IDENTIFICATION OF EPICOCCUM SPECIES ASSOCIATED WITH FALSE SMUT DISEASE AND ITS EFFECT ON SWITCHGRASS DEVELOPMENT

by

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(Under the Direction of Bochra A. Bahri and James Buck)

ABSTRACT

Switchgrass has been selected as a model herbaceous crop for the production of biofuel. Diseases of switchgrass pose a risk to breeding programs and research for increasing biomass yield. A new disease, false smut, was identified on switchgrass in 2019 in Georgia. The research investigated here helped identified four *Epicoccum* species associated with the disease, *Epicoccum andropogonis, E. nigrum, E. sorghinum,* and *E. spegazzinii*. False smut severity accessed on a switchgrass diversity panel located in Watkinsville, GA presented evidence of false smut resistance. Seedling stage and greenhouse trials of switchgrass suggested *E. sorghinum* and *E. spegazzinii* could be classified as pathogens while *E. andropogonis* and *E. nigrum* were classified as endophytes on switchgrass. The relatedness of the *Epicoccum* species was evaluated with the internal transcribed spacer region (503 bp), the β-tubulin gene (278 bp), and elongation factor 1-alpha gene (587 bp) revealing that *E. sorghinum* and *E. spegazzinii* are more closely related and *E.andropogonis* and *E. nigrum* are more distinct.

INDEX WORDS: Switchgrass, *Epicoccum, Claviceps*, Biofuel, Bioenergy, Endophytes, Biomass

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DEDICATION

To Vinny

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

LITERATURE REVIEW

Switchgrass

*Panicum virgatu*m (switchgrass) is a perennial C4 grass native to North America that has been used for animal grazing, soil conservation, and as a bioenergy crop. Perennial forage grasses generally have 5 growth stages: germination, vegetative growth, elongation, reproductive, and seed ripening (Moore, 1991). Vegetative and elongation stages are used to describe the growth of individual tillers within a plant (Mitchell, 1997). The growth stage score is averaged to determine the overall growth for the plant since the tillers of switchgrass can be in different developmental stages. Switchgrass exhibits a determinate flowering habit with the reproductive phase split into three subphases, inflorescence (exsertion from sheath), anthesis, and seed maturation (Sanderson, 1992). The development of switchgrass is related to the photoperiod (Mitchell, 1997). Photoperiodism is the response to seasonal changes in the day length; this would aid in determining the optimal time to plant switchgrass. Switchgrass seedlings exhibit panicoid root development (elongation of the sub-coleoptile internode to place the crown node at the soil's surface) allowing for adventitious root development (Sanderson, 2012). Switchgrass is a highly self-incompatible plant with prefertilization incompatibility that resembles a S-Z system (Martínez, 2002). Postfertilization incompatibly is present in switchgrass that prevents mating of plants with different polyploidy levels resembling an endosperm balance number system (Martínez, 2002). Self-incompatibility of switchgrass poses a challenge to breeding programs.

The Oak Ridge National Laboratory (ORNL) investigated the potential of crops in the production of biofuel in 1992 by supporting 19 projects, 15 at universities and 4 at the United

States Department of Agriculture, to investigate the development of switchgrass as a bioenergy candidate (McLaughlin, 1992). Switchgrass extended root system has beneficial effects on soil, water, and wildlife habitat by soil carbon sequestration (Monti, 2011). Quality and amount of energy obtained in the form of cellulose ethanol are high (Mclaughlin, 1998). Switchgrass has low nutrient inputs that allow it to be grown on marginal lands that can no longer support the growth of conventional crops. The production of switchgrass at 31 different sites in 7 different states was analyzed; 6 of the 7 states found switchgrass gave high biomass yield and switchgrass was the only common bioenergy candidate shared across all 7 states (Wright, 2007). Switchgrass has low economic and net energy input requirements when compared to annual crops which is an advantage for cellulose biomass production (Mitchell, 2008). However, a challenge to switchgrass production is the design and implementation of a production system. In the United States, there are some potential issues in the development of switchgrass fields including cost and space. The average break-even cost, the point where there is no loss or gain in profits, for farms to grow switchgrass in the U.S. was found to be \$113.61 ton^{-1} (Soldavini, 2018). This cost is high for most farmers and may pose challenges in finding land to grow the necessary biomass required to sustain U.S. energy consumption (Fike, 2010).

Switchgrass is slow to establish and requires a large mass of seeds ranging from 4 to 10 kg ha^{-1} and (Parrish, 2007). As a bioenergy crop, environmental impacts are considered when establishing switchgrass and aimed to keep greenhouse emissions low. The establishment year presented the greatest impact value, in factors related to greenhouse gas emissions, when accessing a 4-year growth cycle of switchgrass in the Mediterranean region of Spain (Escobar et al., 2017). The establishment of switchgrass is most effective when 1 to 2 years of preparation in perennial weed management to control competition and quantify soil health (Sanderson, 2012).

The quantification of soil health can reduce the likelihood of over-fertilizing with nitrogen that results in an increase of lodging and run-off (Monti, 2011). Another strategy for improved establishment is to select switchgrass accession with improved germination rates. The optimal temperature and pH ranges for seed germination are 25-35 ° C and 6-8 and there is a correlated relationship between pH and temperature influencing seed germination success (Hanson, 2005). 'Heavy' seeds (45.5 mg/ 50 seeds) had doubled the germination rate after treatment with acid scarification (8 M H2So4; 5 min), sodium hypochlorite (5.25% NaOCl; 15 min), and moist chilling (prechilling in 0.2% KNO3; 14 days) (Haynes, 1997).

Switchgrass production and management are focused on quantifying the economic and environmental impact of switchgrass on both farmland and conservation lands (Sanderson, 2006). Overfertilization with nitrogen can offset the environmental benefits of switchgrass-based biofuel with no increase in biomass production (Mbonimpa, 2016). Nitrogen fertilization was found to have no effect on the density of switchgrass roots and as a deep-root crop, it could potentially be used to capture nonpoint pollution (Ma, 2000). Switchgrass was found to recover 65% of applied nitrogen compared to wheat and corn (50%) indicating it could be superior (Bransby, 1998). Potential benefit of switchgrass in the environment contributes to its use desired use as a bioenergy crop in addition to the high biomass yield when compared to other crops such as corn.

Phenotypic and genetic diversity of Switchgrass

Switchgrass has the diversity to be grown in most regions of North America since it has two distinct ecotypes and is polyploidy. In switchgrass, the difference between ecotypes is associated with regional differences. The two distinct ecotypes are lowland commonly tetraploid (2n = 4× = 36) and upland commonly octoploid (2n = 8× = 72) and at times hexaploid (2n = 6× =

54) or tetraploid found in the southern U.S. and southern Canada and, northern U.S., respectively (Hultquist, 1996; Lewandowski, 2003).

The upland and lowland ecotypes exhibit different phenotypic traits. The lowland ecotype is adapted to humid, warm environments, has a longer growth period, later flowering, thick stems, and grows taller than the upland. The upland is adapted to cold semi-arid environments, and exhibits opposite growth behavior (Porter, 1966). The vegetative growth period differs between the two ecotypes with the lowland starting growth 5 days sooner and a flower heading 18 days later when compared to the upland (Jiang, 2019). Lowland ecotypes have a longer growing season and produce more biomass (Stroup, 2003). A gene ontology annotation of transcripts showed that the lowland ecotypes had an increase of chloroplast that allowed for more light perception and carbon fixation resulting in higher photosynthetic rate (Serba, 2015). Switchgrass ecotype traits, including height and flowering time, can differ drastically in different latitudinal and longitudinal planar axes (Mcmillan, 1959).

Switchgrass accessions (115), assembled into collection subsets, were assigned based on chromosome number, ecotype, and morphological similarities, and 9 core groups were revealed in cluster analysis (Taliaferro, 2003). Several molecular markers associated with the lowland and upland ecotypes were developed. Random amplified polymorphic DNA (RAPD) were identified in switchgrass and have been used to distinguish switchgrass populations by ecotype (Gunter, 1996). RAPD markers were later used to access genetic variation between switchgrass from central and northern America to determine the limits that gene pools can be exchanged without contamination (Casler et al, 2007). Restriction fragment length polymorphisms (RFLP) (Hultquist, 1996; Missaoui, 2006) and expressed sequence tag-simple sequence repeats (EST-SSRs) (Narasimhamoorthy, 2008) have also been used to differentiate between the lowland and

upland ecotypes. In addition, genetic and genomic analysis have identified different switchgrass populations within the two ecotypes (Okada, 2010; Zhu, 2013; Wang, 2013; Evans, 2015; Bahri, 2018; Fiedler, 2018).

Lowland and upland ecotypes were used to develop biparental mapping populations to identify quantitative trait loci (QTL) associated with biomass production and other developmental traits such as delayed flowering time and resistant to biotic and abiotic stressors (Serba, 2013; Missaoui, 2005; Okada, 2010; Lu, 2013; Casler, 2011; Milano, 2016; Lowry, 2019; Razar, 2020; Serba, 2015). Mapping of switchgrass populations within the ecotypes also revealed populations to be highly diverse (Martínez, 2002).

