year and treatment (p -value < 0.001) were found to significantly shift fungal diversity (Table 2.3, Figure 2.1). The treatment itself did not have a major impact on alpha diversity. These results suggest that temporal variation (year) strongly influenced the diversity and that the effect of treatments on diversity may vary by year. However, the treatment itself does not have a major impact on its own.

From 2021 to 2023, the Shannon diversity index ranged from 0 to 1.99, indicating a variation in fungal diversity across root samples. Additionally, the number of observed species ranged from 1 to 9 across samples, with some samples showing low species richness while others showed more species. The Kruskal-Wallis results showed that all factors affected alpha diversity: year (p-value < 0.050), treatment (p-value = 0.002), location (p-value = 0.013), the interactions between year and treatment (p-value = 0.018), year and location (p-value < 0.001), treatment and location (p-value < 0.001), and the interaction between year, treatment and location (p-value < 0.001) (Table 2.4. Figure 2.1). These results suggest that fungal diversity in the root samples from 2021 and 2023 was influenced by the treatment, location, and their interactions over the years.

The OTUs belong to 6 phyla, 15 classes, 32 orders, 58 families, 76 genera, and 89 species after classification. There were three dominant phyla with relative abundances of more than 1%: Ascomycota was the most abundant phylum, followed by *Basidiomycota*, then *Mortierellomycota*. In 2019, the dominant families with a relative abundance of more than 1% of fungi were *Phaffomycetaceae* (33.7%), *Mrakiaceae* in 2020 (46.3%) and 2021 (30.4%), *Nectriaceae* in 2022 (19.4%), and *Plectosphaerellaceae* in 2023 (35.7%).

3.2. Beta Diversity

Since sample type was a significant driver of differences in fungal diversity, we ran separate comparisons for each type. Data from 2019-2020 were analyzed separately from 2021–2023 due to the availability of only one location (Judd Hill) in the earlier years.

To examine differences in fungal diversity among sample types within each group (year, treatment, and location), a Principal Coordinate Analysis (PCoA) was performed. For soil samples from 2019-2020, PCoA revealed that the first axis explained 25.3% of the variation, while the second axis explained 18.5% (Figure 2.5a). Notably, samples from different years did not form

distinct clusters, indicating no clear treatment-driven separation in microbial communities. For root samples from 2019-2020, PCoA revealed that the first axis explained 28.1% of the variation, while the second axis accounted for only 8.3% of the variation (Figure 2.5b). There was a noticeable clustering of samples of the same year, but no clear separation was observed among treatments.

For soil samples from 2021-2023, PCoA revealed that the first axis explained 20.7% of the variation, while the second axis explained only 10.1% (2.5c). Samples from the same year clustered, but no clear separation was observed among treatments or locations. For root samples from 2021-2023, PCoA revealed that the first axis explained 14.8% of the variation, while the second axis explained only 5.3% (Figure 2.5d). Similar to soil, samples from the same year clustered, but no clear separation was observed among treatments or locations.

The visual patterns observed in the PCoA were further statistically confirmed by permutational multivariate analysis of variance (PERMANOVA; adonis test) (Anderson, 2001), using 999 permutations and a Bray-Curtis distance matrix. This analysis tested whether the compared groups were compositionally distinct, with a significance level set at $\alpha = 0.05$.

For soil fungal composition, in 2019-2020, there were no significant differences in the factors, indicating that soil microbial communities remained stable across years and treatments (Table 2.5). For root fungal composition in 2019-2020, year ($R^2 = 0.718$, $P = 0.001$) significantly explained the differences in the fungal community (Table 2.8). No significance was observed for treatment nor the interaction between year and treatment. The year effect was further explored with pairwise PERMANOVA, where the pair across years was significantly different in fungal communities ($R^2 = 0.719$, $P = 0.001$) (Table 2.9).

For soil fungal composition in 2021-2023, location ($R^2 = 0.110$, $P = 0.043$) significantly

explained the community variances (Table 2.6). The effect of location was further explored with pairwise PERMANOVA, where the pair across Judd Hill and Marianna was significantly different ($R^2 = 0.089$, $P = 0.031$) (Table 2.7). For root fungal composition in 2021-2023, year ($R^2 = 0.062$, $P = 0.010$), treatment ($R^2 = 0.201$, $P = 0.001$), and the interactions between year and treatment ($R^2 = 0.161$, $P = 0.003$), year and location ($R^2 = 0.113$, $P = 0.001$), and treatment and location ($R^2 = 0.082$, $P = 0.008$) all significantly explained the differences in the fungal community (Table 2.10). The effects of year, treatment, and interactions were further explored with pairwise PERMANOVA, where the pairs “treatment 1 x treatment 3” ($R^2 = 0.300$, $P = 0.006$) (Table 2.11), “treatment 2 x treatment 3” ($R^2 = 0.259$, $P = 0.012$), “2021:Marianna vs 2023:Marianna” ($R^2 = 0.302$, $P = 0.030$) (Table 2.12), and “treatment 3:Marianna vs treatment 4:Marianna” ($R^2 = 0.415$, $P = 0.028$) were significantly different (Table 2.13). The comparisons between the years themselves and between the interaction year and treatment and location were not significant after multiple testing corrections.

3.3. Shift in the Relative Abundance of Dominant Fungal Families

The top 10 most abundant families in soil samples from 2019-2020 included *Mortierellaceae*, *Plectosphaerellaceae*, *Nectriaceae*, *Bartaliniaceae*, *Herpotrichiellaceae*, *Agaricaceae*, *Lasiosphaeriaceae*, *Trichocomaceae*, *Hydnaceae*, and *Bombardiaceae* (Figure 2.3). Within the top 10, *Mortierellaceae*, *Plectosphaerellaceae*, and *Nectriaceae* comprised more than 80% of the fungal composition. In 2019, the relative abundance of *Mortierellaceae* was the highest in treatment 4 (65%, control: 48.8%) and the lowest in treatment 2 (42.6%, control: 48.8%). *Plectosphaerellaceae* had a slight increase in treatment 2 (30.7%) and a decrease in treatment 3 (18.8%) when compared to treatment 1 (control, 27.7%). *Nectriaceae* was higher in treatment 2 (20.2%) and slightly lower in treatments 3 (10%) and 4 (10%) when compared to the control

(13.4%). *Bartaliniaceae* was similar to the control during that year, at a low level of relative abundance (lower than 2%). Other families, such as *Agaricaceae* (treatments 2 and 3), *Herpotrichiellaceae* (treatment 3), and *Bombardiaceae* (treatment 4), were present in other treatments but not in the control. In 2020, the relative abundance of *Mortierellaceae* was higher in treatment 4 (49.3%), and lower in treatment 3 (22.7%) compared to the control (39.4%). *Plectosphaerellaceae* was higher in treatment 3 (31.9%) and slightly lower in treatment 4 (22.5%) when compared to treatment 1 (control, 23.5%). *Nectriaceae* was slightly higher in treatment 2 (14.4%) and lower in treatment 4 (9.8%) compared to the control (12.7%). *Bartaliniaceae* had a decrease in all treatments (4%, 7%, and 7.9%, respectively) compared to the control (9.2%). *Lasiosphaeriaceae* was only present in the control in that year (6%). *Trichocomaceae* was absent in treatments 3 and 4, but had an increase in treatment 2 (5.5%) compared to the control (1.6%). Other families, such as *Herpotrichiellaceae* (treatment 3) and *Hydnaceae* (treatment 4), were present in other treatments but not in the control.

At Judd Hill, from 2019 to 2020, the top 10 families in the root-associated community across all treatments included *Mrakiaceae*, *Phaffomycetaceae*, *Filobasidiaceae*, *Nectriaceae*, *Ceratocystidaceae*, *Cystofilobasidiaceae*, *Pleosporaceae*, *Aspergillaceae*, *Bartaliniaceae*, and *Gigasporaceae*. In 2019, the relative abundance of *Phaffomycetaceae* decreased in all treatments (23.3%, 57.8%, and 50%, respectively) compared to treatment 1 (control, 58.7%). This family was only observed that year. *Ceratocystidaceae* was only found in the control (35.8%), with a relatively high relative abundance, but not in any of the other treatments nor 2020. Other families, such as *Nectriaceae* (treatments 2 and 3), *Pleosporaceae* (treatments 2 and 4), *Gigasporaceae* (treatment 2), and *Aspergillaceae* (treatment 4) were present in other treatments but not in the control. In 2020, the relative abundance of *Mrakiaceae* appeared to be constant across the treatments

compared to the treatment (54.1%), except for an increase in treatment 4 (61.8%). This family was only observed in that year. *Filobasidiaceae* had changes in relative abundance, where it was higher in treatment 3 (26.8%) and lower in treatment 4 (11.8%) compared to the control (18.9%). *Nectriaceae* had a slight increase in treatment 4 (9.5%) and a decrease in treatment 3 (5.5%) compared to the control (7.3%). The relative abundance of *Cystofilobasidiaceae* was lower in all the treatments when compared to the control (10%), especially in treatment 2 (3.8%). *Pleosporaceae* had a higher relative abundance in treatment 2 (5.8%), lower in treatment 3 (less than 2%) when compared to the control (2.9%), and was absent in treatment 4. *Bartaliniaceae* had an increase in treatment 2 (5.8%) and a decrease in treatment 4 (less than 2%) compared to the control (4.3%).

At Judd Hill, from 2021 to 2023, the top 10 families in the soil community across all treatments included *Plectosphaerellaceae*, *Mortierellaceae*, *Nectriaceae*, *Bartaliniaceae*, *Nidulariaceae*, *Psathyrellaceae*, *Bombardiaceae*, *Lasiosphaeriaceae*, *Coniochaetaceae*, *Pleosporaceae*. In 2021, the relative abundance of *Plectosphaerellaceae* was the highest in treatment 4 (35%) and the lowest in treatment 3 (10.6%) when compared to the control (21.8%). *Mortierellaceae* had the highest in treatment 3 (60.8%) and the lowest in treatment 4 (30.8%) when compared to the control (56.5%). *Nectriaceae* had the highest relative abundance in treatment 2 (34.9%) and the lowest in treatment 3 (12.1%) when compared to the control (12.8%). The family *Bartaliniaceae* was only present at a very low relative abundance. *Coniochaetaceae* had a decrease in all treatments (4.3% and 5.2%, respectively) compared to the control (7.2%) and was absent in treatment 4. *Bombardiaceae* was present in treatment 3 but it was not found in the control. In 2022, the relative abundance of *Plectosphaerellaceae* was the highest in treatment 4 (18%) and the lowest in treatment 3 (7.7%) when compared to the control (17.8%). *Mortierellaceae* had a

decrease for all treatments that year, with the lowest relative abundance in treatment 4 (32.9%) compared to the control (53.2%). *Nectriaceae* had an increase in all treatments that year when compared to the control (20.1%), with the highest relative abundance in treatment 2 (37.6%). Other families, such as *Pleosporaceae* (treatment 2) and *Coniochaetaceae* (treatment 3), were present in other treatments but not in the control. In 2023, the relative abundance of *Plectosphaerellaceae* was the highest in treatment 2 (60.4%) and the lowest in treatment 3 (53%) compared to the control (53.4%). *Mortierellaceae* seemed to be constant across all treatments that year compared to the control but was absent in treatment 4. *Nectriaceae* had an increase in treatment 3 (10.8%) and a decrease in treatment 3 (3.1%) compared to the control (3.4%), and was absent in treatment 2. *Bartaliniaceae* had an increase in all treatments, with the highest relative abundance in treatment 4 (12.8%) when compared to the control (3.6%). *Nidulariaceae* had the highest relative abundance in treatment 3 (9.2%) and the lowest in treatment 4 (1.7%) when compared to the control (8%). *Psathyrellaceae* had the highest relative abundance in treatment 4 (10.8%) and the lowest in treatment 3 (less than 2%) when compared to the control (5.3%). *Bombardiaceae* had an increase for most of the treatments, especially in treatment 2 (6%) when compared to the control (3%), and was absent in treatment 3. *Lasiosphaeriaceae* had a decrease in all treatments when compared to the control (4%). There was little change in the relative abundances of the other families.

At Judd Hill, from 2021 to 2023, the top 10 families in the root-associated community across all treatments included *Mortierellaceae*, *Nectriaceae*, *Ceratobasidiaceae*, *Phaffomycetaceae*, *Pleosporaceae*, *Mrakiaceae*, *Bartaliniaceae*, *Plectosphaerellaceae*, *Ceratocystidaceae*, and *Sclerotiniaceae*. In 2021, the relative abundance of *Mortierellaceae* had an increase in all treatments, with the highest in treatment 2 (62%) when compared to the control (33.6%). *Nectriaceae* also had a slight increase compared to the control (4.8%), with the highest relative

abundance in treatment 3 (6.5%). *Mrakiaceae* had an increase in treatment 3 (21.8%) and a decrease in treatment 4 (11.9%) compared to the control (14.6%). *Bartaliniaceae* had an increase in treatment 4 (25.7%) and a decrease in treatment 2 (4.9%) compared to the control (11.9%). *Sclerotiniaceae* was absent in treatment 4 and had a decrease in all treatments when compared to the control (24.1%). Other families, such as *Pleosporaceae* and *Plectosphaerellaceae*, were only present in treatment 2, but not in the control. In 2022, *Nectriaceae* had an increase in relative abundance in all treatments, with the highest in treatment 3 (44.4%) when compared to the control (17.9%). *Pleosporaceae* had a decrease in all treatments, with the lowest relative abundance in treatment 4 (18.9%) when compared to the control (33.1%). *Ceratocystidaceae* had an increase in treatment 2 (23.7%) when compared to the control (12.9%), and was absent in treatments 3 and 4. Other families, such as *Mortierellaceae* (treatment 2) and *Phaffomycetaceae* (treatments 3 and 4), were present in other treatments but not in the control.

In 2023, *Mortierellaceae* was only observed in the control, at a very low relative abundance level (1.3%). *Nectriaceae* had a decrease in all treatments, with the lowest in treatment 2 (13.4%) when compared to the control (29.6%) and was absent in treatment 4. *Ceratobasidiaceae* had the highest relative abundance in treatment 2 (52%) and the lowest in treatment 3 (6.8%) when compared to the control (24.5%). *Plectosphaerellaceae* had a decrease in all treatments, with the lowest in treatment 4 (less than 2%) when compared to the control (22.4%). *Ceratocystidaceae* had an increase in treatment 2 (5.7%) when compared to the control (3.1%) but was absent in treatments 3 and 4. *Phaffomycetaceae* was only observed in treatments 3 (25%) and 4 (34.1%).

At Marianna, from 2021 to 2023, at Marianna, the top 10 families in the soil community across all treatments included *Plectosphaerellaceae*, *Mortierellaceae*, *Nectriaceae*, *Strophariaceae*, *Bolbitiaceae*, *Phaeosphaeriaceae*, *Hydnaceae*, *Bombardiaceae*, *Bartaliniaceae*, and *Helotiaceae*.

In 2021, the relative abundance of *Plectosphaerellaceae* had a decrease in all treatments, with the lowest in treatment 3 (22.1%) when compared to the control (38.3%). *Mortierellaceae* had the highest in treatment 3 (58%) and the lowest in treatment 2 (29.7%) when compared to the control (35.1%). *Nectriaceae* was absent in treatment 3, and had a decrease in treatments 2 (8.5%) and 4 (7.3%) when compared to the control (14%). Other families, such as *Hydnaceae* (treatment 2) and *Bartaliniaceae* (treatment 3), were present in other treatments but not in the control. In 2022, there was a decrease in the relative abundance of *Mortierellaceae* in all treatments, with the lowest in treatment 2 (34%) when compared to the control (71.5%). *Nectriaceae* was absent in treatments 3 and 4, and was constant in treatment 2 (18.8%) when compared to the control (18.9%). Other families, such as *Helotiaceae* (treatment 2) and *Hydnaceae* (treatment 4), were present in other treatments but not in the control. In 2023, the relative abundance of *Plectosphaerellaceae* had an increase in all treatments (75.4%, 68.7%, and 69.5%, respectively) when compared to the control (66.6%). *Mortierellaceae* was only observed in the control (6.8%). *Nectriaceae* had an increase in all treatments (15.5%, 13.3%, and 15.5%, respectively) when compared to the control (10%). *Strophariaceae* was absent in treatments 2 and 3 and had an increase in treatment 4 (9.7%) when compared to the control (4.9%). *Phaeosphaeriaceae* had a decrease in all treatments (3.2%, 0.7%, and 0.8%, respectively) compared to the control (3.5%). *Bombardiaceae* had an increase in treatment 2 (3.2%) and decreased in treatment 3 (1.3%) compared to the control (1.6%), and it was not observed in treatment 4. *Bolbitiaceae* was present in treatment 3 but not in the control.

At Marianna, from 2021 to 2023, the top 10 families in the root community across all treatments included *Mrakiaceae*, *Ceratobasidiaceae*, *Nectriaceae*, *Phaffomycetaceae*, *Plectosphaerellaceae*, *Sporidiobolaceae*, *Chaetomiaceae*, *Pleosporaceae*, *Cladosporiaceae*, and *Bulberibasidiaceae*. In 2021, the relative abundance of *Mrakiaceae* had the highest relative

abundance in treatment 3 (72.1%) and the lowest in treatment 4 (53.4%) compared to the control (65%). *Nectriaceae* had an increase in treatment 4 (4.6%) and a decrease in treatment 3 (1.8%) compared to the control (3.6%). *Sporidiobolaceae* was absent in treatment 3 but had an increase in treatments 2 (3.3%) and 4 (10.6%) compared to the control (1.9%). *Cladosporiaceae* had a slight decrease in all treatments compared to the control (3.8%). *Bulberibasidiaceae* had a decrease in all treatments compared to the control (19.4%) and was absent in treatment 3. *Plectosphaerellaceae* was only observed in treatments 3 and 4. In 2022, *Ceratobasidiaceae* was not observed in treatment 3, and had a decrease in relative abundance in treatments 2 (8.5%) and 4 (19.3%) when compared to the control (33%). *Nectriaceae* was absent in treatment 3, but had an increase in treatments 2 (28.5%) and 4 (13.6%) compared to the control (3.5%). *Plectosphaerellaceae* was also absent in treatment 3 but had an increase in treatments 2 (18.1%) and 4 (11.9%) compared to the control (7.1%). *Sporidiobolaceae* was not observed in treatment 3, but had an increase in treatments 2 (7%) and 4 (24.1%) compared to the control (4.7%). *Chaetomiaceae* was only present in the control (19.2%). *Pleosporaceae* was not observed in treatment 3, but had an increase in treatments 2 (15.1%) and 4 (15.6%) when compared to the control (9.8%). *Cladosporiaceae* was absent in treatment 3, and had an increase in treatment 2 (12.5%) and a decrease in treatment 4 (4%) when compared to the control (5.8%). *Phaffomycetaceae* was only present in treatment 3 (50%). In 2023, the relative abundance of *Ceratobasidiaceae* had an increase in all treatments (53.8%, 65.9%, and 33%, respectively) compared to the control (32.2%). *Nectriaceae* was absent in treatment 3, but had an increase in treatments 2 (10.3%) and 4 (11.4%) when compared to the control (5.7%). *Phaffomycetaceae* was only present in the control (13.8%). *Plectosphaerellaceae* had a decrease in all treatments (1.1%, 1.9%, and 5%, respectively) when compared to the control (8.4%). *Chaetomiaceae* had an increase

in all treatments (5.6%, 10.5%, and 5%, respectively) when compared to the control (5.3%).

3.4. Plant Pathogens Shifts over Time, Treatment and Location

Each fungicide seed treatment was used to target specific cotton seedling pathogens, including *Rhizoctonia solani*, *Berkeleyomyces basicola*, and *Fusarium* spp. We aimed to assess the efficacy of these treatments against their targets and their broader impact on other fungal communities.

Based on the FungalTraits database, plant pathogens, soil saprotrophs, mycoparasites, and wood saprotrophs were some of the common fungal guilds in our dataset. Fungal plant pathogens were separated from other functional groups and used in this study. In soil samples from 2019-2020, the dominant plant pathogens families *Bartalliniaceae* (all treatments), *Nectriaceae* (all treatments), and *Plectosphaerellaceae* (all treatments) were found across all treatments (Figure 2.3). At the genus level, *Truncatella* (all treatments), *Fusarium* (treatment 2 – metalaxyl), *Neonectria* (treatment 1 - control), and *Plectosphaerella* (treatment 3 - penflufen) were the predominant plant pathogens found (Figure 2.4).

In roots samples from 2019-2020, the plant pathogens families were *Pleosporaceae* (all treatments), *Bartalliniaceae* (all treatments), *Nectriaceae* (all treatments), *Pyriculariaceae* (treatments 1 and 4), *Ceratocystidaceae* (treatment 1 - control), and *Cladosporiaceae* (all treatments). *Alternaria* (all treatments), *Fusarium* (treatments 2 and 3), *Truncatella* (all treatments), *Neonectria* (treatment 4 – broad spectrum), *Pyricularia* (treatment 1 – control), *Berkeleyomyces* (treatment 1 – control), and *Cladosporium* (all treatments).

In soil samples from 2021-2023 from Judd Hill, the plant pathogens families were *Pleosporaceae* (treatments 1 and 3), *Bartalliniaceae* (all treatments), *Nectriaceae* (all treatments), *Plectosphaerellaceae* (all treatments), *Phaerosphaeriaceae* (treatment 3), and *Cladosporiaceae* (treatment 1). The relative abundance for each treatment in those years was higher than 50%.

Fusarium (treatment 3), *Truncatella* (all treatments), *Alternaria* (treatments 1, 2, and 3), and *Plectosphaerella* (all treatments).

In root samples from 2021-2023 from Judd Hill, the plant pathogens families were *Ceratobasidiaceae* (all treatments), *Nectriaceae* (all treatments), *Plectosphaerellaceae* (all treatments), *Cladosporiaceae* (all treatments), *Pleosporaceae* (all treatments), *Chaetomiaceae* (treatments 1 and 2), *Bartalliniaceae* (all treatments), *Ceratocystidaceae* (treatments 1 and 2), and *Phaerosphaeriaceae* (treatment 3), representing more than 50% of the relative abundance for each treatment, except for treatment 4. *Acrophialophora* (treatments 1 and 2), *Alternaria* (all treatments), *Berkeleyomyces* (treatment 1 – control), *Cladosporium* (all treatments), *Fusarium* (treatments 1, 2, and 3), *Neonectria* (treatments 2, 3, and 4), *Paraphoma* (treatment 2), *Plectosphaerella* (all treatments), *Rhizoctonia* (treatments 2, 3, and 4), *Thanatephorus* (treatment 1 – control), and *Truncatella* (all treatments), representing more than 50% of the relative abundance for treatments 1 (control) and 4.

In soil samples from 2021-2023 from Marianna, the plant pathogens families were *Nectriaceae* (all treatments), *Plectosphaerellaceae* (all treatments), *Cladosporiaceae* (treatments 1 and 2), *Amphisphaeriaceae* (treatment 4), *Corynesporascaceae* (treatment 3), and *Phaeosphaeriaceae* (all treatments), together represent more than 60% of the relative abundance in each treatment. *Fusarium* (treatments 1, 3, and 4), *Plectosphaerella* (all treatments), *Cladosporium* (treatments 1 and 2), *Paraphoma* (treatments 2 and 4), *Microdochium* (treatment 4), and *Corynespora* (treatment 3), together represent almost 50% of the relative abundance in each treatment.

In root samples from 2021-2023 from Marianna, the plant pathogens families were *Nectriaceae* (all treatments), *Plectosphaerellaceae* (all treatments), *Phaeosphaeriaceae*

(treatments 1, 3, and 4), *Ceratobasidiaceae* (all treatments), *Bartaliniaceae* (treatment 4), *Cladosporiaceae* (all treatments), *Pleosporaceae* (treatments 1, 2, and 4), *Corynesporascaceae* (treatments 2 and 4), *Ceratocystidaceae* (treatment 4), and *Marasmiaceae* (treatment 1). *Alternaria* (treatments 1, 2, and 4), *Berkeleyomyces* (treatment 4), *Cladosporium* (all treatments), *Corynespora* (treatments 2 and 4), *Fusarium* (treatments 1, 2, and 4), *Marasmius* (treatments 1 and 3), *Paraphoma* (treatments 1, 3, and 4), *Plectosphaerellaceae* (all treatments), *Rhizoctonia* (all treatments), and *Truncatella* (treatment 3), together represent less than 50% of the relative abundance in each treatment.

3.5. Environmental Drivers on Soil- and Root-Associated Community Structures

The climate and soil properties data are presented in Table 2.20. Variables including temperature (initial and final), precipitation (initial and final), pH, soil texture (sand, silt, and clay percentages), electrical conductivity (EC), and nutrient levels (Phosphorus, Potassium, Calcium, Magnesium, Sulfur, Sodium, Iron, Manganese, Zinc, Copper, Boron, and available N) were used to explain variations in microbial community structure. Additionally, %LOI (Loss on Ignition) was used as an indicator of soil organic matter content. The initial and final terms mean months of planting and sample collection, respectively.

To evaluate the relationship of fungal community structure with environmental factors, redundancy analysis (RDA) of the fungal community and the Mantel test was used. From 2019-2020, soil-associated communities were not significantly affected by environmental factors, confirming results from PERMANOVA. For root-associated communities in 2019-2020, however, there was a significant influence from initial temperature, consistent with the PERMANOVA results (data not shown). Redundancy analysis (RDA) indicated that 100% of the variability was significantly explained by the first axis (RDA1). In these years, the fungal family

Phaffomycetaceae was present only in 2019 samples, while *Mrakiaceae* appeared exclusively in 2020 samples. We hypothesize that these abundance shifts, along with the short study period and the lack of a second location, may have contributed to these findings. Based on these considerations, we opted to focus the analysis on environmental factors for the 2021-2023 soil- and root-associated communities.

For soil-associated communities from 2021-2023, the RDA plot revealed that the first axis significantly explained 88.8% of the variation, while the second axis also significantly explained 8% (Table 2.15, Figure 2.6). Final temperature and initial and final precipitation significantly influenced community structure (Table 2.14). To explore the factors driving these compositional differences, Mantel's test was performed to assess the relationships between microbial and functional composition with environmental variables. Based on the Mantel test, initial and final precipitation, sand, silt, and clay percentages, EC, P, K, Ca, Mg, S, Zn, and %LOI significantly affected soil-associated communities (Table 2.16). For root-associated communities from 2021-2023, the RDA plot revealed that the first axis significantly explained 63.1% of the variation, while the second axis accounted for 16.9% (Table 2.18, Figure 2.6). Initial temperature and precipitation had significant effects on community structure (Table 2.17). The Mantel test showed that all environmental factors tested significantly affected root-associated communities (Table 2.19, Figure 2.7). Overall, initial precipitation was a key factor shaping community structures, regardless of whether factors were analyzed individually or collectively. Additionally, soil properties contributed to variations in community composition.

4. DISCUSSION

Fungicide seed treatment (FST) is a widely used strategy to manage fungal pathogens affecting seeds and seedlings. It helps protect against seed surface-borne, seedborne, and soilborne fungi,

reducing infections that occur both pre- and post-emergence (McMullen and Lamey, 2000; Paveley et al., 1996). For cotton, fungicide seed treatments are a key tool in managing seedling diseases, offering critical protection against soilborne and seedborne pathogens (Minton et al., 1986). Each year, nationwide trials evaluate the efficacy of different fungicides, but their success often depends on the composition and prevalence of soilborne pathogen populations, which vary annually and regionally (Garber et al., 1980). While FST aims to reduce pathogen loads while preserving seed viability and seedling vigor, they are frequently linked to off-target effects (Nettles et al., 2016; Yuan et al., 2019). In this study, we aimed to investigate the impact of different active ingredients on soil- and root-associated fungal communities across multiple years and locations.

For soil samples collected from 2019 to 2020, when our study included only one location (Judd Hill), soil fungal diversity was not significantly affected by treatments or years. This lack of significance may be due to the short duration of the study, suggesting that most of the shifts in soil fungal communities occur over longer time scales. This observation is consistent with Sun et al. 's (2017) findings, where fungal community structures remained largely unchanged over a six-month period. However, from 2021 to 2023, when we decided to include another location (Marianna), both year and location were found to influence the soil fungal diversity significantly. Since treatment was not significant, it indicates that the different fungicide seed treatments used in this study did not influence the overall diversity. These findings suggest that although soil fungal communities exhibited seasonal shifts throughout our sampling period, their overall structure could not be predicted by the year itself. Instead, these patterns likely reflect the interaction between temporal and environmental factors in shaping soil microbial diversity. Our results align with previous studies, which showed that even when sampling occurred in the same season, the specific fungal taxa present and their relative abundances varied from year to year (Burke, 2015).

This suggests that factors beyond just the season, such as inter-annual environmental fluctuations (e.g., precipitation, temperature, and soil properties), may have influenced soil fungal diversity in our study. PCoA analysis showed that fungal communities varied more by year than by treatment or location. Samples from the same year tended to cluster, but no clear separation was observed for treatment. Furthermore, PERMANOVA analysis indicated that soil fungal community composition varied significantly between locations but was not significantly influenced by fungicide treatments or year. This supports our alpha diversity findings, reinforcing that while the fungicide seed treatments may not have caused significant shifts in overall community structure or diversity, other factors, such as environmental variations, were more influential in shaping soil fungal composition.

For root samples from 2019 to 2020, when our study only included one location (Judd Hill), root-associated fungal diversity was significantly influenced by year and the interaction between year and treatment, but not by treatments alone. This suggests that temporal variations played a key role in shaping root-associated fungal communities, potentially driven by environmental fluctuations such as temperature, precipitation, or soil conditions. We hypothesize that the lack of a strong treatment effect during this period may be related to how fungicide seed coatings influence root microbiomes differently than other fungicide application methods. Unlike foliar sprays or soil-applied fungicides, seed coatings are translocated from the seed into the roots (Sartori et al., 2020), affecting endophytic microbes more than those in the soil (Vasanthakumari et al., 2018; Chen et al., 2020). However, because root-associated microbial communities are also influenced by environmental factors, their response to seed treatments may vary across years rather than showing a consistent treatment-driven pattern. PCoA analysis showed that fungal communities varied more by year than by treatment. This aligns with PERMANOVA analysis, which showed

that root fungal community composition varied significantly between years but was not significantly affected by fungicide treatments or the interaction between year and location.

For root samples from 2021 to 2023, when we decided to include another location (Marianna), all factors (year, location, treatment, and their interactions) significantly influenced fungal diversity. PCoA analysis clearly showed that fungal communities varied by year. The PERMANOVA analysis confirmed that fungal community composition varied significantly over the years. However, it also varied between fungicide seed treatments, and between the interactions of year and treatment, year and location, and treatment and location. This change suggests that environmental conditions unique to each location, along with temporal variations and treatment effects, may have had a more significant influence on shaping root-associated fungal communities over time.

The effects of year, treatment, and interactions for root-associated communities in 2021-2023 were further explored with pairwise PERMANOVA. The significant differences between treatments 1 vs. 3 and 2 vs. 3 suggest that these specific fungicide treatments influenced the root-associated fungal community structure, with treatment 3 (penflufen) differing more substantially from the control (treatment 1) and treatment 2 (metalaxyl). Moreover, the significant interaction between ‘treatment 3:Marianna vs. treatment 4:Marianna’ highlights that at Marianna, the fungal community composition differed between treatment 3 (penflufen) and the broad-spectrum fungicide combination (treatment 4: prothioconazole + myclobutanil + penflufen + metalaxyl). This suggests that in certain environmental conditions, treatment 4 had a more pronounced effect on fungal community composition compared to treatment 3 alone, possibly because of multiple active ingredients. Penflufen is an important fungicide seed treatment used to control seed- and soil-borne pathogens. It is highly effective at low dosages against economically significant fungi,

including *Rhizoctonia solani* (Adam et al., 2012). During seed germination, penflufen is absorbed by the roots and translocated via the xylem to other parts of the plant, providing systemic protection (Tian et al., 2016). Metalaxyl is a broad-spectrum fungicide widely used to protect various crops, including horticultural crops, vegetables, and fruits, from fungal diseases such as damping-off, late blight, stem, downy mildew, and fruit rots (Celis et al., 2015; Wang et al., 2014). Its favorable physicochemical properties, such as nonvolatility and high stability under varying pH, temperature, and light conditions, have contributed to its widespread global use (Malhat, 2017).

Additionally, the significant interaction between ‘2021:Marianna vs. 2023:Marianna’ indicates that fungal communities at Marianna changed significantly between these years, possibly due to environmental fluctuations such as temperature, precipitation, and soil properties. This reinforces the idea that root-associated fungal communities are dynamic and responsive to annual environmental changes. Unlike soil fungal communities, root-associated fungal communities are controlled by both plant influences and environmental conditions, making them particularly sensitive to climate perturbations (Fu et al., 2022).

Across years and treatments, fungal community composition in soil and root-associated samples varied. In soil samples from 2019-2020, *Mortierellaceae*, *Plectosphaerellaceae*, and *Nectriaceae* dominated, comprising over 80% of the fungal composition, with treatment 4 generally showing higher *Mortierellaceae* abundance. Root-associated communities at Judd Hill included *Mrakiaceae*, *Phaffomycetaceae*, and *Filobasidiaceae*, with notable fluctuations in *Phaffomycetaceae* (only 2019), *Mrakiaceae* (only 2020), and *Ceratocystidaceae* (only treatment 1 in 2019) between years. From 2021-2023, *Plectosphaerellaceae*, *Mortierellaceae*, and *Nectriaceae* remained dominant in soil, with *Mortierellaceae* decreasing over time, and *Plectosphaerellaceae* increasing over time, while root-associated communities included

Mortierellaceae, *Nectriaceae*, and *Ceratobasidiaceae*, showing shifts in relative abundance across treatments.

When we analyzed the plant pathogens separately, it revealed shifts in dominant taxa over time, treatment, and location. In 2019-2020, *Bartalliniaceae*, *Nectriaceae*, and *Plectosphaerellaceae* dominated soil samples, while *Pleosporaceae*, *Bartalliniaceae*, and *Nectriaceae* were prevalent in roots. From 2021-2023, Judd Hill and Marianna showed distinct pathogen profiles, with *Fusarium*, *Truncatella*, *Alternaria*, and *Plectosphaerella* commonly found in soil, while roots had diverse genera, including *Rhizoctonia*, *Neonectria*, *Fusarium*, and *Cladosporium*. These shifts reflect the impact of fungicide treatments and environmental factors on pathogen dynamics. *Berkeleyomyces* was found predominantly at Judd Hill rather than at Marianna.

These shifts highlight the impact of fungicide treatments on fungal community dynamics over time. However, they reinforce that both fungicide treatments and environmental conditions interact to shape root-associated fungal communities, with some treatments applying a more pronounced effect depending on location and year. This underscores the importance of considering both spatial and temporal variability when evaluating the long-term effects of fungicide seed treatments on microbial communities.

It is well-known that soil- and root-associated communities can be influenced by a range of factors, including climatic variables such as temperature and precipitation, geographical conditions, soil chemical properties like pH, texture, nutrient levels, and organic matter content, as well as the composition of microbial populations and other biological factors (Yu et al., 2020; Wen et al., 2020).

Regarding the impact of individual environmental factors on the soil- and root-associated

fungus communities from Judd Hill and Marianna over the years (2021-2023), the Redundancy Analysis (RDA) results showed that final temperature and initial and final precipitation had a significant impact on soil-associated fungus communities ($P = 0.001$, $P = 0.005$, and $P = 0.001$, respectively). To determine which specific taxa were strongly correlated with environmental factors, a heatmap correlation analysis was performed. For soil samples from 2021-2023, the heatmap did not indicate a strong correlation between temperature (temp1 and temp2) and individual taxa, suggesting that temperature's effect on community composition may be more diffuse rather than driving distinct changes in specific taxa. However, it was significant for initial and final precipitation, sand, silt and clay %, EC, P, K, Ca, Mg, S, Zn, and %LOI. For root samples in 2021-2023, initial temperature and precipitation were significant ($P = 0.045$, and $P = 0.026$, respectively). The heatmap was significant for all environmental factors we tested.

Seasonal climate change directly influences soil fungus diversity and indirectly shapes it by altering soil properties and root-associated factors (Xie and Yin, 2022). In this study, the initial temperature was consistently lower than the final temperature. For soil samples from 2021-2023, the lowest temperature occurred in 2021 at Judd Hill (initial: 21.9°C, final: 27.2°C) and the highest in 2023 (initial: 24.4°C, final: 28.7°C). None of the temperatures had a significant positive or negative correlation with the families. However, for root-associated communities, initial and final temperatures (temp1 and temp2, respectively) were significantly negatively correlated with *Mrakiaceae*, *Bulleribasidiaceae*, *Cystofilobasidiaceae*, and significantly positively with *Pleosporaceae*, and *Nectriaceae*.

Precipitation levels were generally higher than finals, except at Judd Hill in 2023 (initial: 0.1 in, final: 0.2 in) and Marianna in both 2021 (initial: 0.18 in, final: 0.23 in) and 2023 (initial: 0.04 in, final: 0.07 in), where final precipitation exceeded initial levels. The highest precipitation level

was recorded in 2021 at Judd Hill (initial: 0.19 in, final: 0.14 in) and in 2021 at Marianna (initial: 0.18 in, final: 0.23 in). For soil-associated communities from 2021-2023, initial precipitation (prec1) was significantly positively correlated with *Mortierellaceae* and significantly negatively with *Plectosphaerellaceae*, while final precipitation was significantly positively correlated with *Bartaliniaceae* and significantly negatively with *Nectriaceae*. For root-associated communities from 2021-2023, initial precipitation (prec1) was significantly negatively correlated with *Ceratobasidiaceae* and *Exidiaceae* and significantly positively with *Sporidiobolaceae* and *Mrakiaceae*, while final precipitation was significantly positively correlated with *Bartaliniaceae* and significantly negatively with *Nectriaceae* and *Mortierellaceae*. Wang et al. (2020) also observed that when precipitation was increased, it decreased *Nectriaceae* abundance. Xue et al. (2022) observed that when the precipitation was reduced to a normal level, *Ceratobasidiaceae* again predominated, indicating that *Ceratobasidiaceae* were more likely to survive in low-precipitation habitats, confirming our results.

Several previous studies have also assessed the effects of soil pH on microbial diversity and community structure (Zhou et al., 2020; Rousk et al., 2010; Waldrop et al., 2017; Zeng et al., 2016). It ranged from 6.1 to 6.3 at Judd Hill and 6.3 to 6.6 at Marianna. For soil-associated communities from 2021-2023, pH was significantly negatively correlated with *Coniochaetaceae* and *Mortierellaceae* and significantly positively with *Plectosphaerellaceae*. For root-associated communities from 2021-2023, pH was significantly positively correlated with *Ceratobasidiaceae*, *Exidiaceae*, and *Chaetomiaceae*, and significantly negatively with *Mortierellaceae*, *Bartaliniaceae*, and *Sclerotiniaceae*. One possible explanation for pH driver differences in fungal community structures is that soil pH affects the shift in community structure through environmental factors (such as nutrient availability, organic C, and soil water condition), which

often changes synchronously with changes in soil pH (Rousk et al., 2010; Waldrop et al., 2017). Another reason is that soil pH can affect the plant community and soil fauna, which can further lead to shifts in the soil microbial community (Johnston and Sibly, 2020; Zeng et al., 2016).

According to Lauber et al. (2008), the composition of the fungal community is most closely related to the change in soil nutrients. In this study, soil nutrients drove the community structure of soil microorganisms. For soil-associated communities in 2021-2023, Fe and P were significantly positively correlated with *Nectriaceae*, while Na was significantly negatively correlated with *Nectriaceae*. For root-associated communities, N and EC were significantly negatively correlated with *Nectriaceae*. S, K, sand %, and P were significantly negatively correlated with *Ceratobasidiaceae*, while B, Ca, clay %, %LOI, EC, silt %, Mg, and Mn were significantly positively correlated with *Ceratobasidiaceae*.

Overall, our study concluded that fungicide seed treatments play a significant role in changing microbial communities and vary with active ingredients, years of sampling, and environmental conditions. Complex interactions exist between treated seed and microbial communities, with these effects being context dependent. Some treatments effectively control their target pathogens (metalaxyl and penflufen, for example) but also induce shifts in some non-target fungal populations (penflufen seemed to increase *Berkeleyomyces* population), possibly having long-lasting implications on soil health and plant-microbe interactions. Understanding these dynamics is crucial for optimizing seed treatment strategies to control pathogens while maintaining a functionally diverse and resilient microbiome.

TABLES AND FIGURES

Table 2.1. ANOVA results of the Shannon diversity index for soil samples from 2019-2020.

Variable	Metric	Method	DF	SS	Mean Sq	F	Pr(>F)
year	Shannon	Anova	1	0.44	0.44	2.57	0.17
treatment			3	0.02	0.01	0.03	0.99
year:treatment			3	0.29	0.10	0.58	0.65
Residuals			5	0.85	0.17		

Table 2.2. ANOVA results of the Shannon diversity index for soil samples from 2021-2023.

Variable	Metric	Method	DF	SS	Mean Sq	F	Pr(>F)
year	Shannon	Anova	2	1.74	0.87	4.69	0.019*
treatment			3	0.26	0.09	0.47	0.71
location			1	1.65	1.65	8.86	0.007**
year:treatment			6	0.41	0.07	0.37	0.89
year:location			2	0.27	0.14	0.74	0.49
treatment:location			3	0.35	0.12	0.62	0.61
year:treat:loc			6	0.58	0.10	0.53	0.78
Residuals			24	4.46	0.19		

*indicates statistical significance at $P < 0.05$.

**indicates statistical significance at $P < 0.01$.

Table 2.3. Kruskal-Wallis results of the Shannon diversity index for root samples from 2019-2020.

Variable	Metric	Method	DF	Chi-Squared	p-value
year	Shannon	Kruskal-Wallis	1	21.85	<0.001***
treatment			3	2.83	0.419
year:treatment			7	24.98	<0.001***

***indicates statistical significance at $P < 0.001$.

Table 2.4. Kruskal-Wallis results of the Shannon diversity index for root samples from 2021-2023.

Variable	Metric	Method	DF	Chi-Squared	p-value
year	Shannon	Kruskal-Wallis	2	5.95	0.050*
treatment			3	14.77	0.002**
location			1	6.10	0.013*
year:treatment			11	22.82	0.018*
year:location			5	24.98	<0.001***
treatment:location			7	29.74	<0.001***
year:treat:loc			23	54.20	0.001***

*indicates statistical significance at $P < 0.05$.

**indicates statistical significance at $P < 0.01$.

***indicates statistical significance at $P < 0.001$.

Table 2.5. PERMANOVA analysis of soil-associated communities from 2019-2020.

Variable	DF	SS	R ²	F	Pr(>F)
year	1	0.03	0.25	2.31	0.22
treatment	3	0.01	0.07	0.21	0.89
year:treatment	3	0.03	0.25	0.77	0.59
Residuals	4	0.05	0.43		
Total	11	0.11	1.00		

Table 2.6. PERMANOVA analysis of soil-associated communities from 2021-2023.