The switchgrass genome, from AP13 (a tetraploid lowland accession), was sequenced at the chromosomal level (1130 Mb; assembly GCA_016808335) by the Joint Genome Institute (US Department of Energy). The genome has 57% repetitive sequences and is constituted of 2 diploid genomes N and K that diverged approximatively 5.7 Mya (Bahri, 2018).

Selecting improved switchgrass cultivars that benefit the conversion of biomass to biofuel consider traits that increase biomass yield and reduce lignification (Sanderson, 2006). Lignification is the polymerization that strengthens the cell wall; the enzyme required to break down lignin in the biofuel conversion process can result in a higher cost (Sharma, 2021). Primary selection quality focuses on biomass yield for the upland ecotype, winter survival for the lowland ecotype, and developing an upland x lowland hybrid with advanced-generation-heterosis effects (Casler, 2014). Cultivar selection for switchgrass differs by geographical area with the main factor being an accession latitude origin (Elbersen, 2001). Biomass yield for a single accession of switchgrass can be difficult to predict when grown outside their respective region of optimal adaptation (Casler, 2003). Cultivar selection will be different by region and a unique accession

evaluation is required for each region of North America and other parts of the world. Cultivar selection is estimated to take 10 years but can be sped up with marker-assisted selection of genes that are desirable in switchgrass cultivars (Sanderson, 2006). Switchgrass grown in monoculture show an increase in biomass yield with N fertilization, but no increase in biomass yield was observed in mixtures (Wang, 2010). Overall, there is a benefit in developing specific regional monocultures of switchgrass.

Plant endophytes

Endophytes are microorganisms that form a symbiotic relationship with a host plant for part of the host life cycle. Endophytes can improve the growth and overall health of the host plant by means of osmotic balance, regulation of stomata, root morphology modification, mineral uptake enhancement, and alteration of metabolism (Anyasi, 2019). Endophytes can be used to support plants used in bioremediation by removing harmful chemicals, such as *Pseudomonas putida* removing 2,4 dichlorophenoxyacetic acid from the soil (Burragoni, 2021). The diversity of endophyte populations is dependent on host genotype, regional environment, and climate (Nair, 2014). Detection of endophytes has been done with light or electron microscopy, cultivation of endophyte surface and sterilization followed by plating (Ahmad, 2019). Cultivation of endophytes has been performed to access the full endophyte population within a plant population. Since the development of next-generation sequencing techniques endophyte detection has become more rapid (Ahmad, 2019).

Endophytes are separated into two main classifications, clavicipitaceous and non-clavicipitaceous. Non- clavicipitaceous endophytes are spilt into three subgroups: dikarya, horizontal transmitted fungi, and mycorrhizal (Jalgaonwala, 2011). Endophyte classification is based on mode of interaction between the endophyte and their host plant

Dikarya endophytes penetrate the host plant by piercing the tissue with its hyphae and grow intercellularly without altering the appearance of the plant (Rodriguez, 2009). Dikarya endophytes confer host plant resistance in a stressed environment through the production of reactive oxygen species. The third class of endophytes (horizontally transmitted fungi) colonize a localized part of the host shoot, while class 4 is primarily comprised of mycorrhizal fungi (Rodriguez, 2009).

Clavicipitaceous fungi primarily infect the intercellular space of the host plant shoots and produce secondary compounds that can provide some disease protection (Kuldau, 2008). *Clavicipitaceous* is a fungi family with many endophytic species including Endophytes, *Neotyphodium, Epichloe,* and *Balansia* that have been reported to produce ergot-alkloids (Glenn, 1997). Defense mutualism of *Clavicipitaceous* endophytes *(Neotyphodium, Epichloe,* and *Balansia*) evolved prior to endophytism (Torres, 2007).

Endophytes in switchgrass influence plant biomass

Exploration of the endophytes in switchgrass is required to understand the potential benefits that could be provided by these fungi to biomass production. Endophytes from 18 different taxonomic groups were isolated from switchgrass in Oklahoma including species of *Alternaria*, *Codinaeopsis*, *Fusarium*, *Gibberella*, *Hypocrea* and *Periconia.* Endophytes constituted 50% and 58% of the shoot and root fungal communities, respectively (Ghimire, 2011). Endophytic *Alternaria, Epicoccum, Phoma, Phaeosphaeria,* and *Stagonospora* isolated from switchgrass in Illinois and Indiana were reinoculated in switchgrass seeds (Midwest genotype; sourced from native prairie remnants in north-central Iowa) in greenhouse conditions to determine their impact on biomass production. *Epicoccum nigrum* increased total shoot and root biomass production by 25-33% of the infected switchgrass (Kleczewski et al. 2012). In a

similar study, 86% of fungal endophyte strains isolated from a monoculture switchgrass field in Kentucky, and reinoculated in Alamo switchgrass, increased plant height; the most effective strains belonged to genera of *Pleosporalessp*. *Hypoxylon*, *Fusarium*, and *Meyerozyma* (Xia, 2018).

The fungal microbes and endophyte population in switchgrass can vary due to environmental conditions. Leaf (established in plant from local sources) and seed (established in plant at seed) fungal endophytes of switchgrass were evaluated and it was observed that over time there would be a greater portion of leaf fungal microbes present in plants suggesting most microbes that infect the plant were derived from the environment (Bell-Dereske, 2021). Fungal microbiomes on switchgrass accessions Madison, Fermi, and Cave-in-Rock were observed to originate from the local environment (Whitaker, 2018).

Endophytic bacteria have also been reported in switchgrass and can impact biomass production. Strains of *Bacillus*, as well as *Flavobacterium* sp., *Brevibacillus* sp., *Paenibacillus* sp., *Paenibacillus polymyxa*, *Lysinibacillus fusiformis*, *Pseudomonas* sp., *Pseudomonas putida*, *Micrococcus* sp. and *Burkholderia gladioli* were found to increase lamina expansion by 25% when reintroduced into switchgrass native to Kentucky (Xia, 2013). Alamo switchgrass inoculated with *Burkholderia phytofirmans* strain PsJN grew 21.7% taller and had 54% enhanced photosynthetic rates when compared to non-inoculated plants (Wang, 2015).

Diseases of switchgrass

Wider planting of switchgrass through the U.S. has resulted in the observation of more diseases since 2009. These include rust (*Puccinia* spp.), sharp eyespot (*Rhizoctonia cerealis*), helminthosporium spot blotch (*Bipolaris sorokiniana*), leaf spot (*B. victoriae*), bipolaris seed rot and leaf spot *(B. oryzae*) and smut (*Tilletia maclagani*) (Parrish, 2009). By far, rust is the most

prevalent disease in switchgrass. Five species of rust, *P. amari*, *P. graminicola*, *P. novopanici*, *P. pammelii*, and *P. pascua* were found to infect switchgrass and its relative, bitter panicgrass (*Panicum amarum*) (Demers et al. 2017). *Puccinia novopanici*, a switchgrass rust, was characterized on upland (Summer and Cave-in-Rock) and lowland (Alamo and Kanlow) in the field (Uppalapati, 2013). More recently, two new emerging diseases, ergot and false smut, were reported in switchgrass in several states in the US.

Ergot and mycoparasites

Ergot is a fungal disease caused by *Claviceps* spp. that infects the ovaries of grasses and forms sclerotia in place of the seed. About 40 *Claviceps* species have been confirmed to cause ergot symptoms. *Claviceps purpurea* is the most frequently studied species with the broadest host range (rye, ryegrass, barley, oats, triticale, wheat, sorghum, and other species in the subfamily *Pooideae*) and production of alkaloids (Miedaner, 2015). Historically *Claviceps purpurea* infected economically important crops, like rye, wheat, and barley used to make flour for bread. *Claviceps purpurea* alkaloids poisoned flour causing restricted blood flow in humans and livestock resulting in sickness, ergotismus gangrenosus and ergotismus convulsivus (Smakosz et al., 2021). These same alkaloids have more recently been used for treating diseases of the nervous system (Haarmann et al., 2009). Ergot alkaloid contamination is estimated to cause losses of more than $860 million per year (Coufal-Majewski, 2016).

In early spring, *Claviceps* ascospores, are wind-dispersed and land on grass stigmas, thus acting as the primary inoculum. Once on the stigma, the ascospore germinates and its hyphae grows down through the style into the ovary. A specialized stroma develops and produces haploid, one-celled conidia. This asexual reproduction is observed through the growth and spread of mycelium found within the honeydew. Honeydew is a sticky sap that spreads between

neighboring plants with airborne spores or by the sap sticking to other plants in more heavily compact grasses. Towards the end of the growing season, *Claviceps* will form a sclerotium from the honeydew; a survival structure that allows for over-wintering. Sclerotia will not germinate in the spring until the temperature is between 0°C and 10°C for a period of 4 to 8 weeks (Miedaner, 2015; White, 2003). Sclerotia germinate and give rise to stroma where sexual reproduction occurs through fusion of a female ascogonia and a male antheridium, to form diploid nuclei. The diploid nuclei then undergo meiosis to return to a haploid state and produces sexual fruiting bodies, perithecia, that houses asci with 8 ascospores that are in perithecia that ejects the spores into the air (Miedaner, 2015; White, 2003).