Variable	DF	SS	R ²	F	Pr(>F)
year	2	0.22	0.14	2.86	0.064
treatment	3	0.06	0.04	0.52	0.707
location	1	0.18	0.11	4.63	0.043 *
year : treatment	6	0.13	0.08	0.55	0.755
year : location	2	0.04	0.02	0.47	0.691
treatment : location	3	0.07	0.05	0.64	0.627
year : treatment : location	6	0.15	0.09	0.64	0.699
Residual	20	0.77	0.48		
Total	43	1.61	1.00		

*indicates statistical significance at $P < 0.05$.

Table 2.7. Pairwise PERMANOVA analysis of soil-associated communities from 2021-2023.

Pairs	DF	SS	F. Model	R ²	p-value	p-value adjusted
Judd Hill vs Marianna	1	0.14	4.12	0.09	0.03	0.031 *

*indicates statistical significance at $P < 0.05$.

Table 2.8. PERMANOVA analysis of root-associated communities from 2019-2020.

Variable	DF	SS	R ²	F	Pr(>F)
year	1	1.92	0.72	85.73	0.001 **
treatment	3	0.08	0.03	1.17	0.331
year : treatment	3	0.07	0.03	1.01	0.425
Residual	27	0.61	0.23		
Total	34	2.68	1.00		

**indicates statistical significance at $P < 0.01$.

Table 2.9. Pairwise PERMANOVA analysis of root-associated communities from 2019-2020.

Pairs	DF	SS	F. Model	R²	p-value	p-value adjusted
2019 vs 2020	1	1.92	84.31	0.72	0.001	0.001 ***

***indicates statistical significance at $P < 0.001$.

Table 2.10. PERMANOVA analysis of root-associated communities from 2021-2023.

Variable	DF	SS	R²	F	Pr(>F)
year	2	0.25	0.06	4.84	0.010 **
treatment	3	0.79	0.20	10.34	0.001***
location	1	0.03	0.01	1.26	0.264
year : treatment	6	0.63	0.16	4.14	0.003 **
year : location	2	0.44	0.11	8.71	0.001***
treatment : location	3	0.32	0.08	4.24	0.008 **
year : treatment : location	5	0.15	0.04	1.18	0.293
Residual	51	1.30	0.33		
Total	73	3.92	1.00		

**indicates statistical significance at $P < 0.01$.

***indicates statistical significance at $P < 0.001$.

Table 2.11. Pairwise PERMANOVA analysis of root-associated communities from 2021-2023.

Pairs	DF	SS	F. Model	R²	p-value	p-value adjusted
1 vs 2	1	0.010	0.515	0.010	0.515	1.000
1 vs 3	1	0.619	13.274	0.300	0.001	0.006 **
1 vs 4	1	0.042	0.949	0.028	0.400	1.000
2 vs 3	1	0.571	12.901	0.259	0.002	0.012 *
2 vs 4	1	0.030	0.820	0.020	0.387	1.000
3 vs 4	1	0.420	6.140	0.150	0.018	0.108

*indicates statistical significance at $P < 0.05$.

**indicates statistical significance at $P < 0.01$.

Table 2.12. Pairwise PERMANOVA analysis of root-associated communities from 2021-2023.

Pairs	DF	SS	F. Model	R²	p-value	p-value adjusted
2021_Judd Hill vs 2021_Marianna	1.00	0.14	5.25	0.16	0.028	0.420
2021_Judd Hill vs 2022_Judd Hill	1.00	0.24	6.93	0.28	0.011	0.165
2021_Judd Hill vs 2022_Marianna	1.00	0.14	2.51	0.09	0.115	1.000
2021_Judd Hill vs 2023_Judd Hill	1.00	0.15	3.42	0.13	0.066	0.990
2021_Judd Hill vs 2023_Marianna	1.00	0.03	1.86	0.06	0.156	1.000
2021_Marianna vs 2022_Judd Hill	1.00	0.07	1.54	0.08	0.225	1.000
2021_Marianna vs 2022_Marianna	1.00	0.07	1.06	0.04	0.320	1.000
2021_Marianna vs 2023_Judd Hill	1.00	0.02	0.46	0.02	0.540	1.000
2021_Marianna vs 2023_Marianna	1.00	0.30	12.10	0.30	0.002	0.030 *
2022_Judd Hill vs 2022_Marianna	1.00	0.06	0.68	0.04	0.389	1.000
2022_Judd Hill vs 2023_Judd Hill	1.00	0.04	0.46	0.03	0.443	1.000
2022_Judd Hill vs 2023_Marianna	1.00	0.36	10.97	0.38	0.005	0.075
2022_Marianna vs 2023_Judd Hill	1.00	0.01	0.16	0.01	0.719	1.000
2022_Marianna vs 2023_Marianna	1.00	0.23	4.42	0.15	0.040	0.600
2023_Judd Hill vs 2023_Marianna	1.00	0.27	6.62	0.22	0.006	0.090

*indicates statistical significance at $P < 0.05$.

Table 2.13. Pairwise PERMANOVA analysis of root-associated communities from 2021-2023.

Pairs	DF	SS	F. Model	R ²	p-value	p-value adjusted
1_Judd Hill vs 2_Judd Hill	1.00	0.001	0.07	0.01	0.822	1.000
1_Judd Hill vs 3_Judd Hill	1.00	0.18	4.04	0.29	0.051	1.000
1_Judd Hill vs 4_Judd Hill	1.00	0.15	2.38	0.18	0.137	1.000
1_Judd Hill vs 2_Marianna	1.00	0.02	0.77	0.05	0.420	1.000
1_Judd Hill vs 3_Marianna	1.00	0.33	5.89	0.31	0.024	0.672
1_Judd Hill vs 1_Marianna	1.00	0.003	0.13	0.01	0.868	1.000
1_Judd Hill vs 4_Marianna	1.00	0.01	0.88	0.06	0.375	1.000
2_Judd Hill vs 3_Judd Hill	1.00	0.24	7.10	0.31	0.006	0.168
2_Judd Hill vs 4_Judd Hill	1.00	0.21	4.37	0.21	0.059	1.000
2_Judd Hill vs 2_Marianna	1.00	0.02	0.96	0.05	0.341	1.000
2_Judd Hill vs 3_Marianna	1.00	0.48	11.05	0.37	0.002	0.056
2_Judd Hill vs 1_Marianna	1.00	0.002	0.12	0.01	0.924	1.000
2_Judd Hill vs 4_Marianna	1.00	0.03	1.75	0.08	0.192	1.000
3_Judd Hill vs 4_Judd Hill	1.00	0.01	0.12	0.01	0.846	1.000
3_Judd Hill vs 2_Marianna	1.00	0.13	2.71	0.15	0.104	1.000
3_Judd Hill vs 3_Marianna	1.00	0.02	0.25	0.02	0.663	1.000
3_Judd Hill vs 1_Marianna	1.00	0.23	5.27	0.25	0.030	0.840
3_Judd Hill vs 4_Marianna	1.00	0.35	9.22	0.37	0.005	0.140
4_Judd Hill vs 2_Marianna	1.00	0.11	1.77	0.09	0.188	1.000
4_Judd Hill vs 3_Marianna	1.00	0.04	0.47	0.03	0.528	1.000
4_Judd Hill vs 1_Marianna	1.00	0.19	3.35	0.17	0.089	1.000
4_Judd Hill vs 4_Marianna	1.00	0.30	5.75	0.25	0.021	0.588
2_Marianna vs 3_Marianna	1.00	0.30	5.45	0.22	0.019	0.532
2_Marianna vs 1_Marianna	1.00	0.02	0.66	0.03	0.457	1.000
2_Marianna vs 4_Marianna	1.00	0.08	3.07	0.13	0.084	1.000
3_Marianna vs 1_Marianna	1.00	0.45	8.85	0.32	0.010	0.280
3_Marianna vs 4_Marianna	1.00	0.63	13.49	0.42	0.001	0.028 *
1_Marianna vs 4_Marianna	1.00	0.02	0.93	0.04	0.368	1.000

*indicates statistical significance at $P < 0.05$.

Table 2.14. RDA analysis of soil-associated communities from 2021-2023.

Variable	DF	Variance	F	Pr(>F)
temp1	1	326508	2.37	0.082
temp2	1	2973311	21.60	0.001 ***
prec1	1	1120554	8.14	0.005 **
prec2	1	3217825	23.38	0.001 ***
ph	1	210731	1.53	0.193
Residual	42	5780774		

**indicates statistical significance at $P < 0.01$.

*** indicates statistical significance at $P < 0.001$.

Table 2.15. Explanatory response (soil 2021-2023).

Variable	DF	Variance	F	Pr(>F)
RDA1	1	6972895	50.66	0.001 ***
RDA2	1	629836	4.58	0.035 *
RDA3	1	159404	1.16	0.808
RDA4	1	67751	0.49	0.968
RDA5	1	19043	0.14	0.996
Residual	42	5780774		

* indicates statistical significance at $P < 0.05$.

*** indicates statistical significance at $P < 0.001$.

Table 2.16. The Spearman correlation heatmap for soil-associated communities from 2021-2023.

Group	Variables	mantel	Type	method	Coefficient	p-value	p-adjusted
A11	temp1	mantel	test	pearson	0.064	0.113	0.140
A11	terr2	mantel	test	pearson	0.026	0.138	0.153
A11	prec1	mantel	test	pearson	0.296	0.001	0.004
A11	prec2	mantel	test	pearson	0.081	0.029	0.047
A11	ph	mantel	test	pearson	0.086	0.059	0.085
A11	Sand %	mantel	test	pearson	0.107	0.010	0.021
A11	Silt %	mantel	test	pearson	0.104	0.006	0.014
A11	Clay %	mantel	test	pearson	0.297	0.001	0.004
A11	EC	mantel	test	pearson	0.147	0.004	0.011
A11	P	mantel	test	pearson	0.224	0.001	0.004
A11	K	mantel	test	pearson	0.221	0.002	0.007
A11	Ca	mantel	test	pearson	0.340	0.001	0.004
A11	Mg	mantel	test	pearson	0.086	0.017	0.030
A11	S	mantel	test	pearson	0.102	0.016	0.030
A11	Na	mantel	test	pearson	0.059	0.061	0.085
A11	Fe	mantel	test	pearson	0.032	0.202	0.212
A11	Mn	mantel	test	pearson	0.025	0.128	0.149
A11	Zn	mantel	test	pearson	0.178	0.004	0.011
A11	Cu	mantel	test	pearson	0.007	0.366	0.366
A11	B	mantel	test	pearson	0.080	0.068	0.089
A11	%LOI	mantel	test	pearson	0.223	0.001	0.004

* indicates statistical significance at $P < 0.05$.

** indicates statistical significance at $P < 0.01$.

Table 2.17. RDA analysis of root-associated communities from 2021-2023.

Variab1e	DF	Variance	F	Pr(>F)
temp1	1	2875756	2.96	0.045 *
temp2	1	2279453	2.35	0.081
prec1	1	3493118	3.60	0.026 *
prec2	1	1279392	1.32	0.258
ph	1	1214671	1.25	0.295
Residual	94	91286710		

* indicates statistical significance at $P < 0.05$.

Table 2.18. Explanatory response (roots 2021-2023).

Variab1e	DF	Variance	F	Pr(>F)
RDA1	1	7026587	7.24	0.046 *
RDA2	1	1881085	1.94	0.77
RDA3	1	1263953	1.30	0.78
RDA4	1	709973	0.73	0.83
RDA5	1	260791	0.27	0.83
Residual	94	91286710		

* indicates statistical significance at $P < 0.05$.

Table 2.19. The Spearman correlation heatmap for root-associated communities from 2021-2023.

Group	Variables	mantel	Type	method	Coefficient	p-value	p-adjusted
A11	templ	mantel	test	pearson	0.190	0.001	0.001
A11	temp2	mantel	test	pearson	0.178	0.001	0.001
A11	precl	mantel	test	pearson	0.245	0.001	0.001
A11	prec2	mantel	test	pearson	0.211	0.001	0.001
A11	ph	mantel	test	pearson	0.168	0.001	0.001
A11	Sand %	mantel	test	pearson	0.110	0.001	0.001
A11	Silt %	mantel	test	pearson	0.101	0.001	0.001
A11	Clay %	mantel	test	pearson	0.258	0.001	0.001
A11	EC	mantel	test	pearson	0.150	0.001	0.001
A11	P	mantel	test	pearson	0.227	0.001	0.001
A11	K	mantel	test	pearson	0.158	0.001	0.001
A11	Ca	mantel	test	pearson	0.287	0.001	0.001
A11	Mg	mantel	test	pearson	0.141	0.001	0.001
A11	S	mantel	test	pearson	0.222	0.001	0.001
A11	Na	mantel	test	pearson	0.154	0.001	0.001
A11	Fe	mantel	test	pearson	0.216	0.001	0.001
A11	Mn	mantel	test	pearson	0.155	0.001	0.001
A11	Zn	mantel	test	pearson	0.169	0.001	0.001
A11	Cu	mantel	test	pearson	0.233	0.001	0.001
A11	B	mantel	test	pearson	0.167	0.001	0.001
A11	%LOI	mantel	test	pearson	0.162	0.001	0.001

*** indicates statistical significance at $P < 0.001$.

Table 2.20. Environmental factors for Judd Hill and Marianna over the years.

Site	Year	°C		in		µmhos/cm		mg/kg (scooped)								Zn	Cu	B	mg/kg				
		temp1	temp2	prec1	prec2	pH	EC	P	K	Ca	Mg	S	Na	Fe	Mn				%LOI	NO ₃ -N&NO ₂ -N	Sand %	Silt %	Clay %
Judd Hill	2019	24.2	27.7	0.28	0.14	5.9	347	42	93	1186	170	8.0	7.5	201	49	2.4	0.9	1.2	1.36	80.6	57.2	32.7	10.1
	2020	27.6	30.4	0.07	0.1	5.3	589	82	218	1197	161	9.0	8.7	283	33	2.7	0.7	1.4	1.84	193.0	44.5	47.9	7.6
	2021	21.9	27.2	0.19	0.14	6.1	119	54	129	1028	117	5.3	5.5	165	85	2.5	1.1	0.9	1.19	24.3	52.2	40.2	7.6
	2022	23.9	28.5	0.16	0.02	6.3	98	71	143	1082	124	4.7	6.2	192	81	2.3	1.1	1.2	1.19	13.9	49.7	42.7	7.6
	2023	24.4	28.7	0.1	0.2	6.3	149	18	120	1157	165	5.2	11.7	119	77	1.1	0.9	1.1	1.64	22.4	38.2	51.7	10.1
Marianna	2021	20.2	25.3	0.18	0.23	6.3	201	42	148	1012	274	5.7	9.2	157	124	4.3	1.1	1.1	1.59	33.8	9.1	83.3	7.6
	2022	22.4	26.7	0.2	0.08	6.4	165	44	148	1063	277	5.0	9.4	174	129	5.2	1.3	1.1	1.65	21.0	6.6	84.5	8.9
	2023	22	25.7	0.04	0.07	6.6	212	35	131	1149	341	4.6	6.9	172	144	2.8	1.3	1.4	1.91	19.2	6.2	82.4	11.4

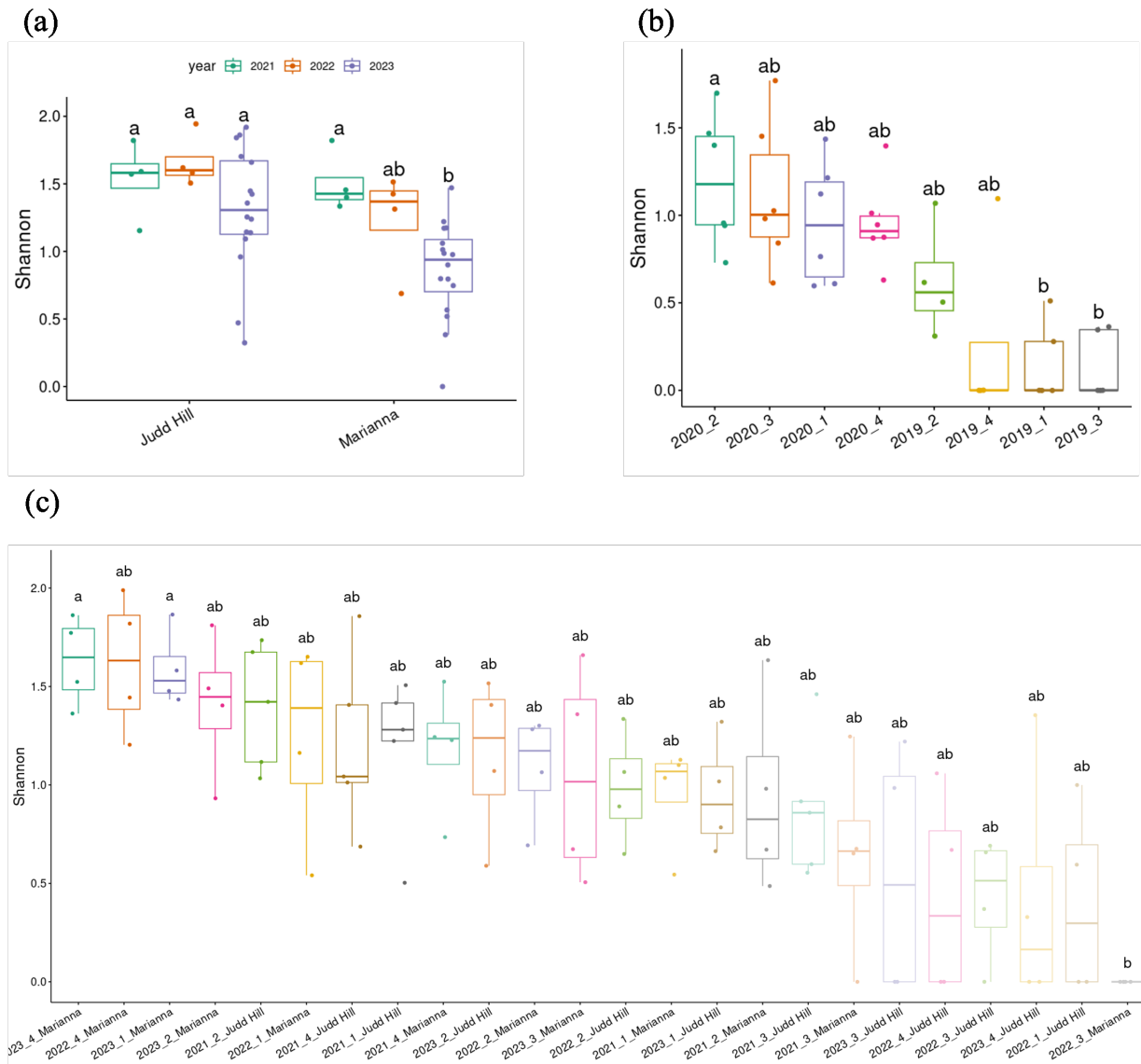


Figure 2.1. Alpha diversity box plot displaying the Shannon index. Panel (a) shows the alpha diversity between location and year, (b) shows the alpha diversity index between year and treatment, and (c) shows the alpha diversity between year and treatment. The letters a and b are used to clarify whether the difference between any pair of groups calculated by ANOVA and Kruskal-Wallis was statistically significant ($P < 0.05$) and whether there was a significant difference in diversity between the two sharing no common letter markers.

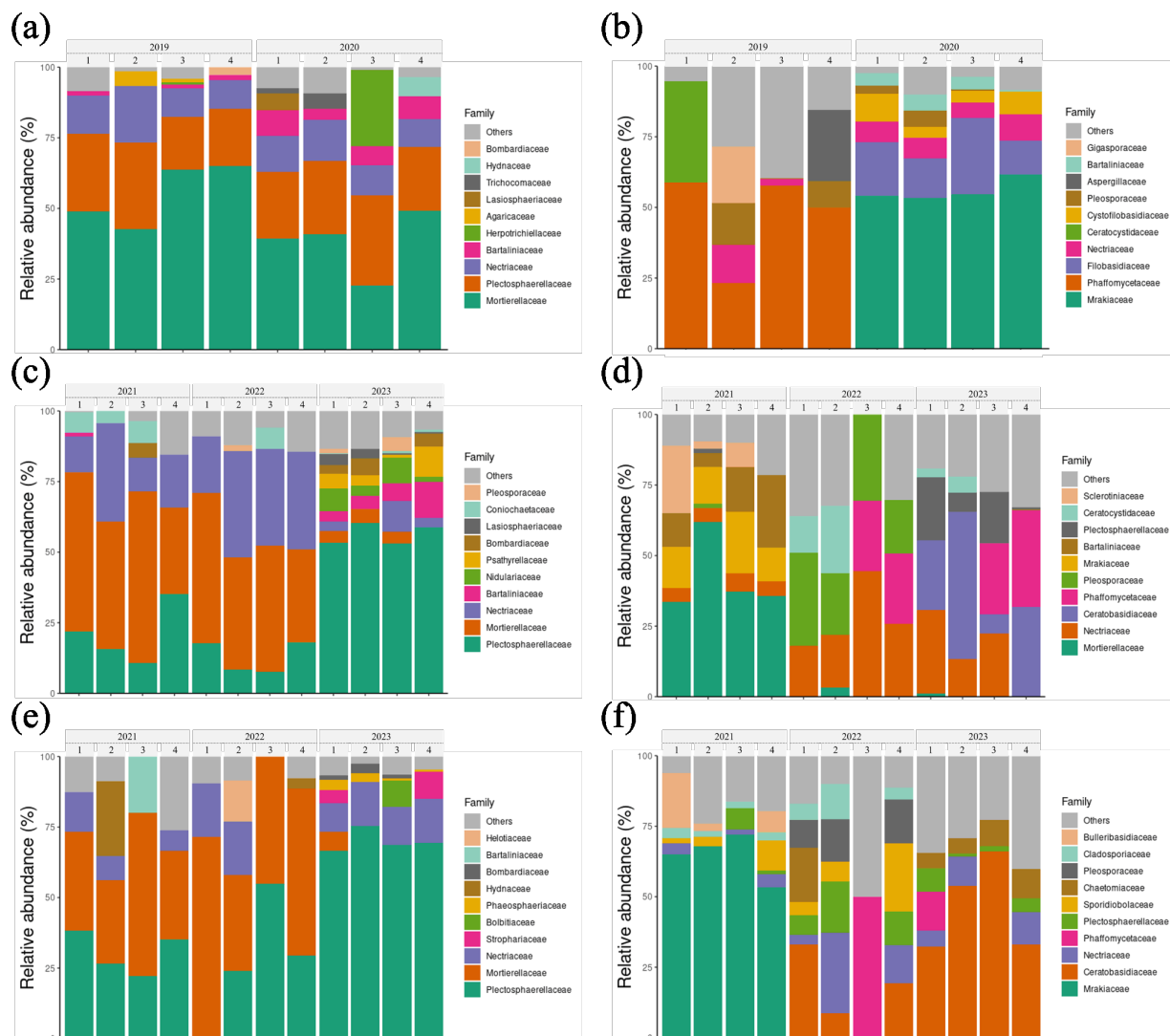


Figure 2.2. Top 10 relative abundance (%) of fungi at the family level in soil and root samples for each treatment and year (2019 – 2020). Panel (a) displays the top 10 most abundant fungal families at Judd Hill in each treatment by year in soil samples, (b) displays the top 10 most abundant fungal families at Judd Hill in each treatment by year in root samples, (c) top 10 most abundant families at Judd Hill in each treatment by year in soil samples, (d) top 10 most abundant families at Judd Hill in each treatment by year in root samples, (e) top 10 most abundant families at Marianna in each treatment by year in soil samples, and (f) top 10 most abundant families at Marianna in each treatment by year in root samples.