The best management strategy for ergot is preventative, including seed cleaning, sanitation, and burning plant residues when disease is present in the field. Host resistance is the most economically feasible management option. Nine QTL linked to molecular markers were identified in sorghum for reduced infection of ergot caused by *Claviceps africana* and were used for marker-assisted selection screening (Parh et al., 2008). The restorer gene (IRAN IX), expressed during pollination, was tested in ryegrass at gene level activity of 25% (SC25), 50% (SC50), and 100% (SC100) and ergot infection was reduced by 62%, 81%, and 94%, respectively in artificial infection and then 60%, 37%, and 94% respectively in natural infection (Kodisch et al., 2020). In wheat, 4 QTLs were identified that were responsible for the reduction of the weight and size of sclerotia formed by *Claviceps* on infected plants (Gordon et al., 2015). Two of these QTLs were also observed in wheat cultivars in Canada and the UK (Gordon et al., 2020). Further genetic mapping of plant populations is required to determine ergot resistance lines for each respective host.

Mycoparasites on *Claviceps* spp. have been reported including *Clonostachys rosea* (Ondřej, 2010). Symptoms of false smut have also observed in switchgrass since 2019 in Georgia (Bahri, unpublished data). False smut on switchgrass replaces the seed and presents as a black brain-like sclerotia on the flower. These symptoms were similar to false smut disease reported in sugarcane that was associated with *Epicoccum andropogonis* that colonized on ergot (Singh, 1976). *C. fusiformis* sclerotia were inoculated with *Trichoderma viride, T. harzianum* and *Gliocladium virens* reduced sclerotia germination from 58% to 3.3%, 4.0% and 3.3%, respectively (Mohan, 1990).

Epicoccum spp. potentially beneficial or pathogenic

Epicoccum is a genus of fungus that is a saprotrophic mold in the Dothideomycetes class and Didymellaceae family that has been reported as being associated with many different plant species including grasses (Braga, 2018). It has been difficult to decipher the different species that make up this genus. A study conducted in 2017 analyzed the genus *Epicoccum* with 3 other genera and found that there may be up to 12 different species in this genus (Jayasiri, 2017). Most research to date in the *Epicoccum* genus has been done with *E. nigrum,* which has been widely reported as an endophyte in various plant species (Fávaro, 2011). A phylogenetic analysis of 112 *E. nigrum* strains (2 reference strains; CBS 318.83 and CBS 161.73) found that the highly variable *E. nigrum* species could be separated into 2 different clades suggesting there are two distinct species (Fávaro, 2011). A mixture of genotypic and phenotypic identification techniques was found to be beneficial when studying *E. nigrum* (Arenal, 2002).

Epicoccum nigrum was also reported in switchgrass and its co-occurrence increased the biomass production of both the plant shoots and the roots (Kleczewski et al. 2012). However,

Cerebella andropogonis, now *Epicoccum andropogonis* (Arenal, 2000), isolated from switchgrass microbiome, did not impact the development of the plant (Gravert, 2002).

In addition to reports of *Epicoccum* spp. acting as beneficial endophyte for plant growth, it also inhibits the growth of several plant pathogens. *E. nigrum* was shown to reduce *Fusarium graminearum* growth by 52.8–68.9% in wheat grains (Jensen, 2016). Application of *E. nigrum* seven days before inoculation of *Claviceps africana*, and an additional application after 3 days, significantly reduced the severity of ergot disease on sorghum bicolor in greenhouse trials (Bhuiyan, 2003). Antimicrobial properties have been extensively reported in *E. nigrum* and a few other *Epicoccum* species. The secondary compounds produced by *Epicoccum spp*. give promise to *Epicoccum* use as a biocontrol agent. Some of these secondary compounds include polyketides, polyketide hybrids, epicolactone, and diketopiperazines (Braga, 2018). Further analysis of secondary compounds produced by *E. nigrum* found that epicotripeptin limited the growth of gram-positive (55–70%) and gram-negative (20–30%) bacteria in an antibiofilm plate study. Two other compounds, cyclo (L-Pro-L-Ile) and cyclo (L-Pro-L-Tyr) were found to limit the growth of gram-positive bacteria, but did not impact gram-negative bacteria (Qader, 2021).

The ability of *Epicoccum* spp. to act as a biocontrol agent requires more than the production of secondary compounds. It is important that the fungus acts as an endophyte, meaning it does not cause any disease symptoms in the host plant and benefit the host plant. Some species of *Epicoccum* spp., including *E. nigrum*, have potential as biocontrol agents on some host plants. However, some *E. nigrum* was reported to act as a weak pathogen on at least 46 host plants (Taguiam, 2021). In addition, plant-fungal interactions may vary between different plant hosts. For example, *E. nigrum* inoculation resulted in disease on both sugar beet (*Beta vulgaris ssp. Vulgaris*) and red clover (*Trifolium pratense*) (Ogórek, 2020).

RESEARCH OBJECTIVES

This project is funded by the Center for Bioenergy Innovation (CBI) and is part of an effort to identify, quantify and evaluate the risk of switchgrass pathogens. False smut is a new disease that was reported in switchgrass in Georgia (Bahri, unpublished data).

This project aims to:

Objective 1:

Identify the *Epicoccum* species associated with false smut symptoms in switchgrass, assess the genetic variation between the *Epicoccum* species and develop species-specific markers for detection.

Objective 2:

Evaluate the impact of the different *Epicoccum* species on switchgrass seedling survival and phenotypic traits at maturity.

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CHAPTER II

IDENTIFICATION OF EPICOCCUM SPECIES ASSOCIATED WITH FALSE SMUT DISEASE IN SWITCHGRASS

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ABSTRACT

Switchgrass is a perennial C4 grass native to North America that is a leading candidate in the production of cellulosic ethanol. Switchgrass (*Panicum virgatum)* diversity panels were established in Watkinsville and Tifton, GA where false smut has been observed in the field annually since its establishment in 2019. False smut is a fungal disease of flowers that is caused by *Epicoccum* spp. that colonized sclerotia of ergot (*Claviceps* spp.) and produced brain-like sporodochia on florets. The objectives of this research were to evaluate the prevalence of the false smut in the diversity panels, identify the *Epicoccum* species associated with false smut, and determine the genetic differences between the *Epicoccum* species for diagnosis. *Epicoccum* species, *E. andropogonis, E. nigrum, E. sorghinum* and *E. spegazzinii,* were identified from 20 switchgrass leaf samples from the diversity panel. Phylogenetic analysis of Sanger sequenced partial DNA fragments of the internal transcribed spacer region (503 bp), the β-tubulin gene (278 bp), and elongation factor 1-α gene (587 bp) revealed that across 1,337 bp *E. sorghinum* and *E. spegazziniii* differed by only 6 SNPs making them more genetically related to each other. *E. andropogonis* and *E. nigrum* presented 18 and 14 unique SNPs, respectively. This research is the first to evaluate genetic similarities between *Epicoccum* species isolated off false smut infecting switchgrass. Nine pairs of species-specific primers based on the ITS region were designed. Four primer pairs were shown to successfully and specifically amplify DNA of *Epicoccum* spp. extracted from pure cultures at a concentration as low as 0.5 ng/μL.

INTRODUCTION

Switchgrass was selected as a bioenergy crop because the low nutrient inputs that allow it to be grown on marginal land that can no longer support conventional crops (Mclaughlin, 1998). Switchgrass production in the United States aims to industrialize production to reduce cost to farmers and finding the necessary land to support the U.S energy consumption (Fike, 2010). Switchgrass has the diversity to be grown in most regions of North America since it has two distinct ecotypes and is polyploidy (Serba, 2015). The two ecotypes are lowland adapted to the south and upland adapted to the north. However, a comparison of tetraploid switchgrass originating on the coast of Rhode Island was shown to be distinct from tetraploid lowland ecotype (Ecker, 2015). Breeding programs have worked to develop biparental mapping populations for the identification of quantitative trait loci (QTL) related to biomass production, delayed flowering time, and make the plant more resistant to biotic and abiotic stressors (Lowry, 2019; Razar, 2020; Tornqvist, 2018).

Wider planting of switchgrass throughout the U.S. since 2009 has resulted in the observation of more diseases which includes rust (*Puccinia* spp.), sharp eyespot (*Rhizoctonia cerealis*), leaf spot (*B. victoriae*), *Bipolaris* seed rot and leaf spot *(B. oryzae*) and smut (*Tilletia maclaganii*) (Gravert, 2002). Among diseases infecting switchgrass florets, smut resulted in premature flowering, replacement of seeds by fungal sori, and biomass yield loss estimated at 17% in Iowa (Thomsen et al., 2008). More recently, other inflorescence diseases, Ergot and false smut, were observed for the first time in 2019 in switchgrass diversity panels in Watkinsville and Tifton, GA, and Knoxville, TN (Figure 2.1). Ergot is a fungal disease caused by *Claviceps* spp.

that infects the ovaries of grasses producing a sticky sap called honeydew and forms an overwintering fungal structure, sclerotia, in place of the seed (Miedaner, 2015).