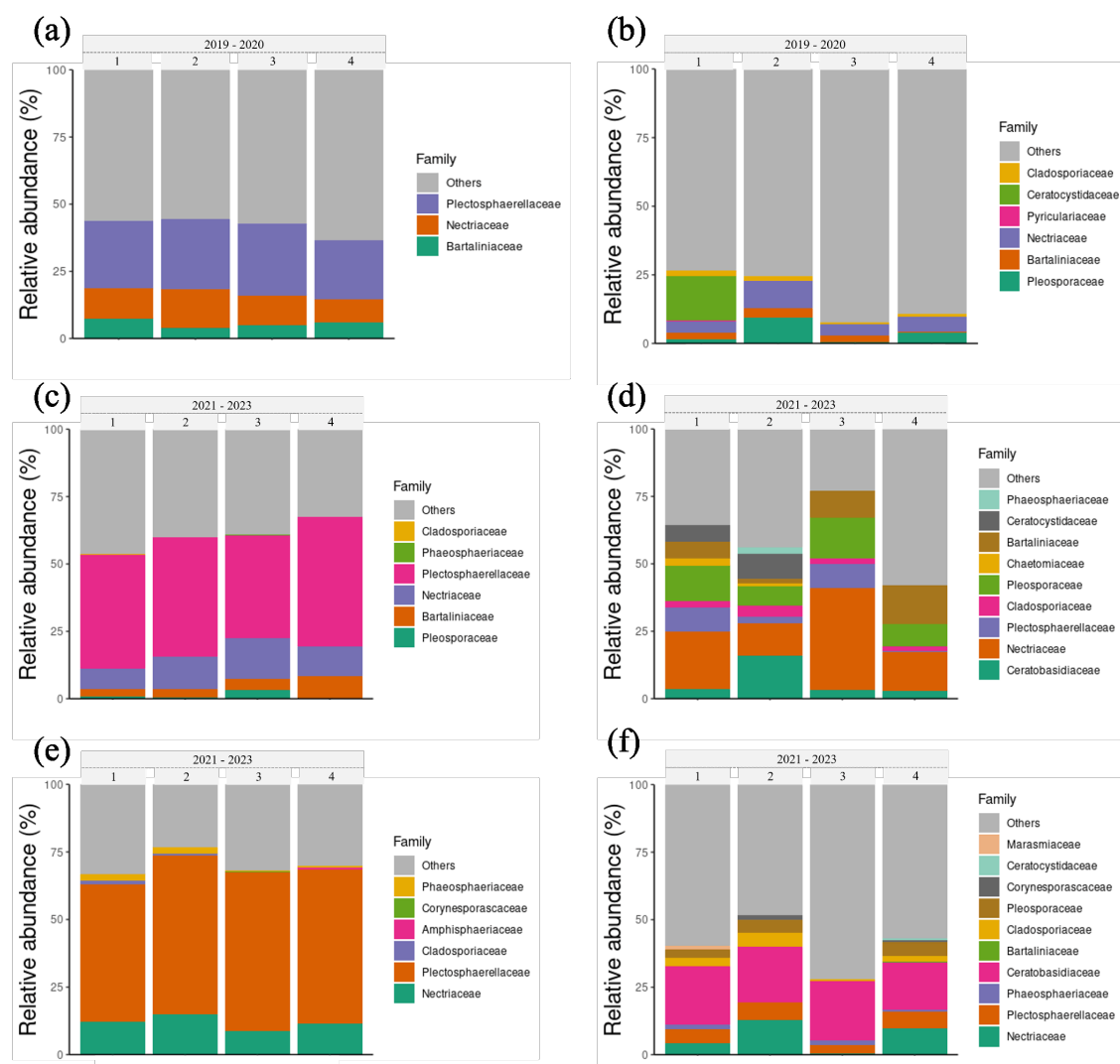


Figure 2.3. Plant pathogen lifestyles were identified using the FungalTraits database. Panel (a) shows the relative abundance of plant pathogens for each treatment from soil samples from 2019-2020, (b) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020, (c) shows the relative abundance of plant pathogens for each treatment from soil samples from 2021-2023 at Judd Hill, (d) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020 at Judd Hill, (e) shows the relative abundance of plant pathogens for each treatment from soil samples from 2019-2020 at Marianna, and (f) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020 at Marianna.

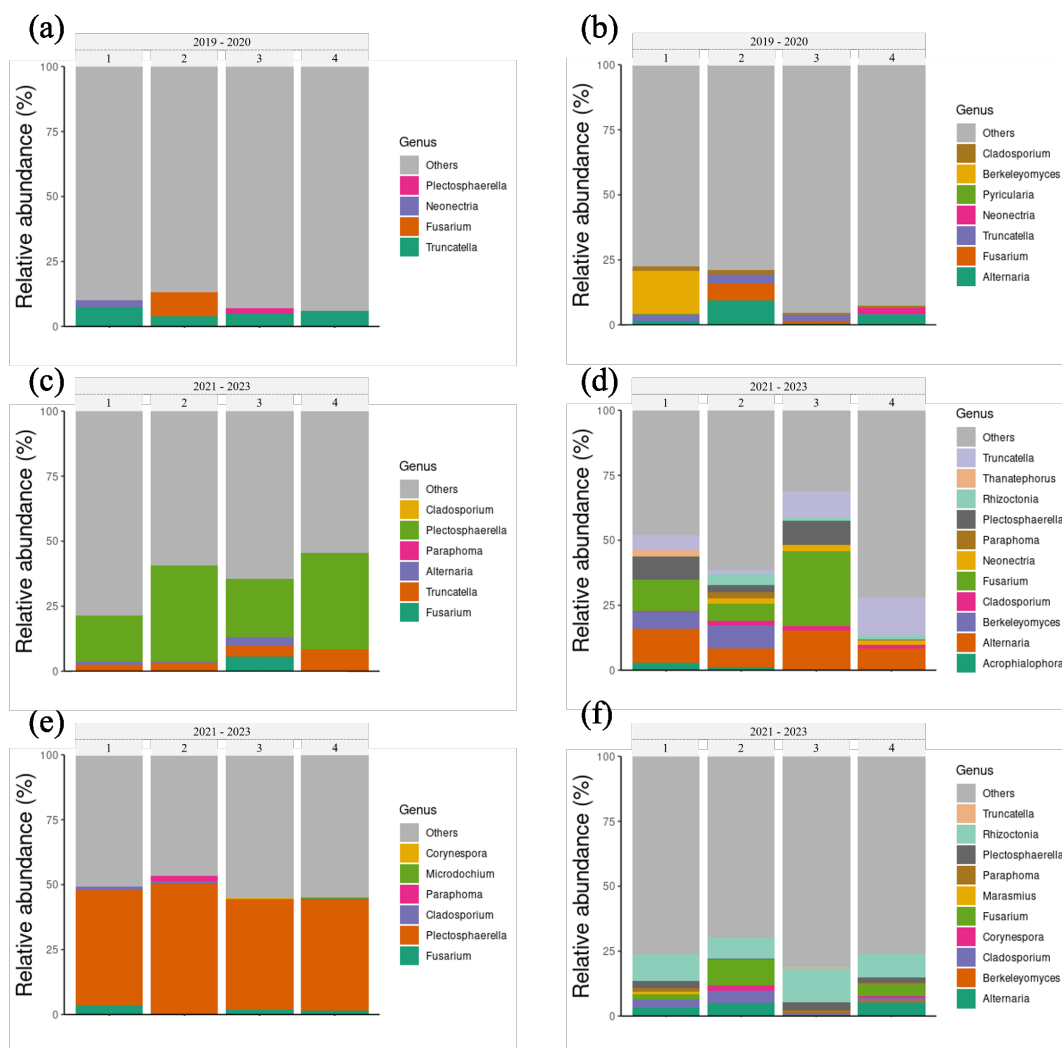


Figure 2.4. Plant pathogen lifestyles were identified using the FungalTraits database. Panel (a) shows the relative abundance at genus level of plant pathogens for each treatment from soil samples from 2019-2020, (b) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020, (c) shows the relative abundance of plant pathogens for each treatment from soil samples from 2021-2023 at Judd Hill, (d) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020 at Judd Hill, (e) shows the relative abundance at genus level of plant pathogens for each treatment from soil samples from 2019-2020 at Marianna, and (f) shows the relative abundance of plant pathogens for each treatment from roots samples from 2019-2020 at Marianna.

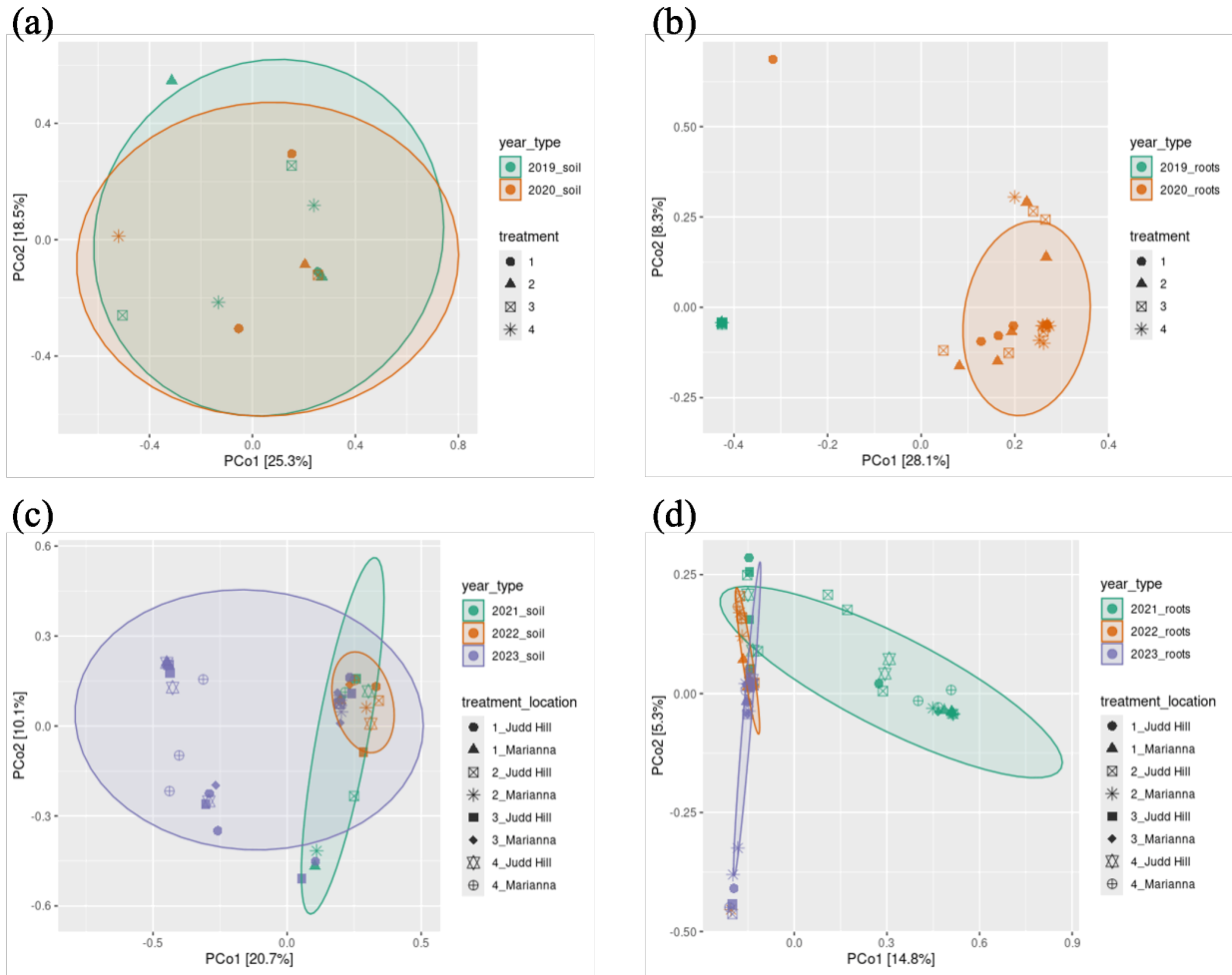


Figure 2.5. Principal coordinate analysis (PCoA) plot based on the Bray-Curtis dissimilarity matrix comparing the treatment distribution between years at (a) soil samples from 2019-2020, (b) and root samples from 2019-2020. PCoA plot comparing the combination of treatment and location by year for (c) soil from 2021-2023, and (d) roots from 2021-2023.

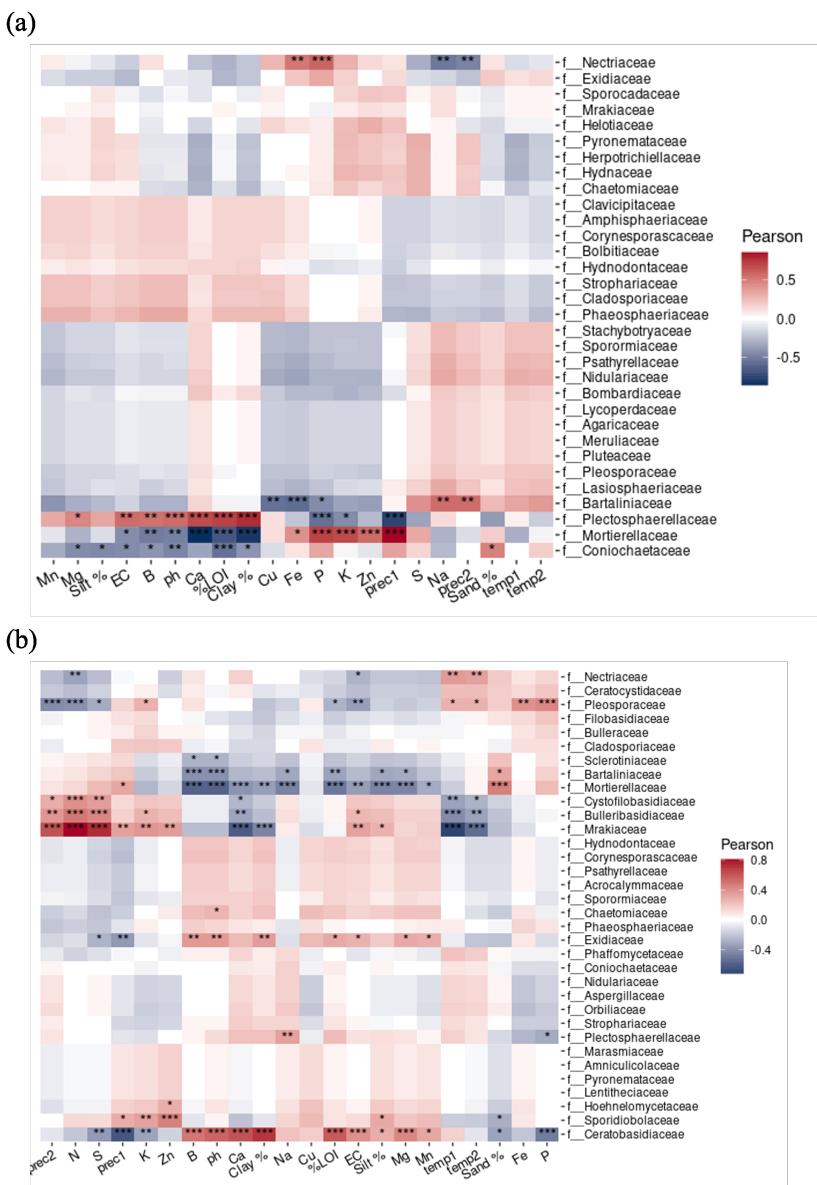


Figure 2.7. The Spearman correlation heatmap, showing the correlations with environmental conditions and fungal families from (a) soil samples, and (b) root sample.

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CHAPTER 3: EFFECTS OF TEMPERATURE ON COTTON SEEDLING DISEASE, ROOT-ASSOCIATED FUNGAL COMMUNITIES, AND THEIR INTERACTION WITH FUNGICIDE TREATMENTS

ABSTRACT

Cotton seedling diseases pose a significant threat to stand establishment and overall cotton production worldwide, impacting germination, emergence, and early-season growth. To mitigate disease severity and enhance stand establishment, fungicide seed treatments are widely utilized. In the United States, cotton is cultivated across diverse regions within the Cotton Belt, where temperature fluctuations throughout the growing season can create conditions that favor seedling disease development. Cool temperatures, in particular, promote pathogen activity and increase disease risk, highlighting the need for effective disease management strategies tailored to these challenging conditions. The objectives of this study were to evaluate the performance of seed treatments under two different temperature conditions, to assess whether seed treatments enhance seedling tolerance to cold stress in an early planting production system for cotton, and to investigate the effects of temperature on root and soil fungal communities and their interactions with cotton-treated seeds. A bioassay under controlled conditions using soil collected from Judd Hill and Marianna, Arkansas, was conducted to evaluate the effect of two temperatures, 18 and 25°C, on seed germination. Treatments consisted of no fungicide (T1), metalaxyl (T2), penflufen (T3), and a mix of prothioconazole, myclobutanil, penflufen, metalaxyl (T4). All the treatments contained insecticide (imidacloprid). Additionally, a combination of culturing methods and metabarcoding approaches was used to assess fungal diversity. Results indicated that different fungicide seed treatments did not significantly affect germination rates at lower temperatures by seven days after planting but contributed to seedling establishment over time by reducing disease

pressure. At soil samples from Marianna, treatments 2 and 4 had significantly higher germination rates than the control, whereas no differences were observed at soil samples from Judd Hill, suggesting that *Pythium* was a greater issue at Marianna. These results suggest that at both temperatures, all active ingredients were efficient in limiting target fungi at low levels.

1. INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is the world's leading natural fiber crop (FAO, 2023), with over 4 million hectares planted in the United States alone in 2023 (USDA NASS, 2023). Each growing season, key management decisions, such as variety selection, seedling vigor, seeding rate, and planting date, are critical for optimizing stand establishment and yield. Due to annual and regional variability in environmental conditions at planting and during early growth, growers must tailor their management practices to ensure optimal plant populations and a healthy, productive crop (Holladay et al., 2024).

Weather and temperature conditions vary across cotton-growing regions and throughout the planting season. In the United States, cotton experiences various temperatures across the Cotton Belt during its growth cycle, often encountering conditions above and below optimal levels (Reddy et al., 2017). In Arkansas, cotton is usually planted between April 20 and May 20 most years, or when mid-morning soil temperatures are at least 20°C (68°F) at a planting depth for three consecutive days, and a favorable five-day forecast following planting is best (Robertson et al., 2022). Ensuring adequate soil moisture at planting is crucial, particularly when little rainfall is expected, to allow seeds to imbibe enough water for germination and emergence. Planting into soils without surface crusting is also beneficial, as crusting can hinder seedling emergence and reduce plant stands (Jones et al., 2021).

Temperature is a key factor influencing the timing and success of cotton planting, directly

impacting both plant health and crop productivity. Early planting is a common strategy among growers that can help cotton avoid late-season insect pressure and, in some cases, lead to higher lint yields (Davidonis et al., 2004; Pettigrew, 2002; Rothrock et al., 2017). However, it often exposes seedlings to low temperatures and excess rainfall, which can slow germination and increase the window of susceptibility to seedling diseases (Rothrock et al., 2017; Bradow & Bauer, 2010; Collins, 2015; Jones et al., 2021).

When temperatures are below 12-15°C, growth is significantly inhibited, resulting in slower development, delayed maturation, and an increased likelihood of chilling injuries (Reddy et al., 1991; Bange and Milroy, 2004). Early-season chilling slows biomass accumulation and, in severe cases, can prevent seedling emergence altogether (Christiansen and Thomas, 1969). Soil temperature is equally crucial, as it directly affects root system development, as root penetration and expansion in the soil are closely tied to temperature conditions (Kaspar and Bland, 1992).

Conversely, late planting reduces the risks of cold stress but may compromise fiber quality and yield due to a shortened growing season and the need for early crop termination (Rothrock et al., 2017; Bauer et al., 2000; Davidonis et al., 2004). Both soil temperature and moisture, therefore, are key factors in balancing cotton's planting window, ensuring that seedlings establish quickly in favorable conditions that support growth and limit pathogen activity (Johnson et al., 1969; Minton et al., 1982; Riley et al., 1969).

Cotton seedling diseases significantly impact stand establishment and overall cotton production worldwide (DeVay, 2001; Hillocks, 1992; Melero-Vara & Jiménez-Díaz, 1990; Ogle et al., 1993). These diseases impact germination, emergence, and early-season growth. Key pathogens associated with cotton seedling diseases include *Pythium* spp., *Rhizoctonia solani* Kühn, *Thielaviopsis basicola*, and *Fusarium* spp. (Colyer et al., 1991; Davis, 1975; DeVay, 2001; Fulton

& Bollenbacher, 1959; Johnson et al., 1978; Melero-Vara & Jiménez-Díaz, 1990; Roy & Bourland, 1972; Rude, 1984). These pathogens can cause a range of symptoms, particularly affecting the roots and hypocotyls, ultimately compromising seedling vigor and survival (DeVay, 2001; Fulton & Bollenbacher, 1959; Johnson et al., 1978; Roy & Bourland, 1972).

To mitigate seedling disease severity and improve stand establishment in cotton, fungicide seed treatments are widely applied (Kelly et al., 2018). In the U.S., cottonseed is typically treated with fungicides before distribution, highlighting both the prevalence of seedling diseases and the effectiveness of these treatments (Rothrock et al., 2007). Metalaxyl is widely used to protect cotton seeds from *Pythium* spp. (Thomson, 1991), while penflufen is specifically effective against *Rhizoctonia solani*, helping to limit infection and enhance seedling survival (Di et al., 2021). The combination treatment (metalaxyl + penflufen + prothioconazole + myclobutanil) is a standard choice among cotton growers, offering broad-spectrum protection against key soilborne pathogens, including *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., and *Thielaviopsis basicola*. Research has shown that this combination improves seedling establishment under field conditions (Kelly et al., 2023).

Effective treatment decisions should consider field history, pathogen prevalence, and environmental conditions (Kelly et al., 2019). The need for disease control measures is particularly evident under cool soil temperatures, which create favorable conditions for seedling disease development (Brown and McCarter, 1976; Colyer et al., 1991; Roncadori and McCarter, 1972). However, the benefits of fungicide seed treatments may be less pronounced in fields with low pathogen inoculum densities or where soil conditions support rapid seedling emergence and growth. While the effect of soil temperatures and emergence has been investigated in field conditions, we aim to dissect the effects of temperature on seed germination and fungal

communities under controlled conditions to better understand these interactions.

The objectives of this study were i) to evaluate the performance of seed treatments under two different temperature conditions, ii) to assess whether seed treatments enhance seedling tolerance to cold stress in an early planting production system for cotton, and iii) to investigate the effects of temperature on root and soil fungal communities and their interactions with cotton-treated seeds.

2. MATERIALS AND METHODS

2.1. Bioassay

2.1.1. Experiment Setup

The soil was collected at two research stations in Arkansas: Judd Hill in Poinsett County (Northeast Research & Extension Center) and Marianna in Lee County (Loan Mann Cotton Research Station). The soil was placed in cups (foam cups, 16 oz. or 473 mL), water saturated overnight and drained before planting. Six cotton seeds were planted in each cup. The standard fungicide seed treatments were as follows: Treatment 1 = Nontreated check, Treatment 2 = metalaxyl (1.5 fl. oz/cwt), Treatment 3 = penflufen (0.64 fl. oz/cwt), and Treatment 4 = metalaxyl (0.75 fl. oz/cwt), penflufen (0.32 fl. oz/cwt), myclobutanil (1.85 fl. oz/cwt), and prothioconazole (0.16 fl. oz/cwt). All treatments contained insecticide (imidacloprid). The cups were placed in two growth chambers, one set to 25°C and the other to 18°C, to evaluate the temperature effect on seed treatment performance under different environmental conditions. Each treatment was replicated five times within each growth chamber, and the entire experiment was repeated three times.

Plants were collected from all temperature conditions 25 days after planting. To remove loosely adhering soil particles, roots were shaken, washed with tap water, and placed on sterilized paper towels. Plants were then weighed for each temperature × location × seed treatment

combination. Following this initial measurement, roots were separated from the aboveground portion and weighed for each combination. The total roots from each cup were divided into two portions: one for DNA extraction and amplicon library preparation and the other for culturing.

2.1.2. Experimental Design and Data Analysis

The bioassay followed a Split-Plot Design. The main plots represented two temperature levels: 25°C and 18°C. Within each growth chamber, the subplots corresponded to two locations: Judd Hill and Marianna. Each subplot was further divided into sub-subplots for the four fungicide standard treatments.

The germination rate was analyzed using a linear mixed-effects model in R Studio (version 4.4.2) using the “lmer” function from the “lme4” package. The model included fixed effects for location (Judd Hill and Marianna), temperature (25°C and 18°C), treatment (four fungicide seed treatments), and day (7, 14, and 21 days after planting), as well as their interactions. To account for variability across experiments, random effects were incorporated. These included a random intercept for each experiment (1|Exp) to capture differences between experimental setups, a random intercept for each experiment within each temperature (1|Temp:Exp) to account for temperature-specific variability, and a random intercept for each experiment within each temperature and location (1|Temp:Exp:Site) to model location-specific differences within temperature-treatment interactions.

The model to analyze stand counts, and fresh weight of cotton seedlings (total and roots) 21 days after planting included fixed effects for location (Judd Hill and Marianna), temperature (25°C and 18°C), and treatment (four fungicide seed treatments), as well as their interactions. Random effects were incorporated to account for variability across experiments. These included a random intercept for each experiment (1|Exp) to capture differences between experimental setups, a

random intercept for each experiment within each temperature (1|Temp:Exp) to account for temperature-specific variability, and a random intercept for each experiment within each temperature and location (1|Temp:Exp:Site) to model location-specific differences within temperature-treatment interactions.

2.2. Culturing-Based Methods

2.2.1. Setup

Root segments, both symptomatic and asymptomatic, were cut and surface disinfected sequentially in 3% bleach for 1 minute and 70% ethanol for 1 minute, followed by a rise in sterile water for 1 minute. The root pieces were then placed on a sterilized paper towel to air-dry. Three pieces of roots, approximately 1 cm each, were plated on three different media types: Potato Dextrose Agar (PDA) with streptomycin (36.0 g/L of agar with 1 mL/L of streptomycin 75 mg/L), Corn Meal agar (CMA) with PARP (17.0 g/L of agar, 10 mg/L of benlate, 1 mL/L of pimaricin 5 mg/mL, 1 mL/L of ampicillin 250 mg/mL, 200 μ L/L of rifampicin 50 mg/mL and 10 mL/L of Pentachloronitrobenzene – PCNB - 5 mg/mL), and Water Agar (WA) with PCNB (15 mg/L of agar and 10 mL/L of Pentachloronitrobenzene – PCNB - 5 mg/mL). The plates were incubated at room temperature, and once the mycelia grew, fungal isolates were transferred to new plates to obtain pure cultures. Samples were categorized based on location, treatment, and temperature, and multiple isolates were recovered from each combination.

2.2.2. DNA Extraction from Pure Isolates

DNA was extracted from each pure isolate using the Amp DNA extraction protocol. Briefly, 20 μ L of extraction buffer (5 mL of 1M Tris, pH 9; 0.93 g KCl; 0.19 g Na₂-EDTA; 50 mL dH₂O; titrated with 1 M NaOH to pH = $\sim$ 9.5 – 10.0) was added to PCR tubes containing a small portion of mycelial tissue. Tubes were incubated at room temperature for 10 minutes, followed by 10

minutes at 95°C in a thermal cycler. After incubation, an equal volume of 3% BSA dilution solution (1.5 g BSA in 50 mL dH₂O) was added to each tube to maintain a 1:1 ratio of extraction buffer to dilution solution. The presence of DNA was confirmed using 1.0% agarose gel electrophoresis.

2.2.3. PCR Amplification of ITS Region

The ITS1 region was amplified using primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993; White et al., 1990), using the following conditions: reactions were carried out in 25 µl containing 2 µl of genomic DNA, 5x PCR Buffer, 25 mM of MgCl₂, 25 mM dNTPs, 10 µM of each primer, 100 x BSA, and 5 U/µl Taq DNA polymerase. The PCR cycling conditions were initial denaturation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes, with a final extension of 72°C for 5:30 minutes. After complete amplification, 4 µl of PCR products were run on 1% (w/v) agarose gel electrophoresis stained with SYBRTM Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) in 1x TBE buffer and visualized under UV light. A 100 bp Plus DNA ladder was used as a marker, and a reaction without a DNA template was used as the negative control.

PCR products were purified using the ExoAP cleanup protocol. To each tube containing approximately 21 µL of PCR product, 6 µL of ExoAP Master Mix (40 µL of AP [5U/µL], 20 µL of Exo I [10U/µL], and 940 µL of molecular biology-grade water) was added. Tubes were incubated in a thermal cycler at 37°C for 40 minutes, followed by 80°C for 10 minutes, and held at 8°C. For Sanger sequencing, 6 µL of cleaned PCR product was mixed with 9 µL of primer mix (6 µL of molecular biology-grade water + 3 µL of ITS1F or ITS4 at 10 µM). Each PCR product was sequenced independently with both primers (two samples per isolate). Sanger sequencing was

performed by the RTSF Genomics Core at Michigan State University.

For long-term storage, 5 mm plugs were taken from the mycelial growth of each isolate using a sterilized straw. These plugs were transferred into cryogenic vials containing 40% autoclaved glycerol and stored at -80°C.

2.3. Root Total DNA Extraction and Amplicon Library Preparation

2.3.1. Setup

The portion of roots designated for DNA extraction was placed in Ziplock bags and stored at -20 °C. When frozen, the samples were transferred to a freeze dryer (Labconco 77530-00 G FreeZone 6 Freeze Dryer System) set at -80°C until completely dry. Subsequently, the dried roots were stored in 50 mL Falcon tubes. Total DNA was extracted from 50 mg of ground root tissue using the Omega Mag-Bind Plant DNA DS 96 kit (M1130, Omega BioTek, Norcross, GA) following the manufacturer's instructions. The integrity of the extracted DNA was confirmed by running DNA extract products on a 1.0% agarose gel with 1.0. × TBE buffer. All DNA samples were stored at -20°C until subsequent processing.

For the fungal community analysis, the internal transcribed spacer (ITS) gene was amplified using the primer sets ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Gardes & Bruns, 1993; White et al., 1990). The PCR amplification was performed in three steps, with a total reaction volume of 25 µL for each step. The first step reaction mix included 7 µL of H₂O, 12.5 µL of 1 × PCR RepliQa Hifi Mix, 4.0 µL of genomic DNA, 0.75 µL of primer ITS1F (10 µM), and 0.75 µL of primer ITS2 (10 µM). After initial denaturation at 98 °C for 10 seconds, amplification was proceeded with 20 cycles of 5 seconds at 58 °C and 5 seconds at 68 °C. The second step reaction comprised 8.5 µL of H₂O, 12.5 µL of 1 × PCR RepliQa Hifi Mix, 2.5 µL of amplified products from step 1, 0.75 µL of frameshift

primer ITS1F (1-6) (10 μ M), and 0.75 μ L of frameshift primers ITS2 (10 μ M) as described in Lundberg et al. (2013). Amplification was conducted with 15 cycles of 10 seconds at 98 °C and 5 seconds at 68 °C. The PCR products from this step were purified using the ExoAP PCR cleanup protocol, where 6 μ L of ExoAP Master Mix (40 μ L of AP [5U/ μ L], 20 μ L of Exo I [10U/ μ L], and 940 μ L of Molecular Biology Grade Water) were pipetted into PCR tubes containing approximately 20 μ L of PCR product each. The tubes were incubated in a thermal cycler for 40 minutes at 37°C, 10 minutes at 80°C, and a hold at 8°C. For the third step, the mix contained 1 μ L of H₂O, 12.5 μ L of 1 $\times$ PCR RepliQa Hifi Mix, 10 μ L of purified products from Step 2, 0.75 μ L of Illumina primer (5'-AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG-3') (10 μ M), and 0.75 μ L of a unique barcode added to each sample. After an initial denaturation at 98 °C for 10 seconds, amplification was performed for 15 cycles with 5 seconds at 63 °C and 5 seconds at 68 °C. PCR products from Step 3 were run alongside those from Step 2 on a 1.5% agarose gel electrophoresis to verify barcode efficiency and were purified using the ExoAP PCR cleanup protocol, as mentioned before. Finally, 2 μ L of each sample was quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA) and the Qubit dsDNA Assay Kit (Invitrogen by Thermo Fisher Scientific) according to the manufacturer's instruction.

Based on quantification results, the DNA samples were pooled for library construction. The library was purified using magnetic beads under the manufacturer's instruction (Mag – Bind® TotalPure NGS, Omega BIO-TEK, Norcross, Georgia), and the final concentration was verified using qPCR assay (CFX Opus 96, BIO-RAD), NanoDrop (2000C, Thermo Scientific), and Qubit Flex™ Fluorometer (Invitrogen by Thermo Fisher Scientific). The libraries were sequenced using an AVITI System platform by the RTSF Genomics Core at Michigan State University.

2.3.2. Data Processing and Taxonomic Annotation

Demultiplexing was performed as if the reads were single-end by importing FASTQ files into QIIME 2 (Bolyen et al., 2019) as ‘SingleEndFastqManifestPhred33V2’ sequences, then using the demux function for demultiplexing. The function demux summarize was then utilized to generate a visualization file (.qzv) to observe the raw sequence counts for each sample. The quality of raw reads was assessed using FastQC (version 0.12.1), with results compiled into an HTML report via MultiQC (version 1.27.1). Cutadapt was utilized to trim adapters and primers from sequences and remove reads that had a low average quality score (Martin, 2011). The minimum read length after trimming was set to 50 base pairs. Sequences were then trimmed to 250 bp during the denoising step to ensure high-quality reads were used for downstream analyses. Low-quality sequences were also filtered out by setting a maximum expected error threshold of 2 (max-ee), a truncation quality score cutoff of 20 (trunc-q), and chimeras were removed. Reads were assigned taxonomy using the “feature-classifier classify-sklearn” command, with the 99% identity threshold for taxonomy assignment with the UNITE database version 9 (Abarenkov et al., 2023).

3. RESULTS

3.1. Effect of Temperature, Treatment, and Location on Cotton Germination

Cotton germination was evaluated at 7, 14, and 21 days after planting to monitor its progress over time. There were statistical differences for days after planting (p-value < 0.001), location (p-value < 0.001), temperature (p-value < 0.001), treatment (p-value < 0.001), and between the interactions day and temperature (p-value < 0.001), day and treatment (p-value = 0.001), location and treatment (p-value < 0.001), temperature and treatment (p-value = 0.043), and the three-way interactions, day, temperature and treatment (p-value = 0.032), and location, temperature and treatment (p-value = 0.015) (Table 3.1).

It was evident that cotton seeds required more time to germinate at 18°C compared to those placed at 25°C (Figure 3.1a). When observed at 7 days after planting, germination was lower than 15% at 18°C, regardless of the treatments and locations (Figure 3.1b). By 14 days after planting, germination at 18°C was above 50% for all treatments at Judd Hill. Treatments 2 (metalaxyl) and 4 (4-way mix fungicide) showed better performance, while treatment 3 (penflufen) performed worse when they were compared to the control. For soil collected from Marianna, only treatments 2 and 4 had germination rates higher than 60%, and both were statistically different from the control. In contrast, treatment 3 (penflufen) had germination rates below 40% and didn't show a different performance than the control. By 21 days after planting for soil collected from Judd Hill, germination at 18°C was above 65% for treatments 2 and 4. However, they were not significantly different from treatment 3 (penflufen) and the control. For soil collected from Marianna, germination at 18°C was above 65% for treatments 2 and 4, showing significantly higher germination rates compared to the control, while treatment 3 (penflufen) did not significantly differ from the control.

At 25°C, germination was higher than 60% regardless of the treatments used at Judd Hill by 7 days after planting. However, their effects on germination were not significantly different from the control. At Marianna, except for the control, all treatments had germination higher than 60%. Treatments 2 and 4 had significantly better germination than the control, while treatment 3 (penflufen) exhibited an intermediate effect that was not significantly different from either the control or treatments 2 and 4. By 14 days after planting, the germination rate did not change much across treatments at Judd Hill. None of the treatments showed a significant difference over the control. At Marianna, all treatments showed an increase in the germination rate compared to the control. However, only treatment 4 (4-way mix fungicide) had a significant difference from the

control, while treatment 2 (metalaxyl) exhibited an intermediate effect that was not significantly different from the control or treatment 3 (penflufen). By 21 days after planting, all treatments showed an increase in germination rate compared to the control. However, none of the treatments was significantly different from the control. In Marianna, the germination rate was higher for treatments 2 and 4, compared to the control. These treatments were relatively similar to each other and differed significantly from treatment 2 (metalaxyl) and the control.

3.2. Effect of Temperature, Treatment, and Location on Cotton Stand

At 21 days after planting, cotton seedlings were harvested and evaluated for stand count, representing the total number of surviving plants. Significant effects were observed for location (p-value = 0.0390), temperature (p-value = 0.023), and treatment (p-value < 0.001), as well as for the interactions between location and treatment (p-value < 0.001), and temperature and treatment (p-value = 0.047; Table 3.2).

At 18°C, all treatments had a higher stand than the control at Judd Hill (Figure 3.2a). However, there were no significant differences between the control and any of the treatments. At Marianna, treatments 2 (Metalaxyl) and 4 (4-way mix fungicide) showed a significantly better cotton stand compared to the control, while treatment 3 (Penflufen) did not differ from the control. Overall, none of the treatments significantly improved the cotton stand at Judd Hill at 18°C. However, at Marianna, treatments 2 (Metalaxyl) and 4 (4-way mix fungicide) seem to be important in that location when temperatures are lower.

At 25°C, similarly, all treatments had a higher stand than the control at Judd Hill, except for treatment 4 (4-way mix fungicide). However, there were no significant differences between the control and any of the treatments. At Marianna, treatments 2 (Metalaxyl) and 4 (4-way mix fungicide) showed a significantly better cotton stand compared to the control, while treatment 3

(Penflufen) had an intermediate performance that did not differ from the control or treatments 2 and 4.

3.3. Effect of Temperature, Treatment, and Location on Cotton Seedling Total Weight

At 21 days after planting, cotton seedlings were harvested and evaluated for total plant weight to assess the impact of temperature, treatment, and location on stands. Significant effects were observed for temperature (p-value = 0.006), and treatment (p-value < 0.001), as well as for the interaction between location and treatment (p-value < 0.001) (Table 3.3).

Overall, at 18°C, the total weight of cotton seedlings did not differ significantly between treatments regardless of the location (Figure 3.2b). At 25°C, plants had a better weight in all treatments, but they were not significantly different from the control at Judd Hill. However, at Marianna, treatments 2 and 4 showed significantly better effects in improving plant weights. These results suggest that the effectiveness of fungicide seed treatments on plant weight improvement is influenced by location-specific factors, possibly related to disease pressure or soil factors.

3.4. Effect of Temperature, Treatment, and Location on Cotton Seedling Root Weight

At 21 days after planting, total root weight was evaluated to assess the influence of temperature, treatment, and location on cotton seedlings. Significant effects were observed for temperature (p-value = 0.007) and treatment (p-value < 0.011), as well as for the interaction between location and treatment (p-value < 0.016) (Table 3.4).

Overall, at 18°C, root weight did not differ significantly between treatments at either location (Figure 3.2c). Similarly, at 25°C, no significant differences were observed between treatments at Judd Hill. However, at Marianna, treatment 4 (4-way mix fungicide) significantly showed better root weight compared to the control (Fig. x).

3.5. Culturing-Based

3.5.1. Fungal Families Recovered from Cotton Seedling Roots through Sanger Sequencing

Three hundred and nineteen isolates were recovered from all temperatures x locations x treatments randomly and identified using Sanger sequencing. The results showed that the top 10 families recovered from the roots across all temperatures and treatments at Judd Hill included *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, *Aspergillaceae*, *Ceratobasidiaceae*, *Hypocreaceae*, *Acrocalymmaceae*, *Glomerellaceae*, and *Bartaliniaceae* (Figure 2.3a). *Nectriaceae* (23.5% at 18°C and 30.6% at 25°C), *Mortierellaceae* (19.8% at 18°C and 23.6% at 25°C), *Ceratocystidaceae* (17.6% at 18°C and 19.4% at 25°C), *Hypocreaceae* (2.2% at 18°C and 4.2% at 25°C), and *Acrocalymmaceae* (1.1% at 18°C and 5.6% at 25°C) were observed at both temperatures, with an increase for all of them at 25°C. *Chaetomiaceae* (8.8% at 18°C and 4.2% at 25°C), *Aspergillaceae* (7.7% at 18°C and 4.2% at 25°C), and *Ceratobasidiaceae* (5.5% at 18°C and 2.8% at 25°C) were observed at both temperatures, but with higher relative abundance at 18°C. *Glomerellaceae* (3.3%) was observed only at 18°C, while *Bartaliniaceae* (2.8%) was only observed at 25°C.