Currently the only management strategy for ergot is preventative including seed cleaning, sanitation, and burning plant residues when disease is present in the field (Schumann, 2017). False smut is a fungal disease of flowers that is caused by *Epicoccum* spp. that colonized the sclerotia of ergot (*Claviceps* spp.) and produced brain-like sporodochia on florets. *Epicoccum* is a genus of fungus that is a saprotrophic mold in the Dothideomycetes class and Didymellaceae family that has been reported as endophytes in many different plant species including grasses (Braga, 2018). It has been difficult to decipher the different species that make up this genus. A study conducted on *Epicoccum* with 3 other genera and found that there are up to 12 different species in this genus (Jayasiri, 2017). The purpose of this research is to compare the prevalence of the false smut development in the different switchgrass ecotypes in Georgia, assess the *Epicoccum* species associated with false smut, determine the genetic variation between the *Epicoccum* species and develop species-specific markers.

MATERIALS AND METHODS

Switchgrass diversity panels

Three switchgrass diversity panels were established at the University of Georgia (UGA) and used in this research. The first switchgrass panel was established by BioEnergy Science Center (BESC) at the Iron Horse Farm in Watkinsville, GA (GPS: 33°43′37″N 83°18′03″W) in 2014 (Figure 2.1A). This switchgrass diversity panel is constituted of 372 genotypes (individual plants) belonging to 36 accessions (17 lowlands; 19 upland) repeated 3 times as blocks in the field in Watkinsville iron horse farm. Among the accessions 45% were tetraploid, 14% were

octoploid and the remaining were a mixture of unknown ploidy levels. The Center of Bioenergy Innovation (CBI) established 2 switchgrass diversity panels, one at the Iron Horse Farm in Watkinsville, GA (GPS: 33°43′37″N 83°18′03″W) in 2019 and one on the Gibbs Farm in Tifton, GA (GPS: 31°23'04.0"N 83°11'33.0"W) in 2020 (Figure 2.1A). The CBI-Watkinsville is constituted of 422 genotypes split among Coastal (29%), Lowland (27%), Upland (19%) and unknown ecotypes in block 1 block 2 with qualitative similar ecotype makeup (Block 1 was established in 2019 and Block 2 in 2020). All the genotypes in the CBI-Watkinsville panel are included in the CBI-Tifton panel repeated three time. Among the accessions 96% were tetraploid, 2% were octoploid and the remainder was a mixture of unknown ploidy levels. For each of the panels, the genotypes were randomly planted within each block following a completely randomized block design. AP13 and Blackwell genotypes were repeated 21 and 18 times within each block respectively.

Morphological characterizations of switchgrass diseases and their causal agents

The CBI switchgrass diversity panel in Watkinsville, GA has been visited annually since the establishment year (2019) to evaluate switchgrass accessions for the presence of diseases. The panicle and leaves of the switchgrass plants within the switchgrass diversity panel that presented disease (leaf spots, rust, ergot, and false smut) symptoms were excised and placed in a zip-lock bag with their accession and date labeled. Samples were transported on ice from the panel location to the laboratory in Griffin, GA, and stored at 4°C. Switchgrass field symptoms were described and samples were observed under 40X magnification to characterize disease signs. Pathogen isolation was performed for the pathogens that could be cultured onto PDA. Symptomatic switchgrass tissue collected from the diversity panels were removed from the plant, surface-sterilized (1.5% sodium hypochlorite for 2 min., 70% ethanol for 2 min., and rinsed with

sterile water three times), plated on Potato Dextrose Agar (PDA) with chloramphenicol (1 µg ml^{-1}) (Fisher Bioreagents, Pittsburgh, PA). Plates were incubated (Isotemp 637F Incubator Oven; Fisher Scientific Pittsburgh, PA) in 24-hour dark at 23°C. Fungal hyphal tips were collected from each individual colony and plated on a new PDA plate at least 3 successive times to obtain a pure culture. *Epicoccum* colony morphology was observed with the naked eye, and spores were observed with a microscope at 40X magnification.

Assessing false smut severity in the CBI-Watkinsville diversity panel

Infection of false smut was characterized in the switchgrass CBI-Watkinsville diversity panel and scored for severity in 2020 (block 1), 2021 (block 1 and 2), and 2022 (block 2). False smut severity on the panicles of the 422 switchgrass plants with different genotypes were assessed based on a scale from 0 to 5 where 0 represented no disease, 1 = 1% to 10% infection, 2 = 11% to 40% infection, 3 = 41% to 60% infection, 4 = 70% to 89% infection, and 5 = 90% infection or above (Figure 2.2). Plants with an infection score of 5 were collected for sampling as described previously.

A binary score of false smut development, present or absent, was assigned to all switchgrass plants for block 1 from 2020 to 2021 and block 2 from 2021 to 2022. Switchgrass was then divided into ecotype categories (upland, lowland, and coastal) and a Z proportions test was used to determine differences in false smut development between the three ecotypes and within ecotype to determine if a block effect (scoring data for 2021 only) and year effect for block 1 (2020 to 2021) and block 2 (2021 to 2022) was present.

Molecular identification of switchgrass pathogens, including *Epicoccum* species

For the molecular identification of the *Epicoccum* species associated with the false smut disease, DNA extraction was performed by the cetyltrimethylammonium bromide (CTAB)

method (Doyle and Doyle 1987) on 19 pure cultures of the *Epicoccum* spp. grown on PDA media. DNA was quantified and quality checked using a nanodrop (C40 NanoPhotometer; Implen Westlake Village, CA) and run on 1.5% agarose gel with Thomas Scientific GelRed 10,000X in DM50 (Swedesboro, NJ) to ensure high quality (1.8 to 2.0 OD 260/ 230) and a concentration of 50 ng/ µL. Regions of the internal transcribed spacer (ITS) (ITS5/ITS4; White et al. 1990), β-tubulin gene (Bt2a/Bt2b; Glass et al. 1995) region, and elongation factor 1-α (EF1F/EF1R; Carbone and Kohn 1999) were polymerase chain reaction (PCR; VeritiPro Thermal Cycler, 384 well; Applied Biosystem Foster City, CA) under specified cycle conditions (Table 2.1). DNA polymerase kit Promega (Madison, WI, USA) were used in all PCR reactions per the manufacturer's instructions. Amplification success was checked on a 1.5% agarose gel and amplicons were Sanger sequenced by Genewiz, USA. Sanger sequences were compared against the NCBI database using the BLAST search of GenBank (http://www.ncbi.nlm.nih.gov/). Similar DNA extraction, PCR amplification and Sanger sequencing procedures were performed on 5 switchgrass samples for the molecular identification of rust pathogens and *Bipolaris.*

SNP variations and phylogenetic analysis between *Epicoccum* spp. associated with false smut

Partial sequences from 19 *Epicoccum* isolates were used to access SNPs variation and perform a phylogenetic analysis in the ITS region, β-tubulin gene, and elongation factor 1- α gene (Table 2.2). SNP variation were identified in the three sequenced regions by comparing the sequences of the 19 *Epicoccum* isolates manually after alignment using MUSCLE in MegaX (Kumar, 2018). A maximum likelihood tree was constructed individually for each of the three regions sequenced (ITS, βtubulin gene and elongation factor 1-alpha gene) as well as for all the three regions concatenated, under MegaX (Kumar, 2018) with 500 bootstrap iterations. The

Tamura-Nei (1993) model was used to judge the best-fit model and provided a maximum likelihood value of -4215.941. No *Epicoccum* reference was used since no published isolates on NCBI had sequences for all three genes.

Epicoccum species-specific primers design

Nine pairs of species-specific primers for *E. andropogonis* (3), *E. nigrum* (3), and *E. sorghinum/ E. spegazzinii* (3) were designed based on SNP positions identified in the partial sequenced ITS region. Primer design was done using Primer 3 (Untergasser A, 2012) and selection was based on melting temperature (T_m) difference less than 5 and GC content close to 50%. Primer were (Louisville, KY) and dissolved in sterile DNase/ RNase free water for a concentration of 100 ng/μL per manufactures instructions.

PCR amplification and primer efficacy tests

The specificity of the primers was first checked on DNA from pure cultures of *Epicoccum andropogonis, E. nigrum, E. sorghinum* and *E. spegazzinii* (Promega Madison, WI, USA) per the manufacturer's instructions. PCR mixtures (10 μL) were made by combining sterile H_2O (5.2 μL), 5X buffer (2 μL), dNTP (0.1 μL), $MgCl_2$ (0.6 μL), GoTaq (0.1 μL), forward primer (0.5 μL), reverse primer (0.5 μL) and respective DNA sample (1 μL) at 50 ng/ μL. A temperature gradient was used to determine optimal annealing temperature for each primer set ranged 52-60 °C and the highest temperature that produced a strong band was selected as the annealing temperature. The thermocycler settings for PCR were initial denaturing of 95 ◦C for 5 min; 35 cycles of 95 ◦C 2 min, 58 and 60◦C for 1 min, 72◦C for 2 min, and final extension of 72◦C for 5 min. PCR products were run on 1.5% agarose gels with Thomas Scientific GelRed 10,000X in DM50 (Swedesboro, NJ) for 30 min at 115V to visualize the presence of amplicons. Specificity was confirmed by testing the primer sets against targeted DNA (respective *Epiccoum*

species) and non-targeted DNA (Table 2.3). Non-targeted DNA constituted of DNA of Summer and Alamo leaf DNA and 5 non-*Epicoccum* fungal isolated from switchgrass. Sterile water was used as a negative control. Functional primers presented a band with target *Epicoccum* DNA and had an absence of a band with switchgrass DNA, non *Epicoccum* fungal DNA and the non-target *Epicoccum* species DNA. In addition, target DNA from *Epicoccum* pure cultures was serially diluted from 50 ng/µL DNA stock to 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL and 0.005 ng/µL to test for sensitivity (Zheng et al., 2019).

RESULTS

Characterization of switchgrass diseases in CBI diversity panels

All switchgrass diseases observed in the Watkinsville, GA diversity panel were described with the naked eye (field symptoms) and under 40X magnification. A dark purple ring surrounding necrotic leaf tissue with black circular bumps in the center was observed and identified to be *Bipolaris spp.* (Figure 2.3 A). The spores were dark brown, elongated, 7-cell and 54.9 µm x 14.4 µm in size. The pathogen was isolated from symptomatic leaf tissue on PDA media. DNA was extracted from a pure culture and partial PCR amplification of the ITS region (ITS4/ITS5) and then blasted on NCBI to confirm the species molecularly as *B. oryzae* (Table 2.4). Anthracnose symptoms were also observed as necrotic leaf tissue surrounded by reddish-brown ring with small condia hair-like structures (Figure 2.3B). In addition, rust was the most prevalent disease observed. Rust presented urediniospores that were globose with a light brown center surrounded by a dark outer ring and is 24.1 µm x 20.7 µm in size. Teliospores were reddish-brown 2-cell cylindric 34.3 µm x 16.4 µm (Figure 2.3C). Rust pustules were scraped off symptomatic leaf tissue and DNA extraction was performed before partial PCR amplification of

the ITS region (ITS4/ITS5). The partial ITS sequence was blasted on NCBI and identified to be *Puccinia emaculata* (Table 2.4).

The causal agent of ergot in Watkinsville, GA was identified as *Claviceps clavispora* by partial PCR amplification of the ITS region (ITS4/ITS5) with DNA extracted from a single sclerotia directly (Table 2.4). Spores of *Claviceps clavispora* were elliptical, hyaline and measured 6 µm x 5.7 µm (Figure 2.4). False smut is black and has a sporodochium with a brain-like appearance condensed on the surface of the spikelet (Figure 2.1B). The morphological and molecular identification of the causal agents of false smut are described below.

False smut severity on switchgrass in CBI-Watkinsville diversity panel

In block 1, most coastal plants (85) did not show developed false smut in 2020, but then the same plants did show developed false smut in 2021 while in block 2 most presented developed false smut in both 2021 and 2022 (Figure 2.5). For both block one and two, most upland plants were either presenting developed false smut symptoms in both year (45 plants) or no false smut in the first year of scoring and then presented developed false smut in the second year of scoring (51 plants) (Figure 2.6). Additionally, an average of 31% of upland plants showed developed false smut in the first year of scoring, but no developed smut in the second year of scoring. In block 1, most lowland plants did not develop false smut in the first year (2020), but did symptoms the second year (2021), while in block 2 most plants (58) developed false smut in both years (Figure 2.7). Also, in block 2, lowland plants (22) had developed false smut in the first year (2021) but did not develop false smut in the second year (2022).

The Z distribution score revealed no significant block effect on false smut prevalence in 2021 for the three ecotypes at the CBI-Watkinsville field (Table 2.5). Block 1 and block 2

percentage of plants with developed false smut was averaged when describing the results of false smut prevalence in the Watkinsville, GA CBI field in 2021.

Overall, there was a significant increase in false smut development from 2020 to 2021 for all three ecotypes. Switchgrass belonging to the coastal and lowland ecotype showed a dramatic increase ($p<0.00001$) of 62% and 79% in false smut prevalence between 2020 and 2021, respectively (Figure 2.8; Table 2.5). Switchgrass belonging to the coastal ecotype saw no significant changes in plants with developed false smut between 2021 and 2022, while the lowland ecotype did have a significant ($p<0.01$) decrease of 17% in plants with developed false smut between 2021 and 2022 (Figure 2.8; Table 2.5). A less dramatic increase ($p<0.05$) of developed false smut, 18%, was observed in the Summer accession between 2020 and 2021 (Figure 2.8; Table 2.5). The frequency distribution and Z distribution showed a continuous increase of false smut prevalence of 11.6% from 2020 to 2022 (Figure 2.8; Table 2.5).

The three ecotypes, upland, lowland, and coastal, were compared within the same block and year. In 2020 (block 1), plants from the upland ecotype were distinct from the lowland and coastal ecotypes ($p<0.00001$) with higher percentage of developed false smut (Table 2.6). However, plants from the lowland and coastal ecotype showed no significant difference from one another. In 2021, neither the lowland or the coastal ecotypes showed a difference in false smut prevalence compared to the upland ecotype in block 1. Coastal plants showed a significantly higher percentage of developed false smut ($p<0.05$) compared to the lowland ecotype (Table 2.6). In 2021 (block 2), only plants from the upland and coastal ecotype were significantly different ($p<0.05$) with plants from the coastal ecotype exhibiting a higher percentage of developed false smut (Table 2.6). Finally, in 2022 (block 2), both coastal ($p<0.0001$) and

lowland ($p<0.001$) ecotypes had a lower percentage of developed false smut compared to plants in the upland ecotype (Table 2.6).

Morphological and molecular identification of *Epicoccum* spp. associated with false smut

Molecular characterization of *Epicocucm* spp. isolated from florets infected with false smut (35) revealed four different species (*E. nigrum, E. andropogonis, E. sorghinum,* and *E. spegazzinii*) across the three panels. *E. spegazzinii* had the highest frequency (35.2%) followed by *E. andropogonis* and *E. nigrum* (26.5%) with the same frequency and lastly *E. sorghinum* (11.8%) had the lowest frequency among the 19 *Epicoccum* isolates studied (Table 2.7).

E. andropogonis and *E. nigrum* plate colony morphology presented irregular margins. *E. andropogonis* had a reddish-brown coloration and formed fruiting bodies in the oldest section of colony. *E. andropogonis* spores are multicell with 4 circular cells overlapping one another, 15.5 µm x 14.1 µm with dark brown pigment (Figure 2.9A). *E. nigrum* had a bright orange coloration and formed spore-dense droplets on the oldest section of colony. *E. nigrum* spores are a single cell, oval shape with a stem branching off, 6.5 µm x 7.3 µm and brown pigmented (Figure 2.9B). Both *E. sorghinum* and *E. spagazzinii* colony morphology presented circular margins and showed dark brown hyphae in the center of the colony while the younger hyphae were a lighter tan color. *E. sorghinum* had a larger darker center that spread further to the edge of the colony compared to *E. spegazzinii.* Spores were not observed from pure cultures of *E. sorghinum* and *E. spegazzinii* under 40X magnification (Figure 2.9C and Figure 2.9D). *Claviceps* spores observed under 40X magnification were single cell, hyaline, oval shape 6 µm x 5.7 µm (Figure 2.4D).

Molecular variation between the *Epicoccum* species

SNP variation was described for the ITS region (471 bp), β-tublin gene (273 bp) and elongation factor 1-α gene (579 bp) using 19 *Epicoccum* isolates. SNP identification showed that

E. spegazzini only had 1 unique SNP in the ITS region and 2 in the elongation factor 1-α sequenced region. *E. sorghinum* had 1 species-specific SNP in the β-tubulin sequenced region and 2 in the elongation factor 1-α sequenced region. *E. andropogonis* showed 5 unique SNPs in the ITS region, 3 in the β-tubulin sequenced region, and 10 in the elongation factor 1-α sequenced region. *E. nigrum* showed 4 unique SNPs in the ITS region, 4 in the β-tubulin sequenced region, and 6 in the elongation factor 1-α sequenced region. *E. andropogonis* and *E. nigrum* shared 22 SNP positions that differed from *E. sorghinum* and *E. spagazzinii* in the ITS region, 7 in the β-tubulin sequenced region, and 9 in the elongation factor 1-α sequenced region. *E. sorghinum* and *E. spegazzinii* shared 22 SNP positions that differed from *E. andropogonis* and *E. nigrum* in the ITS region, 7 in the β-tubulin gene, and 10 in the elongation factor 1-α gene (Table 2.8).

Phylogenetic analysis revealing the relative relatedness between the *Epicoccum* species

The phylogenetic analysis of the combined ITS, β-tubulin sequenced region and the elongation factor 1-α (1,377bp) was performed with 19 *Epioccum* isolates. The tree on the combined sequenced regions, as well as on the trees on the three regions taking individually *E. spegazzinii* and *E. sorghinum* are closely related, while *E. andropogonis* and *E. nigrum* are more distinct when comparing the four species to one another (Figure 2.10).