At Judd Hill, 18°C, the fungal community in the control (treatment 1) was represented by dominant families *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, *Aspergillaceae*, *Ceratobasidiaceae* (Figure 2.3b). For treatment 2 (metalaxyl), the dominant families were *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, *Aspergillaceae*, *Ceratobasidiaceae*, *Hypocreaceae*. For treatment 3 (penflufen), *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, *Aspergillaceae*, *Hypocreaceae*, *Acrocalymmaceae*, and *Glomerellaceae*. For treatment 4 (4-way mix fungicide), *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, and *Aspergillaceae*. At 25°C, the fungal community in the

control (treatment 1) was represented by dominant families *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, *Ceratobasidiaceae*, and *Acrocalymmaeae*. For treatment 2 (metalaxyl), the dominant families were the same as the control, but with higher relative abundance for *Mortierellaceae* and lower for *Ceratocystidaceae*. For treatment 3 (penflufen), *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Chaetomiaceae*, and *Bartalinaceae*. For treatment 4 (4-way mix fungicide), *Nectriaceae*, *Mortierellaceae*, *Ceratocystidaceae*, *Aspergillaceae*, *Hypocreaceae*, and *Acrocalymmaeae*.

At Marianna, the top 10 families recovered from the roots across all temperatures and treatments included *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Bionectriaceae*, *Cordycipitaceae*, *Chaetomiaceae*, *Plectosphaerellaceae*, *Pleosporaceae*, and *Phaeosporiaceae* (Figure 2.3c). *Nectriaceae* (16.4% at 18°C and 26.9% at 25°C), *Aspergillaceae* (16.4% at 18°C and 25.4% at 25°C), *Mortierellaceae* (13.7% at 18°C and 26.9% at 25°C), and *Bionectriaceae* (4.1% at 18°C and 6% at 25°C) were observed at both temperatures, with an increase for all of them at 25°C. *Hypocreaceae* (8.2% at 18°C and 4.5% at 25°C) and *Pleosporaceae* (2.7% at 18°C and 1.5% at 25°C) were also observed at both temperatures, but with higher relative abundance at 18°C. *Cordycipitaceae* (6.8%), *Chaetomiaceae* (6.8%), and *Plectosphaerellaceae* (4.1%) were only observed at 18°C, while *Phaeosporiaceae* (1.5%) was only observed at 25°C.

At Marianna, 18°C, the fungal community in the control (treatment 1) was represented by dominant families *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Chaetomiaceae*, and *Plectosphaerellaceae* (Figure 2.3d). For treatment 2 (metalaxyl), the dominant families were *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Bionectriaceae*, *Cordycipitaceae*, *Chaetomiaceae*, *Plectosphaerellaceae*, and *Pleosporaceae*. For treatment 3 (penflufen), only

Mortierellaceae and *Cordycipitaceae* were recovered. For treatment 4 (4-way mix fungicide), *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Bionectriaceae*, *Cordycipitaceae*, *Chaetomiaceae*, and *Pleosporaceae*. At 25°C, the fungal community in the control (treatment 1) was represented by dominant families *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, and *Bionectriaceae*. For treatment 2 (metalaxyl), the dominant families were *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, and *Hypocreaceae*. For treatment 3 (penflufen), *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Bionectriaceae*, *Pleosporaceae*, and *Phaeosphariaceae*. For treatment 4 (4-way mix fungicide), *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, *Hypocreaceae*, *Bionectriaceae*, *Cordycipitaceae*, *Chaetomiaceae*, and *Bionectriaceae*.

Overall, fungal communities isolated from cotton seedling roots varied across Judd Hill and Marianna, with differences in the dominant families recovered between temperatures. *Nectriaceae* and *Mortierellaceae* were two of the most prevalent families at both locations and showed increases in relative abundance at 25°C, suggesting that these fungi may thrive under warmer temperatures. Families such as *Chaetomiaceae*, *Aspergillaceae*, and *Ceratobasidiaceae* at Judd Hill and *Hypocreaceae* and *Pleosporaceae* at Marianna were more abundant at 18°C, indicating a possible preference for lower temperatures. Additionally, site-specific differences were observed, with *Glomerellaceae* and *Bartaliniaceae* only found at Judd Hill and, *Cordycipitaceae*, *Plectosphaerellaceae*, and *Phaeosphariaceae* only recovered at Marianna.

3.5.2. Effects of Fungicide Seed Treatments on Targeted Cotton Seedling Disease Pathogens

3.5.2.1. Fungal Families

In this study, we tracked key fungal pathogen families associated with cotton seedling diseases, including *Nectriaceae*, *Ceratocystidaceae*, and *Ceratobasidiaceae* (Figure 2.4). At Judd

Hill, *Nectriaceae* was recovered from all treatments at both temperatures, with a higher relative abundance at 25°C than at 18°C (Figure 2.4a). Treatment 2 (Metalaxyl; 34.8%) had a higher relative abundance, while treatment 4 (4-way mix fungicide; 11.1%) had a lower compared to the control (25%) at 18°C. Treatment 3 (Penflufen; 60%) had a higher relative abundance, while treatment 4 (4-way mix fungicide; 7.1%) had a lower compared to the control at 25°C. *Ceratocystidaceae* was also recovered from all treatments at both temperatures, with a higher relative abundance at 25°C than at 18°C. All treatments had higher relative abundance than the control at 18°C (8.3%), especially treatment 4 (4-way mix fungicide; 38.9%), while at 25°C all treatments (14.3%, 15%, and 14.3%, respectively) had a lower relative abundance than the control (35.3%). *Ceratobasidiaceae* was recovered from both temperatures, with a higher relative abundance at 18°C than at 25°C. At 18°C, this family was recovered only for treatment 2 (Metalaxyl; 13%), and when compared to the control (8.3%) had a higher relative abundance. At 25°C, was also recovered only from treatment 2 (Metalaxyl; 4.8%), with a slight decrease compared to the control (5.9%).

At Marianna, *Nectriaceae* was recovered from both temperatures, with a higher relative abundance at 25°C than at 18°C (Figure 2.4c). Except for treatment 3 (Penflufen), which was absent from this family, treatments 2 (29%) and 4 (10.5%) had a higher relative abundance compared to the control (6.7%) at 18°C. At 25°C, treatment 2 (Metalaxyl; 56.2%) had the highest relative abundance, while treatments 3 (10%) and 4 (26.3%) had the lowest compared to the control (16.7%). *Ceratocystidaceae* was only recovered from treatment 3 (Penflufen; 12.5%) at 18°C. *Ceratobasidiaceae* was only recovered from treatment 2 (Metalaxyl; 6.2%) at 25°C.

3.5.2.2. Plant Pathogenic Fungal Genera

At Judd Hill, *Fusarium* was recovered from all treatments at both temperatures, with a

higher relative abundance at 25°C than at 18°C (Figure 2.4b). Treatment 2 (Metalaxyl; 30.4%) had a higher relative abundance, while treatment 3 (Penflufen; 7.7%) had a lower compared to the control (8.3%) at 18°C. Treatment 3 (Penflufen; 60%) had a higher relative abundance, while treatment 4 (4-way mix fungicide; 7.1%) had a lower compared to the control (23.5%) at 25°C. *Berkeleyomyces* was also recovered from all treatments at both temperatures, with a higher relative abundance at 25°C than at 18°C. All treatments had higher relative abundance than the control at 18°C (8.3%), particularly treatment 4 (4-way mix fungicide; 38.9%), while at 25°C, all treatments (14.3%, 15%, and 14.3%, respectively) had a lower relative abundance than the control (35.3%). *Rhizoctonia* was not found in any of the treatments at 18°C, only at 25°C in the control (5.9%).

At Marianna, *Fusarium* was recovered from both temperatures, with a higher relative abundance at 25°C than at 18°C (Figure 2.4d). Except in treatment 3 (Penflufen), which was absent from this genus, all treatments had higher relative abundance than the control (6.7%) at 18°C. At 25°C, all treatments also had higher relative abundance than the control (8.3%), especially treatment 2 (Metalaxyl; 56.2%). *Berkeleyomyces* was only recovered from treatment 3 (Penflufen; 12.5%), but it was not present in any other treatment or temperature. *Rhizoctonia* was not recovered from any of the treatments at 18°C or at 25°C.

3.5.3 Amplicon Approach Based AVITI Sequencing

3.5.3.1. Overall Fungal Families Recovered from Cotton Seedling Roots

In this study, we used the AVITI System to sequence cotton seedling roots that were collected 21 days after planting to evaluate the performance of different active ingredients on root-associated communities. After quality filtering, a total of 7,325,918 high-quality sequences were obtained across 239 samples, with 834 unique features identified. The number of sequences per

sample ranged from 1 to 262,385, with a median of 19,020 and a mean of 30,652.4. The first and third quartiles of sequence distribution were 1,198 and 42,807, respectively.

The results showed that the top 10 families recovered from the roots across all temperatures and treatments at Judd Hill included *Ceratobasidiaceae*, *Aspergillaceae*, *Ceratocystidaceae*, *Plectosphaerellaceae*, *Nectriaceae*, *Chaetomiaceae*, *Mortierellaceae*, *Phaffomycetaceae*, *Cladosporiaceae*, and *Pleosporaceae* (Figure 2.5a).

At Judd Hill, at 18°C, *Ceratobasidiaceae* was recovered from all treatments, with the highest relative abundance in treatment 2 (Metalaxyl; 27.4%) and the lowest in treatment 3 (Penflufen; 10%) compared to the control (22.5%) (Figure 2.5b). *Aspergillaceae* had an increase in the relative abundance in all treatments, particularly in treatment 4 (4-way mix fungicide; 35.2%) compared to the control (7%). *Ceratocystidaceae* was most abundant in treatment 3 (Penflufen; 20%), while it decreased in treatment 4 (4-way mix fungicide) compared to the control (8.3%). *Plectosphaerellaceae* had a higher relative abundance in treatment 3 (Penflufen; 16.4%) and lower in treatment 2 (Metalaxyl; 3.6%) compared to the control (9.1%). *Nectriaceae* showed an overall decline across treatments, especially in treatment 3 (Penflufen; 1.1%) compared to the control (19.9%). *Chaetomiaceae* had the highest relative abundance in treatment 2 (Metalaxyl; 10.5%) compared to the control (1.1%), while it was absent in treatment 4 (4-way mix fungicide). *Mortierellaceae* was more abundant in treatment 3 (Penflufen) but decreased in treatments 2 (0.5%) and 4 (0.3%) compared to the control (8.9%). *Phaffomycetaceae* was recovered only in treatment 3 (Penflufen; 6.7%) and in the control (7.1%). *Cladosporiaceae* had a higher relative abundance in all treatments, especially in treatment 3 (Penflufen; 7.4%) compared to the control (0.1%). *Pleosporaceae* was observed in treatments 2 (0.1%), 3 (0.9%), and 4 (14.4%) but was absent in the control.

At 25°C, *Ceratobasidiaceae* was present in all treatments, with the highest relative abundance in treatment 2 (Metalaxyl; 35.4%) and the lowest in treatment 3 (Penflufen; 6.7%), compared to the control (34.4%). *Aspergillaceae* increased in treatment 3 (Penflufen; 28.4%) but decreased in treatment 4 (4-way mix fungicide; 14.3%) compared to the control (21.4%). *Ceratocystidaceae* had the highest relative abundance in treatment 3 (Penflufen; 36%) compared to the control (10.6%). *Plectosphaerellaceae* increased in treatment 4 (4-way mix fungicide; 17%) but decreased in treatment 3 (Penflufen; 5.6%) compared to the control (7.3%). *Nectriaceae* decreased in all treatments, especially in treatment 4 (4-way mix fungicide; 4.5%) compared to the control (11%). *Chaetomiaceae* showed a decline across all treatments, particularly in treatment 3 (Penflufen; 0.9%) compared to the control (4.9%). *Mortierellaceae* was observed in all treatments but at much lower levels than in the control (0.9%). *Phaffomycetaceae* was not observed at this temperature. *Cladosporiaceae* was found in all treatments, except treatment 3 (Penflufen), but at very low relative abundance. *Pleosporaceae* was also found in treatments but at minimal levels.

The top 10 families recovered from the roots across all temperatures and treatments included at Marianna included *Nectriaceae*, *Glomeraceae*, *Plectosphaerellaceae*, *Ceratobasidiaceae*, *Chaetomiaceae*, *Olpidiaceae*, *Mortierellaceae*, *Aspergillaceae*, *Phaeosphaeriaceae*, and *Lasiosphaeriaceae* (Figure 2.5c).

At Marianna, at 18°C, *Nectriaceae* was found in all treatments, with the highest relative abundance in treatment 2 (Metalaxyl; 25.7%) and lowest in treatment 3 (Penflufen; 1.5%) compared to the control (19.4%) (Figure 2.5d). *Glomeraceae* was found only in treatments 2 (6.8%) and 4 (2.6%) but not in the control. *Plectosphaerellaceae* decreased in all treatments, especially in treatments 2 (3.3%) and 3 (4.7%) compared to the control (28.4%). *Ceratobasidiaceae* was found in all treatments, with the highest relative abundance in treatment 4

(4-way mix fungicide; 12.5%) and lower in treatments 2 (0.4%) and 4 (0.1%) compared to the control (10.9%). *Chaetomiaceae* increased in treatment 3 (Penflufen; 12.5%), but decreased in treatment 2 (Metalaxyl; 4%) compared to the control (5.8%). *Olpidiaceae* had a higher relative abundance in treatment 4 (4-way mix fungicide; 8%) but decreased in treatment 2 (Metalaxyl; 2%) compared to the control (6%). Except for treatment 3 (Penflufen), *Mortierellaceae* was found in all treatments, with the highest relative abundance in treatment 4 (4-way mix fungicide; 17.3%) compared to the control (1.4%). *Aspergillaceae* had a high relative abundance in all treatments (10.7%, 9.8%, and 9.8%, respectively) compared to the control (2.2%). *Phaeosphariaceae* had a high relative abundance in all treatments, especially in treatment 2 (Metalaxyl; 7.6%), but was not observed in treatment 3 (Penflufen). *Lasiosphaeriaceae* was present only in treatments 3 (12.9%) and 4 (2.6%), but not in the control.

At 25°C, *Nectriaceae* was found in all treatments, with the highest abundance in treatment 3 (Penflufen; 17.1%) and the lowest in treatment 2 (Metalaxyl; 5.3%) compared to the control (16.4%). *Glomeraceae* increased in treatment 2 (Metalaxyl; 25.5%) but decreased in treatment 3 (Penflufen; 14.9%) compared to the control (18.2%). *Plectosphaerellaceae* increased in treatments 3 (10.8%) and 4 (10.9%), but decreased in treatment 2 (Metalaxyl; 1.6%) compared to the control (5.7%). *Ceratobasidiaceae* was found in all treatments, but at lower levels in all treatments (19.5%, 7.8%, and 1.8%, respectively) compared to the control (20%). *Chaetomiaceae* had an increased relative abundance in all treatments (4.9%, 9.3%, and 11.4%, respectively) compared to the control (2.4%). *Olpidiaceae* was more abundant in all treatments (13%, 11.2%, and 2.3%, respectively) compared to the control (1.6%). *Mortierellaceae* was found in all treatments (5.8%, 2.8%, and 8.3%, respectively) with a high relative abundance compared to the control (2.2%). *Aspergillaceae* was not observed in any of the treatments. *Phaeosphariaceae* was higher in treatment 2

(Metalaxyl; 8.3%) but lower in treatment 3 (Penflufen; 1.6%) compared to the control (0.2%). Except for treatment 4 (4-way mix fungicide; 5.7%), *Lasiosphaeriaceae* had a very low relative abundance across all treatments, including the control (0.2%).

Overall, these results indicate that fungicide seed treatments influence the fungal community composition associated with cotton seedling roots, with differential effects based on temperature and location. Certain fungal families, such as *Ceratocystidaceae* and *Aspergillaceae*, showed treatment-dependent increases, while *Nectriaceae* and *Plectosphaerellaceae* exhibited reductions across treatments. Treatments 2 (metalaxyl) and 4 (prothioconazole + myclobutanil + penflufen + metalaxyl) were particularly associated with shifts in fungal relative abundance, indicating their impact on root-associated communities. The temperature-dependent variations suggest that environmental conditions play a key role in determining fungal persistence and detection.

3.5.3.2. Effect of Fungicide Seed Treatments on Targeted Fungal Family Pathogens Causing Cotton Seedling Disease (AVIT)

In this study, we also tracked the performance of different active ingredients on families that are associated with cotton seedling disease complex, including *Nectriaceae* (*Fusarium* spp.), *Ceratocystidaceae* (*Berkeleyomyces basicola*), and *Ceratobasidiaceae* (*Rhizoctonia solani*) (Figure 2.6). The number of sequences per sample focusing on these three families ranged from 1 to 262,385, with a median of 19,020 and a mean of 30,652.4. The first and third quartiles of sequence distribution were 1,198 and 42,807, respectively.

At Judd Hill, *Nectriaceae* was found in all treatments at both temperatures, with a higher relative abundance at 18°C than at 25°C (Figure 2.6a). At 18°C, all treatments (18%, 1.1%, and 8.1%, respectively) had a lower relative abundance than the control (19.9%). Similarly, at 25°C,

all treatments (7.3%, 7.3%, and 4.5%, respectively) had a lower relative abundance than the control (11%). *Ceratocystidaceae* was also found in all treatments at both temperatures, with a higher relative abundance at 25°C than at 18°C. At 18°C, treatment 3 (Penflufen) had the highest relative abundance (20%), while treatment 4 (4-way mix fungicide) had the lowest (4.1%) compared to the control (8.3%). At 25°C, all treatments had a higher relative abundance (13.4%, 36%, and 13.5%, respectively) compared to the control (10.6%). *Ceratobasidiaceae* was present in all treatments and temperatures, with higher relative abundance at 25°C than at 18°C. Treatment 2 (Metalaxyl; 27.4%) had a higher relative abundance, while treatment 3 (Penflufen; 10%) had a lower when compared to the control (22.5%) at 18°C. Treatment 2 (Metalaxyl; 35.4%) showed a slight increase, whereas treatment 3 (Penflufen; 6.7%) had a lower relative abundance than the control (34.4%) at 25°C.

At Marianna, *Nectriaceae* was found in all treatments and temperatures, with a higher relative abundance at 18°C than at 25°C (Figure 2.6c). At 18°C, treatment 2 (Metalaxyl; 25.7%) had the highest relative abundance, while treatment 3 (Penflufen; 1.5%) had the lowest compared to the control (19.4%). At 25°C, treatment 3 (Penflufen; 17.1%) showed a slight increase, while treatment 2 (Metalaxyl; 5.3%) had the lowest relative abundance compared to the control (16.4%). *Ceratocystidaceae* was not found at any temperature or treatment. *Ceratobasidiaceae* was present in all treatments and temperatures, with higher relative abundance at 25°C than at 18°C. Treatment 3 (Penflufen; 12.5%) had a higher relative abundance, while treatments 2 (0.4%) and 4 (0.1%) had lower relative abundances compared to the control (10.9%). At 25°C, all treatments (19.5%, 7.8%, and 1.8%) had a lower relative abundance than the control (20%).

3.5.3.3. Effect of Fungicide Seed Treatments on Targeted Fungal Genera Pathogens Causing Cotton Seedling Disease (AVITD)

At Judd Hill, *Fusarium* was found at both temperatures, with a slightly higher relative abundance at 18°C than at 25°C (Figure 2.6b). At 18°C, except for treatment 4 (4-way mix fungicide), all treatments (0.8% and 0.4%, respectively) had a lower relative abundance than the control (6.4%). At 25°C, all treatments (0.6%, 2.3%, and 0.8%, respectively) had higher relative abundance than the control (0.4%). *Berkeleyomyces* was found in all treatments at both temperatures, with a higher relative abundance at 25°C than at 18°C. At 18°C, treatment 3 (Penflufen) had the highest relative abundance (20%), while treatment 4 (4-way mix fungicide) had the lowest (4.1%) compared to the control (8.3%). At 25°C, all treatments had higher relative abundance (13.4%, 36%, and 13.5%, respectively) compared to the control (10.6%), particularly treatment 3 (Penflufen). *Rhizoctonia* was present in all temperatures, with higher relative abundance at 25°C than at 18°C. At 18°C, all treatments had lower relative abundance than the control (13.6%), and was not found in treatment 3 (Penflufen). Similarly, at 25°C, all treatments had lower relative abundance than the control (19%), especially treatment 3 (Penflufen).

At Marianna, *Fusarium* was found in all temperatures, with a higher relative abundance at 25°C than at 18°C (Figure 2.6d). At 18°C, treatment 2 (Metalaxyl; 9%) had the highest relative abundance, while treatments 3 (0%) and 4 (0.9%) had the lowest compared to the control (1%). At 25°C, treatment 3 (Penflufen; 10.4%) had the highest relative abundance, while treatment 2 (Metalaxyl; 1.7%) had the lowest compared to the control (3.7%). *Berkeleyomyces* was not found at any temperature or treatment. *Rhizoctonia* was present in all temperatures, with higher relative abundance at 25°C than at 18°C. All treatments had lower relative abundance at 18°C (0.4%, 0%, and 0.1%, respectively) compared to the control (9.1%). At 25°C, all treatments (4.4%, 5.6%, and

1.3%, respectively) had a lower relative abundance than the control (14.6%).

4. DISCUSSION

For many years, fungicide seed treatments, whether as single formulations or combinations of multiple active ingredients, have been used to manage a diverse array of seedling disease pathogens (Rothrock et al., 2012; Davis et al., 1997; Kaufman et al., 1998; Wheeler et al., 1997). The effectiveness of these treatments is typically assessed by evaluating seedling emergence as an indicator of plant vigor (Munkvold & O'Mara, 2002). The speed of seed emergence and early seedling growth have long been accepted as key parameters for monitoring growth responses (Briggs & Dunn, 2000). However, the efficacy of seed treatment fungicides is not solely determined by the active ingredients; environmental factors, particularly temperature, also play a critical role. Our study evaluated the impact of temperature (18°C and 25°C) on cotton germination under four fungicide seed treatments (Treatment 1 = nontreated check, Treatment 2 = metalaxyl, Treatment 3 = penflufen, and Treatment 4 = prothioconazole + myclobutanil + penflufen + metalaxyl) across two locations (Judd Hill and Marianna).