Efficacy of the designed species-specific primer sets

Nine species-specific primers sets were designed, nine forward primers and one reverse primer to be used with all the forward primers. Three forward primers (EsEsITSF1, EsEsITSF2, and EsEsITSF3) were design to detect both *E. sorghinum* and *E. spegazzinii* based on high genetic similarities. Three forward primers each were designed for *E. andropogonis* (EaITSF1, EaITSF2, and EaITSF3) and *E. nigrum* (EnITSF1, EnITSF2, and EnITSF3). The Tm of the

primers ranged from 60 °C and 69 °C, had a G and C percentage between 45% and 72% and were 18 to 20 base Pairs in length (Table 2.9). The primer sets were tested at 56 °C, 58 °C, 60 °C, 63 °C, and 65 °C and the annealing temperature was selected based on the highest temperature that consistently produced the brightest band (Figure 2.11). The optimal annealing temperature was determined to 63 °C for all 9 primer sets. DNA was extracted from pure cultures of *Epicoccum* species, Alamo and Summer leaf DNA, and non-*Epicoccum* fungal DNA (*Fusarium, Coniochaeta, Alternaria, Curvularia,* and *Bipolaris*) isolated from switchgrass flower tissue. Four of the nine primer sets (EaITSF2, EnITSF2, EnITSF3, and EsEsITSF2) successfully produced a single amplicon from pure cultures of *Epicoccum* spp. without amplifying non-*Epicoccum* DNA (Figure 2.12). However, the four primer sets were unable to differentiate the *Epicoccum* species (Figure 2.13). The sensitivity of the 4 primer sets that successfully amplified *Epicoccum* DNA without amplifying non-Epicoccum DNA or Summer and Alamo leaf DNA were tested. The DNA concentration that the *Epicoccum* primer sets were able to detect *Epicoccum* DNA was at 50 ng/µL (EnITSF2), 5 ng/µL (EnITSF3), and 0.5 ng/µL (EaITSF2, and EsEsITSF2).

DISCUSSION

Prevalence of false smut in switchgrass plants

From 2020 to 2022, false smut severity was scored for 2 blocks consisting of 422 switchgrass plants with different genotypes in the CBI diversity panel in Watkinsville, GA. The plants in the diversity panel were grouped by their respective ecotype, upland, lowland, and coastal, and categorized into plants with developed false smut or absence of false smut symptoms.

An increase of developed false smut was observed on all three ecotype populations, upland, lowland, and coastal, between 2020 to 2021. Ergot (*Claviceps* spp.) develop an overwintering structure known as a sclerotia (Frederickson, 1993). An increase of sclerotia formed and dropped onto the soil of the field would lead to an increase in infection level in the following growing season.

Plants from the lowland ecotype adapted to the pressure of the false smut pathogen and false smut development prevalence decreased by 17% indicating that switchgrass from the lowland ecotype may have more flexible resistance. Quantitative resistance, while only partially effective in inhibiting disease development, is considered more durable compared to major resistance genes (Pilet-Nayel, 2017). Resistant switchgrass genotypes can be used to develop mapping populations to locate false smut-resistant QTLs. In previous studies, mapping populations have been successfully used to locate QTLs related to winter dormancy and switchgrass biomass traits (Razar, 2020; Razar 2022).

Some plants in the diversity panel presented developed false smut in the first scoring year but did not present developed false smut in the second year. This trend was observed in plants from the upland (54), lowland (22), and coastal (23) ecotypes. Change in false smut development may indicate a possible change in virulence of the false smut pathogen disease. Local adaptation of a pathogen population is thought to occur when the fitness of the pathogen changes (Sacristan, 2008). The switchgrass diversity panel in Watkinsville, GA consists of 422 different plants with unique genotypes that increase the number of host plants available to the pathogen population. Changes in the pathogen population may be responsible for the change in false smut development in individual plants between the first and second scoring years.

Epicoccum associated with false smut

Four *Epicoccum* species, *E. andropogonis, E. nigrum, E. sorghinum,* and *E. spegazzinii,* were isolated from false smut symptoms on switchgrass florets. SNP variations on partial ITS, β-tubulin, and elongation factor 1-α gene sequences found that *E. spegazzinii* is more closely related to *E. sorghinum* while *E. andropogonis* and *E. nigrum* are more distinct from *E. spegazzinii* and *E. sorghinum.* Genetic relatedness could indicate similar biological functions. Because *E nigrum* has been reported to act as a biocontrol agent that reduces *Claviceps africana* severity in sorghum (Bhuiyan, 2003; Daba, 2019), *E. andropogonis* could also be a potential biological agent.

The interaction between *Claviceps* spp. and *Epicoccum* spp. resulting in false smut is not understood. Specifically, when inoculation of *Epicoccum* onto switchgrass infected with ergot occurs, location of *Epicoccum* in host plant tissue, and dispersal of *Epicoccum* in the field. Other mycoparasites, *Acremonium strictum,* have been reported in switchgrass and that can reduce the colonization of rice blast, rice sheath blight, cucumber gray mold, tomato late blight, and barley powdery mildew (Choi et al. 2009; Ghimire, 2011). *E. nigrum* and *E. purpurascens* have been reported to act as mycoparasites on *Fusarium graminearum, Phytophnthora* and *pythium* species, reducing disease severity (Jensen, 2016; brown, 1987). Evaluation of *Epicoccum* spp. to act as a mycoparasite on *Claviceps* spp. still needs to be confirmed. Assuming *Epicoccum* acts as a mycoparasite the mechanism of exposure onto *Claviceps* is unknown. *Epicoccum* species-specific primers can confirm the presence or absence of *Epicoccum* on false smut and ergot sclerotia indicating if *Epicoccum* is required for the formation of the false smut complexes. Species-specific primer could identify the location of *Epicoccum* endophyte in the plant and determine if the *Epicoccum* colonizing on ergot originates inside the plant as an endophyte.

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Table 2.1. Primer sets and thermocycler conditions for PCR amplifications for the internal transcribed spacer region, β-tubulin gene, and elongation factor 1- α gene. Forward and reverse primers name, sequence, target, PCR conditions, and manufacturer are listed for all three genes.

Primer	5' to 3' sequence	Target	PCR conditions	manufacturer
ITS4	TCCTCCGCTT ATTGATATGC	Internal transcription region	35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min	eurofin
ITS5	GGAAGTAAAA GTCGTAACAAGG			
Bt2a	GGTAACCAAATC GGTGCT-GCTTTC	B-tubulin	35 cycles of 94 °C for 40 s, 65 °C for 40 s, and 72 °C for 1 min	sigma
Bt2b	ACCCTCAGTGTA GTGACC-CTTGGC			
EF1F	GAYTTCAYCA AGAACATG-AT	Elongation factor 1-alpha	35 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min	sigma
EF1R	GACGTTGAAD CCRACRTT-GTC			

Table 2.2. *Epicoccum* isolates identified from false smut samples collected from BESC and CBI-Watkinsville, GA and CBI-Tifton, GA. Isolate IDs, species, panel, field location, collection year and switchgrass genotype are listed for each isolate.

Isolate ID	Species	Panel	Field location	Collection year	Switchgrass Genotype
L-CBI-W-ESP	*E. spegazzinii*	CBI	Watkinsville	2020	J582.C
C-CBI-T-ESP	*E. spegazzinii*	CBI	Tifton	2020	J271.A
D-CBI-T-ESP	*E. spegazzinii*	CBI	Tifton	2020	J317.A
F4-CBI-W-ESP	*E. spegazzinii*	CBI	Watkinsville	2021	J073.A
CC-CBI-W-ESP	*E. spegazzinii*	CBI	Watkinsville	2020	J582.C
E- CBI-T-ESO	*E. sorghinum*	BESC	Watkinsville	2020	J582.C
K- CBI-T-ESO	*E. sorghinum*	CBI	Tifton	2020	J317.A
PA-CBI-W-ESO	*E. sorghinum*	CBI	Tifton	2020	J041.A
J3-CBI-W-EN	*E. nigrum*	CBI	Watkinsville	2021	Blackwell
J4-CBI-W-EN	*E. nigrum*	CBI	Watkinsville	2021	Blackwell
J6-CBI-W-EN	*E. nigrum*	CBI	Watkinsville	2021	Blackwell
301A-CBI-T-EN	*E. nigrum*	CBI	Tifton	2020	J301.A
AZ-CBI-T-EN	*E. nigrum*	CBI	Tifton	2020	J301.A
A9-CBI-W-EA	*E. andropogonis*	CBI	Watkinsville	2021	J484.B
P1B-BESC-W-EA	*E. andropogonis*	BESC	Watkinsville	2020	J022.B
P1G-BESC-W-EA	*E. andropogonis*	BESC	Watkinsville	2020	J022.B
M-CBI-W-EA	*E. andropogonis*	CBI	Watkinsville	2021	J003.B
AA-CBI-W-EA	*E. andropogonis*	CBI	Watkinsville	2021	Blackwell
R-CBI-W-EA	*E. andropogonis*	CBI	Watkinsville	2021	J181.A
S6-BESC-W-EA	*E. andropogonis*	BESC	Watkinsville	2020	J022.B

S8-BESC-W-EA	*E. andropogonis*	BESC	Watkinsville	2020	J022.B

Table 2.3. Non-*Epicoccum* fungal isolates from switchgrass collected from BESC and CBI-Watkinsville, GA and CBI-Tifton, GA. Isolate ID, genus, panel, collection year and blast results (percent coverage, percent identity, and e-value) are listed for each isolate.