Temperature significantly influenced cotton germination over time (days after planting), where seeds placed at 18°C took longer to germinate than those at 25°C. The temperature thresholds for cotton growth and development range from a minimum of 12–15°C to an optimal range of 20–30°C (Reddy et al., 1999; Singh et al., 2018; Li et al., 2019). Although 18°C is above the minimum threshold for germination, it still significantly delayed the process. These findings align with previous research highlighting the critical role of temperature in early cotton establishment (Ashraf, 2002; Rajjou et al., 2012).

By 7 days after planting, germination remained slow at 18°C, whereas seeds exposed to 25°C germinated more quickly. At 18°C, germination rates were consistently low across all treatments,

regardless of location, with no significant differences observed between treatments. In contrast, at 25°C, germination rates were generally higher across treatments, particularly at Marianna, where all treatments (except the control) showed better germination compared to 18°C. These findings suggest that, even with fungicide treatments, seed germination remained slow at the lower temperature and required more time compared to seeds exposed to the higher temperature.

By 14 days after planting, germination rates began to increase at 18°C, but none of the treatments showed a significant difference from the control at Judd Hill. However, at Marianna, treatments 2 (metalaxyl) and 4 (prothioconazole + myclobutanil + penflufen + metalaxyl) significantly performed better than the control, suggesting that these treatments may be particularly necessary to promote germination at this location. Similar trends were observed at 25°C, reinforcing the importance of these treatments at Marianna. By 21 days after planting, germination at 18°C was above 65% for treatments 2 and 4, but there were no significant differences between the treatments and the control. At Marianna, treatments 2 and 4 continued to show significantly higher germination rates compared to the control.

The significant interaction between location and seed treatment observed in our study indicates that environmental conditions played a critical role in determining when fungicide seed treatments resulted in better stand counts than untreated seeds. Overall, none of the treatments significantly improved cotton stands at Judd Hill, likely due to low disease pressure at this location, which may have minimized the effect of the fungicides, either positive or negative. However, at Marianna, treatments 2 and 4 significantly seem to be important in that location to improve cotton stands, regardless of temperature. This suggests that treatment 2 (metalaxyl) and the combination of active ingredients in treatment 4 (4-way mix fungicide) contributed significantly to better performance at Marianna, potentially due to their ability to manage different pathogens.

The effect of fungicide seed treatments on plant and root weight was also influenced by the interaction between location and treatment. No significant differences in total plant or root weight were observed between treatments at either location, regardless of temperature. At 25°C, plant and root weights improved overall, but no significant differences were detected at Judd Hill. However, at Marianna, treatments 2 and 4 significantly increased plant weight, and treatment 4 (4-way mix fungicide) also improved root weight compared to the control. These findings suggest that location-specific factors, such as disease pressure or environmental conditions, may influence the effectiveness of fungicide seed treatments in promoting plant growth.

To better understand location-specific factors, it is essential to identify the fungi present in cotton seedlings at each location. In this study, we used a combination of culturing methods and metabarcoding approaches to assess fungal diversity. Culturing allowed for the isolation and identification of dominant fungi, while metabarcoding provided a broader view of fungal community composition.

Fungal communities differed between soil from Judd Hill and Marianna, with variations in dominant families detected by each method. At Judd Hill, metabarcoding confirmed the presence of *Ceratobasidiaceae*, *Aspergillaceae*, *Ceratocystidaceae*, *Nectriaceae*, *Chaetomiaceae*, and *Mortierellaceae*, aligning with culturing results. However, families such as *Plectosphaerellaceae*, *Phaffomycetaceae*, *Cladosporiaceae*, and *Pleosporaceae* were only detected through metabarcoding, suggesting these taxa may be difficult to culture under standard conditions. Conversely, *Hypocreaceae*, *Acrocalymnaceae*, *Glomerellaceae*, and *Bartaliniaceae* were recovered through culturing but not detected by metabarcoding, possibly due to sequencing limitations.

At Marianna, metabarcoding confirmed the presence of *Nectriaceae*, *Plectosphaerellaceae*,

Chaetomiaceae, *Mortierellaceae*, *Aspergillaceae*, and *Phaeosphaeriaceae*. However, *Glomeraceae*, *Ceratobasidiaceae*, *Olpidiaceae*, and *Lasiosphaeriaceae* were only detected through metabarcoding, whereas *Hypocreaceae*, *Bionectriaceae*, and *Pleosporaceae* were identified by culturing but absent from the metabarcoding results. These discrepancies highlight the complementary nature of both methods, with culturing potentially favoring fast-growing or easily culturable species, while metabarcoding provides insights into the broader fungal community, including taxa that may be challenging to isolate.

Fungal species within the family *Mortierellaceae* are known to contribute to crop protection and play a role in reducing soil contamination caused by chemical fertilizers and pesticides (Ozimek and Hanaka, 2021). The family *Chaetomiaceae* is known to be plant beneficial. Various *Chaetomium* spp. are identified as biocontrol agents of many pathogenic fungi, such as *Fusarium*, *Helminthosporium*, and *Alternaria*, and oomycetes, such as *Pythium* and *Phytophthora* (Dhingra et al., 2003; Aggarwall et al., 2004; Tomilova and Shternshis, 2006; Phong et al., 2016).

It is known that exposure to cool and moist conditions can increase the likelihood of seedling infections by soil-borne pathogens. Low soil temperatures slow seed germination and seedling emergence, thereby prolonging the window of susceptibility to infection. Many soil-borne pathogens thrive and remain active in cooler temperatures. Previous studies have shown that pathogen aggressiveness can be temperature-dependent. For instance, Matthiesen et al. (2016) demonstrated that certain *Pythium* species vary in aggressiveness depending on temperature, with *P. sylvaticum* being found to be more aggressive at higher temperatures (18 and 23°C), while *P. torulosum* at lower temperatures (13°C). Similarly, Wei et al. (2010) found that while the aggressiveness of eight *Pythium* species recovered from soybeans in Ontario and Quebec fluctuated across a range of temperatures (4°C, 12°C, 20°C, and 28°C), *Pythium ultimum* remained

consistently aggressive at all four tested temperatures. In this study, we aimed to evaluate the efficacy of different active ingredients under different temperatures against targeted fungi using culturing approach and metabarcoding to assess their presence in plant root samples after fungicide seed treatments. The choice of seed treatment fungicide notably impacted the incidence and proportion of targeted fungi in cotton communities.

The discrepancies between these methods likely stem from biases in Sanger sequencing, where dominant taxa overshadowed lower-abundance species. Additionally, the random selection of isolates for Sanger sequencing, along with challenges in antibiotic effectiveness, led to the unintended recovery of non-target organisms. In contrast, AVITI sequencing's ability to detect a broader range of fungal taxa underscores the advantages of metabarcoding as a more reliable approach for capturing community-level shifts following fungicide application. We also acknowledge that our primer selection may have limited our ability to identify fungi at the species complex level.

The higher relative abundance of *Fusarium* in treatment 2 (Metalaxyl) at lower temperatures at Marianna, compared to the control, suggests that the suppression of *Pythium* by metalaxyl may have facilitated *Fusarium* colonization. This aligns with previous research indicating that fungicide treatments induced shifts in microbial communities, allowing other fungal taxa to flourish (Lane et al., 2023). However, it is believed that most of those *Fusarium* isolates were not considered to be pathogenic since their presence did not appear to impact cotton stands. At Judd Hill, the slightly higher relative abundance of *Berkeleyomyces* in treatment 2 (Metalaxyl) at 18°C, compared to the control, suggests that the suppression of *Pythium* by metalaxyl may have facilitated *Berkeleyomyces* colonization.

Treatment 3 included penflufen, an SDHI (succinate dehydrogenase inhibitor) fungicide

widely used to control seed and soil-borne diseases caused by *Rhizoctonia solani*. AVITI sequencing detected *Rhizoctonia* at both temperatures, with higher abundance at 25°C than at 18°C, suggesting that higher temperatures may influence its persistence. Previous studies indicated that indicated that *R. solani* thrives within an optimal temperature range of 20-30°C (Baker and Martinson, 1970; Bolton et al., 2010; Kirk et al., 2008; Windels et al., 2009). Overall, at both locations, treatment 3 (Penflufen) consistently showed a lower presence of *Rhizoctonia* across both temperatures, especially at 18°C. The increased abundance at 25°C suggests that once temperatures rise, *Rhizoctonia* is more effective, but its relative abundance remained lower than the control. These results indicate that penflufen may be particularly effective in limiting *Rhizoctonia* growth, even in higher temperatures. The recommendation to plant early, when soil temperatures are lower, for managing losses from *Rhizoctonia* damping-off assumes that *R. solani* is less active under cool conditions (Harveson, 2008a; Leach, 1986; Leach and Garber, 1970; Windels and Brantner, 2005). Bolton et al. (2010) found that *R. solani* AG 2-2 did not cause disease in sugar beets when maintained at 14.4°C during the day and 8.9°C at night in a controlled growth chamber. However, disease symptoms appeared when temperatures were slightly increased to 15.6°C during the day and 10°C at night. Additionally, temperature also played a critical role in shaping fungal community responses. At 25°C in Judd Hill, *Berkeleyomyces* exhibited an increase (36%) compared to the control (10.6%), while *Fusarium* also increased (2.3% vs. 0.4%). Meanwhile, *Rhizoctonia* remained suppressed (5.6% vs. 19% in the control). At Marianna (25°C), *Fusarium* was enriched in treatment 3 (Penflufen; 10.4% vs. 3.7% in the control), while *Rhizoctonia* remained suppressed (5.6% vs. 14.8% in the control). These findings suggest that penflufen selectively alters fungal community structure, potentially reducing *Rhizoctonia* dominance and allowing other genera, such as *Berkeleyomyces* and *Fusarium*, to thrive.

Treatment 4 consisted of a broad-spectrum mix of metalaxyl, penflufen, myclobutanil, and prothioconazole, targeting multiple pathogens including *Pythium* spp., *Rhizoctonia solani*, *Berkeleyomyces*, and *Fusarium* spp. Myclobutanil is a systemic fungicide labeled on cotton as seed treatment with some efficacy against *Thielaviopsis basicola* (recently reclassified as *Berkeleyomyces* spp.; family: Ceratocystidaceae) (Arthur, 1996; Butler et al., 1996; Kaufman et al., 1998). Previous studies have demonstrated that in soils artificially infested with *Berkeleyomyces*, myclobutanil significantly reduced root necrosis, even at high inoculum levels (300 to 400 CFU/g soil) (Butler et al., 1996). At Judd Hill, at 18°C, this treatment showed to numerically improve cotton stands compared to the control. Metabarcoding results detected a decrease in all treatments compared to the control. However, at 25°C, this treatment was numerically lower than the control, resulting in lower stands. Metabarcoding results detected an increase in *Berkeleyomyces* and *Fusarium* relative abundances compared to the controls. These results suggest that all active ingredients were efficient in limiting target fungi at low levels at 18°C, but whenever the temperature was higher, it was favorable for *Berkeleyomyces* and *Fusarium* to thrive, while *Rhizoctonia* remained lower than the control. We hypothesized that an increase in *Berkeleyomyces* abundance may be related to lower stands at Judd Hill. At Marianna, at 18°C, this treatment was shown to significantly improve cotton stands compared to the control and treatment 3 (penflufen). Metabarcoding results detected a decrease in all treatments compared to the control. At 25°C, this treatment was significantly better than the control, resulting in lower stands. Metabarcoding results detected an increase for *Fusarium* and *Rhizoctonia*, but none of them were higher than the controls. These results suggest that all active ingredients were again efficient in limiting target fungi at low levels at both temperatures, with a slight increase when the temperature was higher.

Cotton is a vital global source of natural textile fibers, but it is highly susceptible to seedling diseases that threaten stand establishment and cotton production worldwide. These diseases can significantly reduce both yield and fiber quality, making effective management crucial for sustainable cotton production. Fungicide seed treatments are widely used to reduce disease severity and improve cotton stand establishment. However, for an effective treatment, factors such as field history, pathogen prevalence, and environmental conditions should be taken into consideration. Cotton is grown across diverse regions within the Cotton Belt, where temperatures fluctuate throughout the growing season, often deviating from optimal conditions, consequently influencing disease development and treatment efficacy. Notably, the need for disease control measures is particularly more evident under cool soil temperatures because it creates favorable conditions for seedling disease development. In this study, we combined growth chamber experiments with culturing methods and metabarcoding analyses to investigate the effects of temperature on root and soil fungal communities and their interactions with cotton-treated seeds. None of the fungicide seed treatments tested were able to enhance cotton germination at a lower temperature. However, over time, they appeared to manage cotton seedlings establishment. Metalaxyl and the combination of metalaxyl + penflufen + prothioconazole + mycobutanil showed significantly better performance, especially at Marianna, regardless of the temperature, whereas no significant difference was observed between treatments at Judd Hill. Penflufen was used to target *Rhizoctonia*, and it exhibited good performance. However, an increase in *Berkeleyomyces* was observed when this active ingredient was used alone or in combination with others, underscoring the need for further research on its effects. Future research should focus on understanding these fungal shifts and optimizing seed treatments for different environmental conditions to improve cotton production.

TABLES AND FIGURES

Table 3.1. Mixed model main effects and interaction of temperature (temp), soil original location (location), and treatment (Treat) factors on the germination of cotton seeds under controlled conditions.

	F	Df	Df.res	Pr(>F)
Day	206.00	2	616	< 0.001 ***
Location	18.16	1	616	< 0.001 ***
Temperature	610.78	1	616	< 0.001 ***
Treatment	16.99	3	42	< 0.001 ***
Day : Location	0.67	2	616	0.512
Day : Temperature	131.83	2	616	< 0.001 ***
Site : Temperature	0.06	1	616	0.811
Day : Treatment	3.76	6	616	0.001 **
Site : Treatment	21.74	3	616	< 0.001 ***
Temperature : Treatment	2.72	3	616	0.044 *
Day : Location : Temperature	1.79	2	616	0.167
Day : Location : Treatment	1.58	6	616	0.151
Day : Temperature : Treatment	2.31	6	616	0.033 *
Location : Temperature : Treatment	3.49	3	616	0.015 **
Day : Location : Temperature : Treatment	0.95	6	616	0.460

* indicates significance at the $P < 0.05$ level.

** indicates significance at the $P < 0.01$ level.

*** indicates significance at the $P < 0.001$ level.

Table 3.2. Mixed model main effects and interaction of soil original location, temperature, and treatment factors for cotton stand.

	F	Df	Pr(>F)
Location	9.14	1	0.0390*
Temperature	41.19	1	0.0234*
Treatment	18.57	3	<0.001***
Location : Temperature	0.63	1	0.47
Location : Treatment	8.18	3	<0.001***
Temperature : Treatment	2.69	3	0.0473*
Location : Temperature : Treatment	0.30	3	0.823

* indicates significance at the $P < 0.05$ level.

*** indicates significance at the $P < 0.001$ level.

Table 3.3. Mixed model main effects and interaction of soil original location, temperature, and treatment factors for total weight of cotton plants.

	F	Df	Df.res	Pr(>F)
Location	0.004	1	4	0.952
Temperature	163.62	1	2.002	0.006 **
Treatment	7.90	3	216	< 0.001 ***
Location : Temperature	2.62	1	4	0.18
Location : Treatment	7.59	3	216	< 0.001 ***
Temperature : Treatment	0.05	3	216	0.985
Location : Temperature : Treatment	1.91	3	216	1.288

** indicates significance at the $P < 0.01$ level.

*** indicates significance at the $P < 0.001$ level.

Table 3.4. Mixed model main effects and interaction of soil original location, temperature, and treatment factors for root weight of cotton plants.

	F	Df	Df.res	Pr(>F)
Location	0.13	1	4	0.738
Temperature	142.00	1	2	0.007 **
Treatment	3.78	3	216	0.011 **
Location : Temperature	1.43	1	4	0.30
Location : Treatment	3.50	3	216	0.016 **
Temperature : Treatment	0.44	3	216	0.728
Location : Temperature : Treatment	1.85	3	216	0.139

** indicates significance at the $P < 0.01$ level.

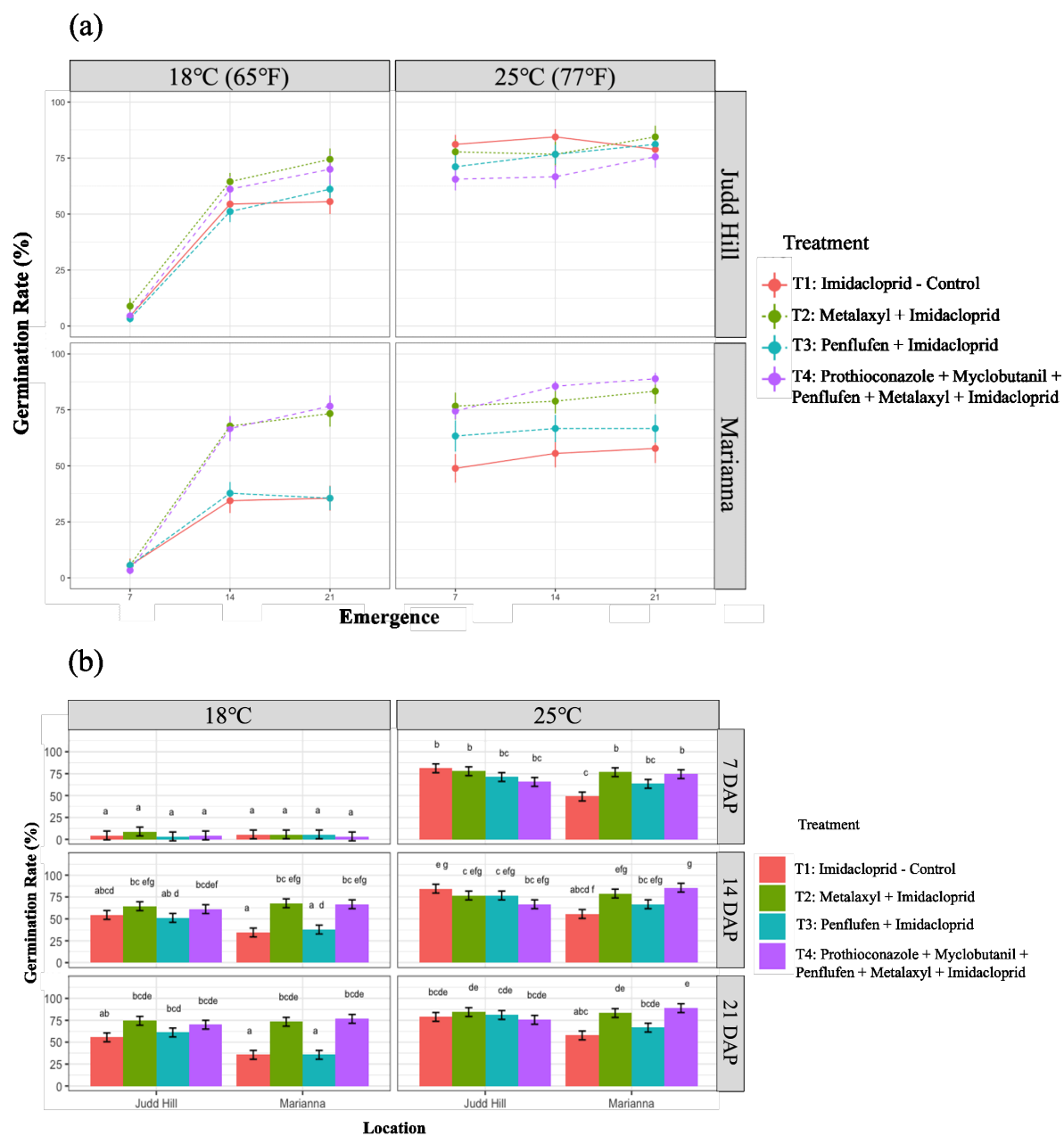


Figure 3.1. Temperature effect on cotton germination over time. Panel (a) shows line plots of cotton seed germination rates across treatments (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) at 7, 14, and 21 days after planting for each temperature and location. Bars represent the estimated marginal means ($\pm$ SE) of the germination rate for each treatment. Each facet is independently tested for significance. Treatments sharing the same letter within a given facet indicate no significant difference was detected between locations for that treatment ($P < 0.05$, Bonferroni-adjusted post hoc tests).