Isolate ID	Genus	Panel	Field location	Collection year	Percent Coverage	Percent identity	e-value
M1-BESC-W-F	*Fusarium*	BESC	Watkinsville	2020	94	99.4	0.0
M3-CBI-T-Co	*Coniochaeta*	CBI	Tifton	2020	94	99.4	0.0
M4-CBI-T-Ni	*Nigrospora*	CBI	Tifton	2020	94	99.8	0.0
M10-CBI-W-Al	*Alternaria*	CBI	Watkinsville	2021	94	99.3	0.0
M11-CBI-W-Cu	*Curvularia*	CBI	Watkinsville	2020	93	100	0.0
M12- CBI-W-Bi	*Bipolaris*	BESC	Watkinsville	2020	95	99.5	0.0

Table 2.4. Identification of switchgrass disease, *Bipolaris* leaf spot, rust, and ergot, and their casual agents, *Bipolaris oryzae, Puccinia emaculata,* and *Claviceps clavispora* respectively. Isolate ID, genus, panel, collection year and blast results (percent coverage, percent identity, and e-value) are listed for each isolate.

Isolate ID	Species	Panel	Field location	Collection year	Percent Coverage	Percent identity	e-value
M12- CBI-W-Bi	*Bipolaris oryzae*	BESC	Watkinsville	2020	95	99.5	0.0
Ru- CBI-W-	*Puccinia emaculata*	CBI	Watkinsville	2021	95	91.7	0.0
Ru- CBI-W-	*Claviceps clavispora*	CBI	Watkinsville	2021	93	97.5	0.0

Table 2.5. Z distribution test results comparing year effect between 2020 and 2021 (block 1) and 2021 and 2022 (block 2) as well as block effect in 2021 (block 1 against block 2).

Block and scoring year comparison	Upland	Lowland	Coastal
Block 1 2020 X Block 1 2021	0.037	0.00001	0.00001
Block 1 2021 X Block 2 2021	NS	NS	NS
Block 2 2021 X Block 2 2022	NS	0.01	NS

Table 2.6. Z distribution test comparing the lowland, upland, and coastal ecotypes within the same block and scoring year.

Block (Scoring year)	Upland X Coastal	Upland X Lowland	Coastal X Lowland
Block 1 (2020)	0.00001	0.00001	NS
Block 1 (2021)	NS	NS	0.015
Block 2 (2021)	0.04	NS	NS
Block 2 (2022)	0.0001	0.001	NS

Table 2.7. *Epicoccum* species, morphologically identified on PDA media after 5-days of incubation and molecularly identified using ITS region, based on 34 isolates purified from 14 samples of switchgrass panicles presenting false smut symptoms collected from BESC, CBI-Watkinsville and CBI-Tifton diversity panels. Numbers between brackets represent *Epicoccum* isolate used in the phylogenetic analysis.

***Epicoccum* Species**	**Watkinsville CBI**	**Tifton CBI**	**Watkinsville BESC**	**Sum of Isolates**
E. andropogonis	4 (4)	0	5 (4)	9
E. nigrum	5 (2)	4 (2)	0	9
E. sorghinum	2 (1)	2 (1)	0	4
E. spegazzinii	9 (2)	3 (2)	0	12
Sum of isolates	20	9	5	34

Table 2.8. Shared and unique SNPs between *E. andropogonis*, *E. nigrum*, *E. sorghinum*, and *E. spegazzinii* in the ITS region, β-tubulin (Beta), and elongation factor α-1 (EF) identified by sanger sequencing.

E. andropogonis	***E. nigrum***	***E. sorghinum***	***E. spegazzinii***	**Beta**	**ITS**	**EF**
Same base pair		Same base pair		7	22	9
Unique SNP		Same base pair		3	5	10
Same base pair	Unique SNP	Same base pair		4	4	6
Same base pair		Unique SNP	Same base pair	1	0	2
	Same base pair		Unique SNP	0	1	2

Table 2.9. Primers designed for the study and efficacy to amplify target DNA. All species-specific primer sets had an optimal annealing temperature of 63 °C

Primer ID	Forward	Reverse	Amplified non-target DNA	*Epicoccum* species detection	Sensitivity (ng/µL)
EnITSF1	CGCGCGCAGACTCGCCTT	CCTACCTGATCCGAGGTCAA	Yes	No	-
EnITSF2	TGGACTTCGGTCTGCTACCT	CCTACCTGATCCGAGGTCAA	No	No	50
EnITSF3	GCCGGTTGGACAACATTCA	CCTACCTGATCCGAGGTCAA	No	No	50
EaITSF1	TGCGTGTAGACTCGCCTTAA	CCTACCTGATCCGAGGTCAA	Yes	No	-
EaITSF2	TAGACTTCGGTCTGCTACCTCTT	CCTACCTGATCCGAGGTCAA	No	No	0.5
EaITSF3	GCCGACTGGACAACATTCA	CCTACCTGATCCGAGGTCAA	Yes	No	-
EsEsITSF1	GTTTGTCTCCTGTAGACTCGCC	CCTACCTGATCCGAGGTCAA	Yes	No	-
EsEsITSF2	GTTGTAGGCTTTGCCTGCTAT	CCTACCTGATCCGAGGTCAA	No	Yes	0.5
EsEsITSF3	CCACCGATTGGACAAACTTA	CCTACCTGATCCGAGGTCAA	Yes	No	-

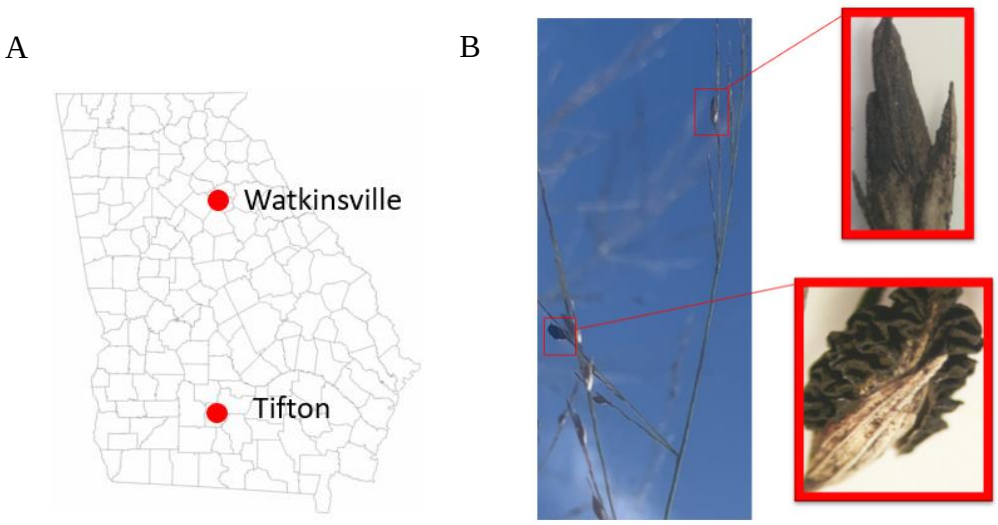


Figure 2.1. Map of Georgia indicating the location of BESC and CBI-Watkinsville and CBI-Tifton diversity panels (A). Switchgrass florets infected with ergot sclerotia, top image, and false smut, brain-like sporodochia, bottom image (B).

Figure 2.2. False smut severity scores based on a scale from 0 to 5 used in the CBI-Watkinsville diversity panel. No infection (0), 1% to 10% infection (1), 11% to 40% infection (2), 41% to 60% infection (3), 70% to 89% infection (4) and 90% or above infection (5).

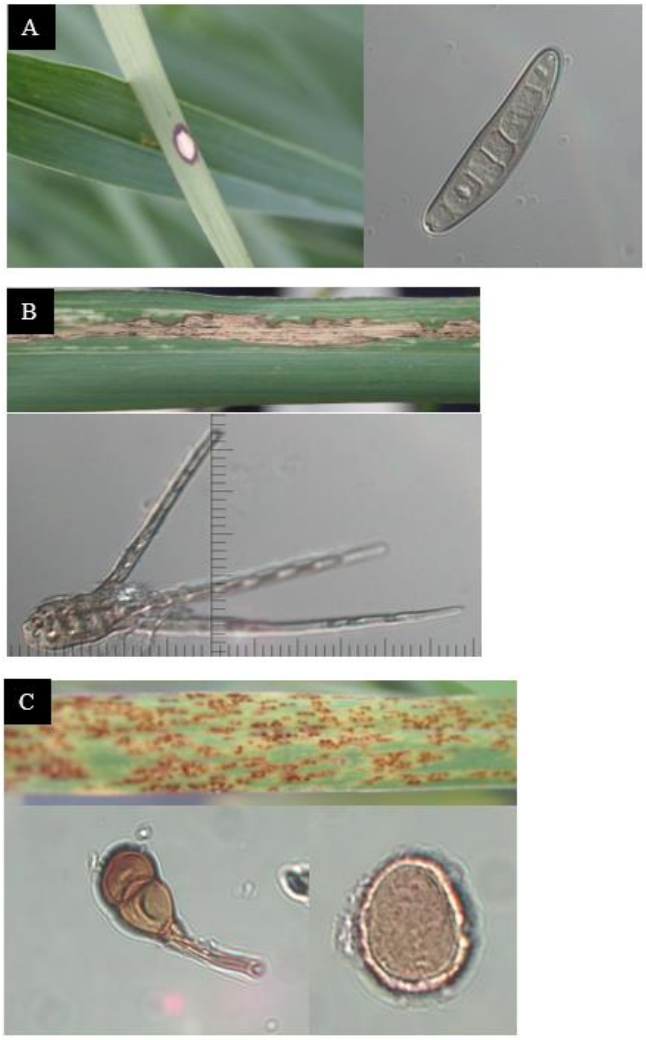


Figure 2.3. *Bipolaris:* leaf spot presented as a dark purple ring surrounding necrotic leaf tissue and dark brown, elongated, 7-cell and 54.9 µm x 14.4 µm spores (A). Anthracnose leaf spot presented as necrotic leaf tissue surrounded by reddish-brown ring with small conidia hair-like structures (B). Rust presented orange pustules (top) with a light brown, globose, 24.1 µm x 20.7 urediniospores (bottom right) and reddish-brown 2-cell cylindric 34.3 µm x 16.4 µm teliospores (bottom left).