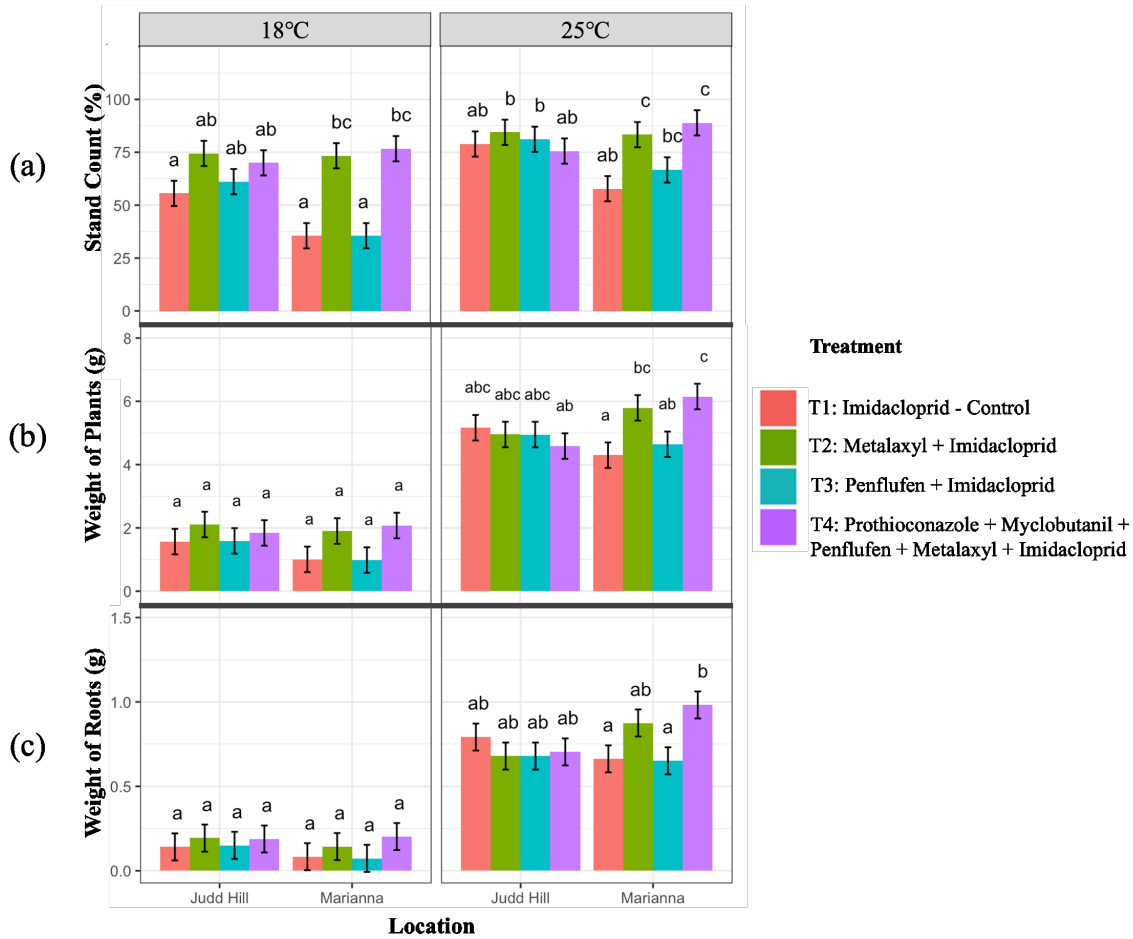


Figure 3.2. Temperature effect on cotton seedlings 21 days after planting. Panel 2 (a) compares stand counts (%) between treatments (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) at two temperatures (18°C and 25°C) and locations (Judd Hill and Marianna), (b) shows plant weight (g), and (c) roots weight (g) for the same treatments 21 days after planting. Bars represent estimated marginal means ($\pm$ SE) of stand count, plant weight, and roots weight for each treatment. Each facet is independently testing tested for significance. Treatments with the same letter within a given facet indicate no significant difference was detected between locations for that treatment ($P < 0.05$, Bonferroni-adjusted post hoc tests).

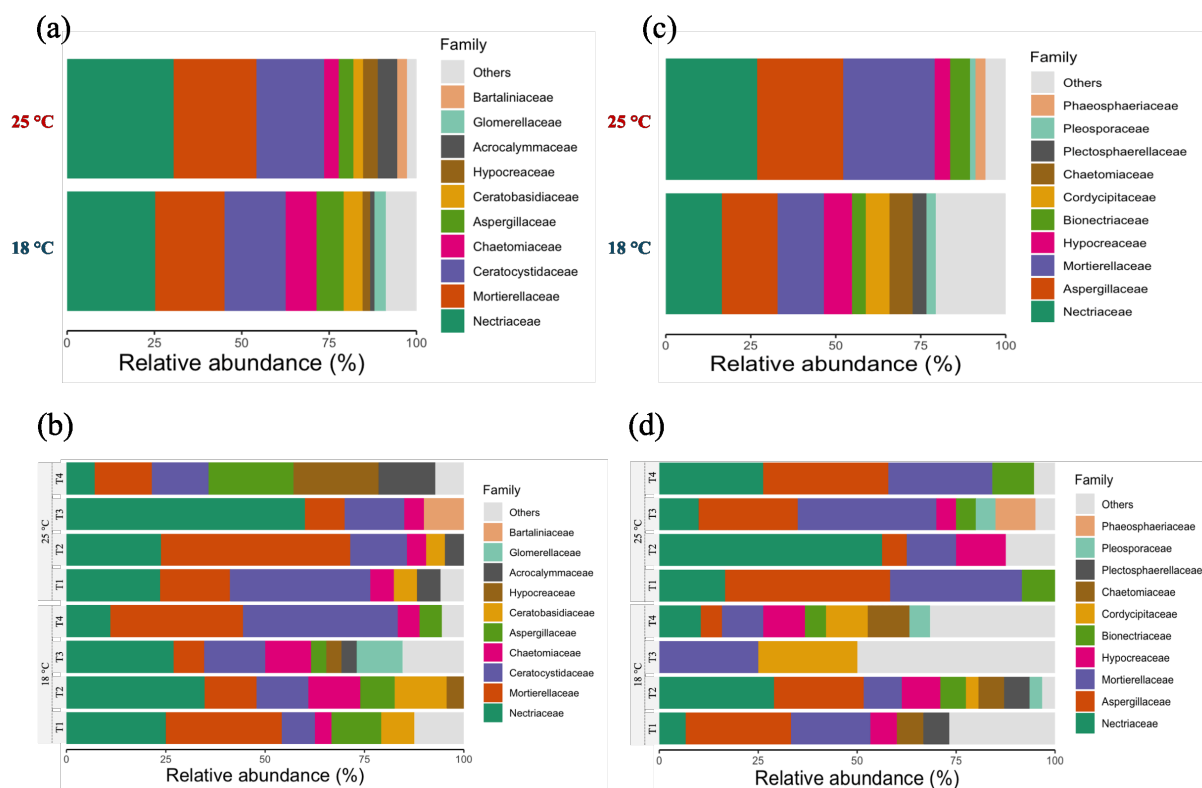


Figure 3.3. Top 10 relative abundance (%) of fungi at the family level isolated from cotton root plants. Panel (a) displays the top 10 most abundant fungal families at Judd Hill at 18°C and 25°C, isolated from roots and sequenced using Sanger. (b) shows the relative abundance for each treatment (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) within each temperature at Judd Hill. (c) displays the top 10 most abundant families at Marianna at 18°C and 25°C, isolated from roots and sequenced using Sanger. (d) shows the relative abundance for each treatment within each temperature at Marianna.

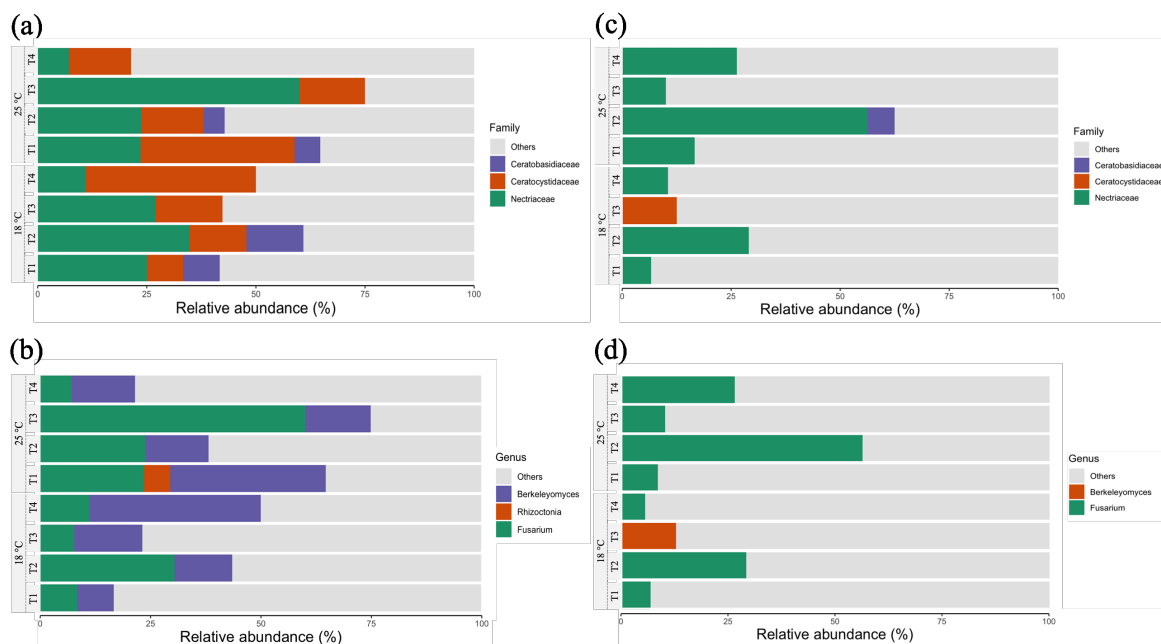


Figure 3.4. Cotton seedling disease-targeted pathogens isolated from cotton root plants at family and genus levels. Panel (a) displays the relative abundance of *Nectriaceae*, *Ceratocystidaceae*, and *Ceratobasidiaceae* across treatments (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) within each temperature (18°C and 25°C) at Judd Hill, isolated from cotton roots and sequenced using Sanger. (b) shows the relative abundance of *Fusarium*, *Rhizoctonia*, and *Berkeleyomyces* across treatments at Judd Hill, with the same temperature conditions and sequencing methods. (c) displays *Nectriaceae*, *Ceratocystidaceae*, and *Ceratobasidiaceae* across treatments at Marianna, isolated from cotton roots and sequenced using Sanger. (d) shows the relative abundance of *Fusarium* and *Berkeleyomyces* across treatments at Marianna, following the same temperature conditions and sequencing methods.

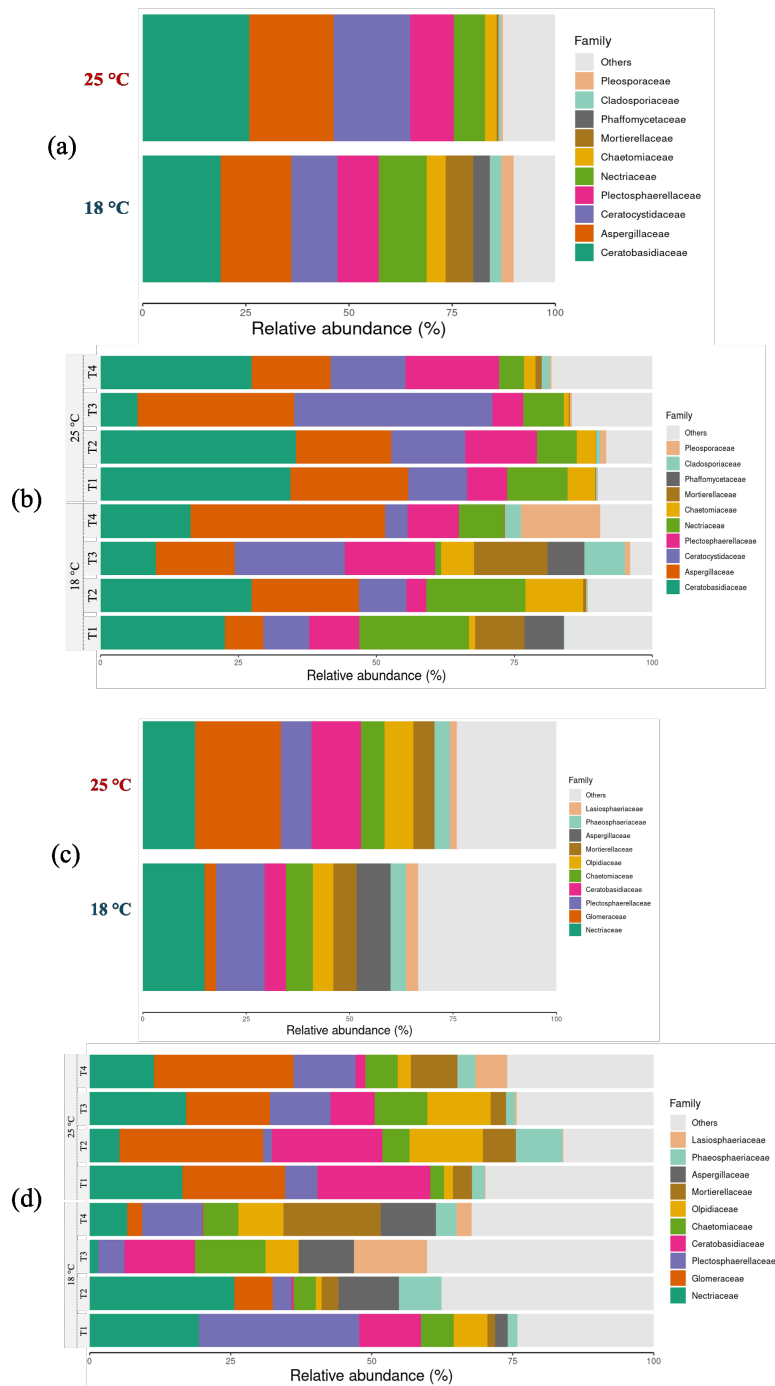


Figure 3.5. Top 10 relative abundance (%) of fungal families extracted from DNA of cotton root plants. Panel (a) displays the top 10 most abundant fungal families at Judd Hill at 18°C and 25°C, extracted from cotton root DNA and sequenced using AVITI. (b) shows the relative abundance for each treatment (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) within each temperature at Judd Hill. (c) displays the top 10 most abundant fungal families at Marianna at 18°C and 25°C, extracted from cotton root DNA and sequenced using AVITI. (d) shows the relative abundance for each treatment within each temperature at Marianna.

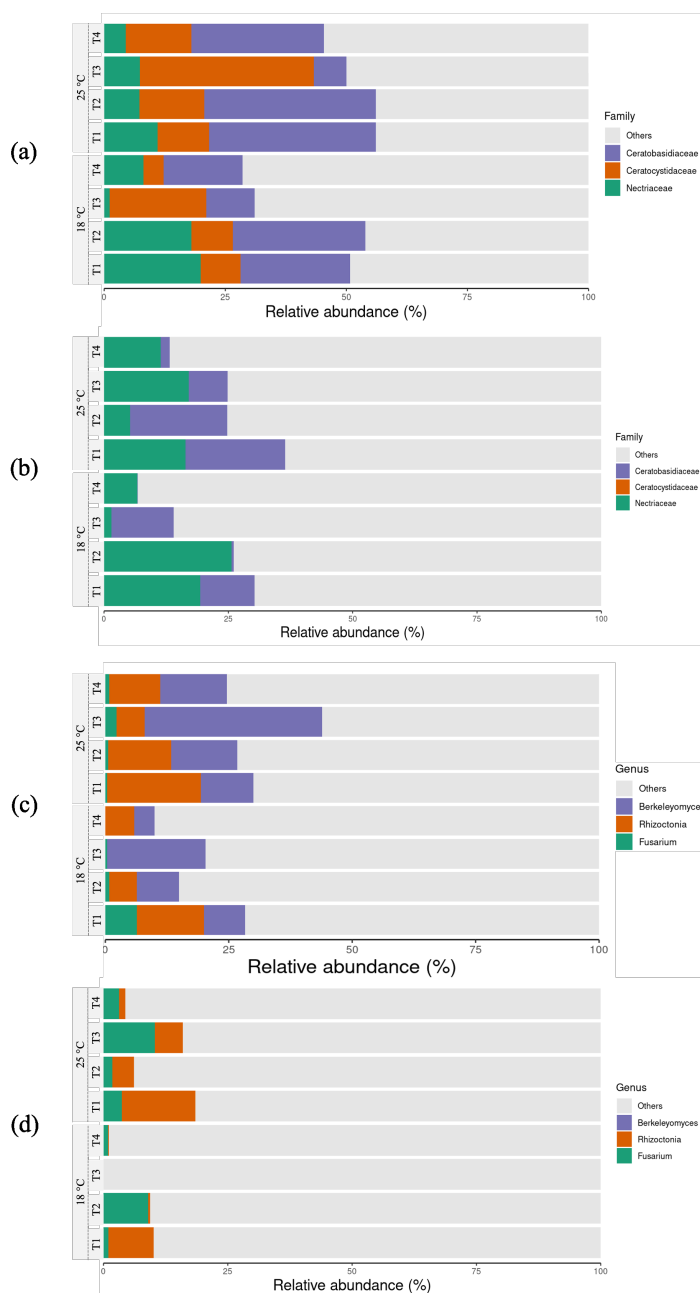


Figure 3.6. Cotton seedling disease-targeted pathogens extracted from cotton root DNA at family and genus levels. Panel (a) displays the relative abundance of *Nectriaceae*, *Ceratocystidaceae*, and *Ceratobasidiaceae* across treatments (T1 = control; T2 = metalaxyl; T3 = penflufen, and T4 = metalaxyl, penflufen, myclobutanil, and prothioconazole) within each temperature (18°C and 25°C) at Judd Hill, extracted from cotton root DNA and sequenced using AVITI. (b) shows the relative abundance of *Fusarium*, *Rhizoctonia*, and *Berkeleyomyces* across treatments at Judd Hill, under the same temperature conditions and sequencing methods. (c) displays the relative abundance of *Nectriaceae*, *Ceratocystidaceae*, and *Ceratobasidiaceae* across treatments at Marianna, extracted from cotton root DNA and sequenced using AVITI. (d) shows the relative abundance of *Fusarium* and *Berkeleyomyces* across treatments at Marianna, following the same temperature conditions and sequencing methods.

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CHAPTER 4: CONCLUSION

This master's thesis explored the effects of fungicide seed treatments on cotton seedling health and microbial communities through multi-year field trials, high-throughput sequencing, and controlled environment assays. Each chapter approached the problem from a different perspective: agronomic performance (Chapter 1), microbial community dynamics (Chapter 2), and the combined influence of temperature, culturing, and molecular approaches (Chapter 3). Together, these studies offer a comprehensive picture of how environmental factors, fungicide seed treatments, and microbial interactions shape plant health and microbial communities.

In Chapter 1, field trials conducted in Judd Hill and Marianna, Arkansas, evaluated plant stands under different seed treatments over years and locations. Stand counts were recorded from 2019 to 2023 at Judd Hill and from 2021 to 2023 at Marianna. Four treatments, each containing a base insecticide (imidacloprid), were evaluated: no fungicide (T1), metalaxyl (T2), penflufen (T3), and a combination of prothioconazole, myclobutanil, penflufen, and metalaxyl (T4). No significant differences were observed between treatments at Judd Hill over the five-year period. However, at Marianna in 2023, fungicide seed treatments notably improved stand count, particularly with Treatments 2 (metalaxyl) and 4 (the combination of prothioconazole, myclobutanil, penflufen, and metalaxyl). These findings emphasize that the effectiveness of fungicide seed treatments is significantly influenced by both location and year, underlining the critical role of environmental factors in treatment performance. This variability highlights the need for region-specific recommendations and further research to fully understand the intricate interactions between fungicides, pathogens, and environmental conditions.

Building on this, Chapter 2 investigated how fungal communities in soil and roots varied between two locations, across multiple years, and in response to different seed treatments. Using

high-throughput sequencing, we found that while soil communities were primarily shaped by location, root-associated fungi were more sensitive to fungicide treatments and environmental variation. Overall, fungicide seed treatments played a significant role in changing microbial communities and varied with active ingredients, years of sampling, and environmental conditions.

The use of Illumina MiSeq in Chapter 2 allowed us to detect a wide range of fungal taxa and assess how these communities responded to different seed treatments and environmental conditions. This approach revealed treatment-induced shifts in both target and non-target fungi, which were not detectable through isolation alone. For example, some treatments effectively controlled their target pathogens (metalaxyl and penflufen, for example). However, increases in *Berkeleyomyces* and *Fusarium* spp. associated with certain treatments were not evident in the culturing data but were captured through sequencing. Thus, the MiSeq approach complemented the isolation data by providing a comprehensive view of fungal dynamics, which is essential for understanding the full impact of fungicide use. This broader perspective is crucial for optimizing seed treatment strategies that not only target specific pathogens, but also preserve a diverse and resilient microbial community critical to plant health.

In Chapter 3, we combined growth chamber experiments with culturing methods and metabarcoding analyses to investigate the effects of temperature on root and soil fungal communities and their interactions with cotton-treated seeds. We observed that the cooler temperature (18°C) initially delayed seedling emergence. However, treatments containing metalaxyl (treatment 2) and a combination of metalaxyl + penflufen + prothioconazole + mycobutanil (treatment 4) appeared to have a better establishment over time, especially at Marianna, regardless of the temperature, whereas no significant difference was observed between treatments at Judd Hill, confirming the findings of Chapter 1.

All active ingredients effectively limited target fungi to low levels at both temperatures, though a slight increase in pathogen relative abundance was observed when the temperature was higher. When penflufen was used to target *Rhizoctonia*, it was associated with an increase in *Berkeleyomyces* at Judd Hill, both alone and in combination with other treatments, and an increase in *Fusarium* spp. at Marianna, underscoring the need for further research on its effects. These findings emphasize the role of temperature and location in shaping fungicide effectiveness and microbial community responses. Future research should focus on understanding these fungal shifts and optimizing seed treatments for different environmental conditions to improve cotton production.

In conclusion, our study highlights the significant influence of location, year, and environmental factors on the effectiveness of fungicide seed treatments. The variability observed across different conditions underscores the importance of region-specific recommendations for fungicide use. Furthermore, understanding the dynamics between fungicides, pathogens, and the surrounding microbiome is essential for optimizing seed treatment strategies that both control pathogens and support a diverse, resilient microbial community. Additionally, the findings emphasize the critical role of temperature and location in shaping fungicide effectiveness and microbial responses. To improve cotton production and make more informed decisions on seed treatments, future research should focus on understanding these environmental interactions and how they affect fungal populations, ultimately guiding strategies for different agricultural conditions.