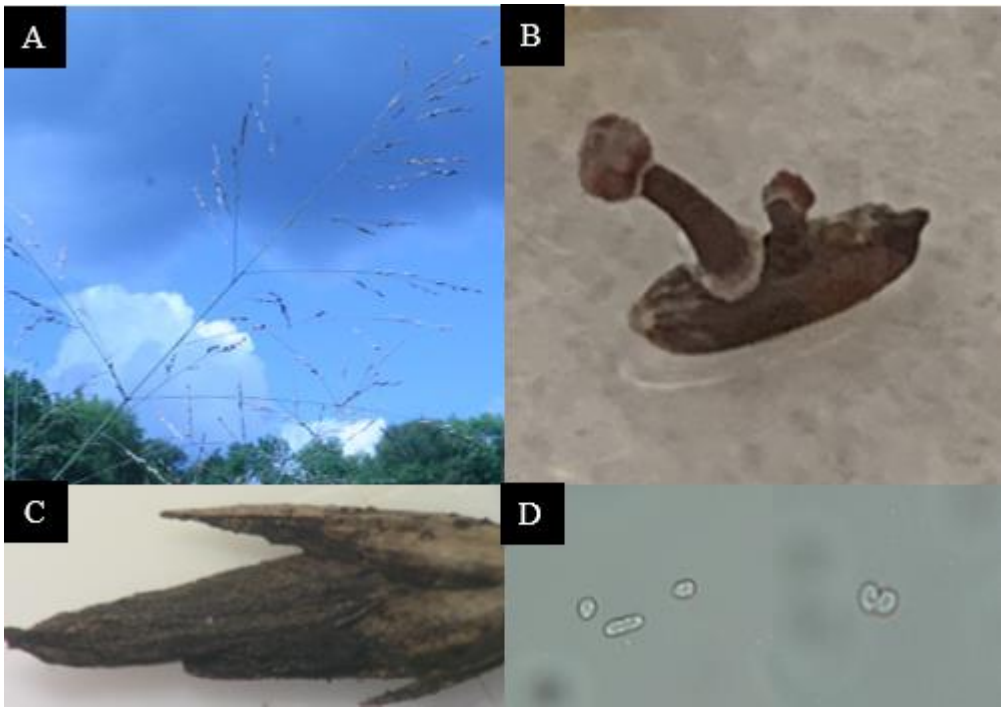


Figure 2.4. Ergot field symptoms on switchgrass panicle in Watkinsville, GA (A). Ergot sclerotia on switchgrass spikelet (B). Germinating sclerotia (C). *Claviceps* spores at 40X magnification (D).

Fig. 2.5. Changes in false smut infection on switchgrass coastal ecotype (123 genotypes) between 2020 to 2021 (Block 1) and 2021 to 2022 (Block 2).

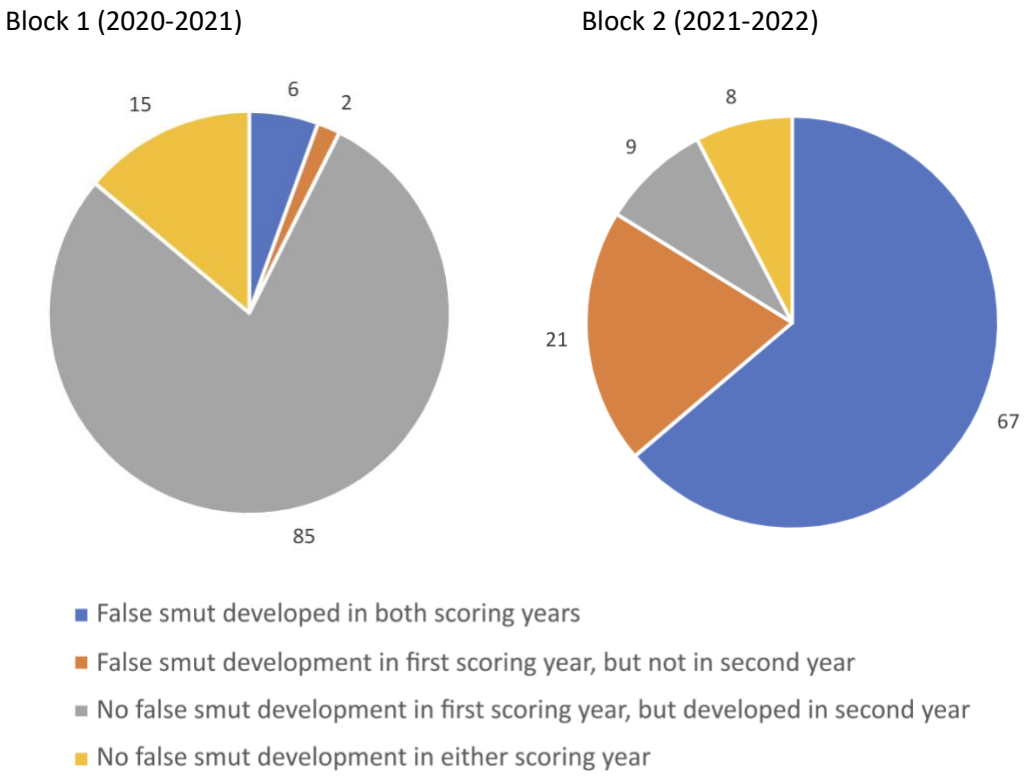


Fig. 2.6. Changes in false smut infection on switchgrass upland ecotype (88 genotypes) between 2020 to 2021 (Block 1) and 2021 to 2022 (Block 2).

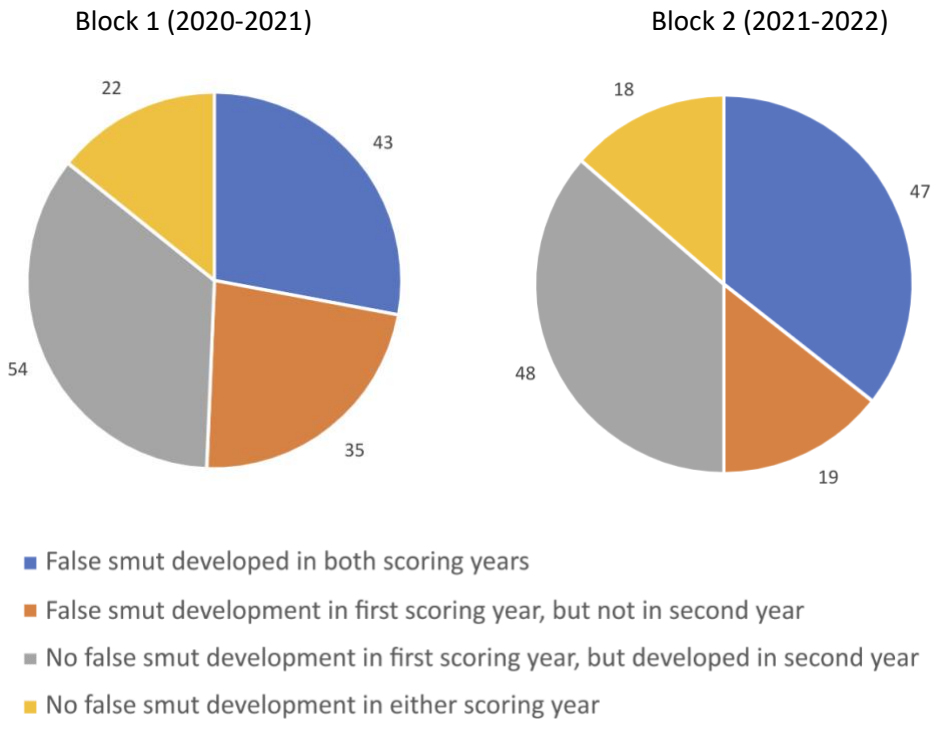


Figure 2.7. Changes in false smut infection on switchgrass lowland ecotype (102 genotypes) between 2020 to 2021 (Block 1) and 2021 to 2022 (Block 2).

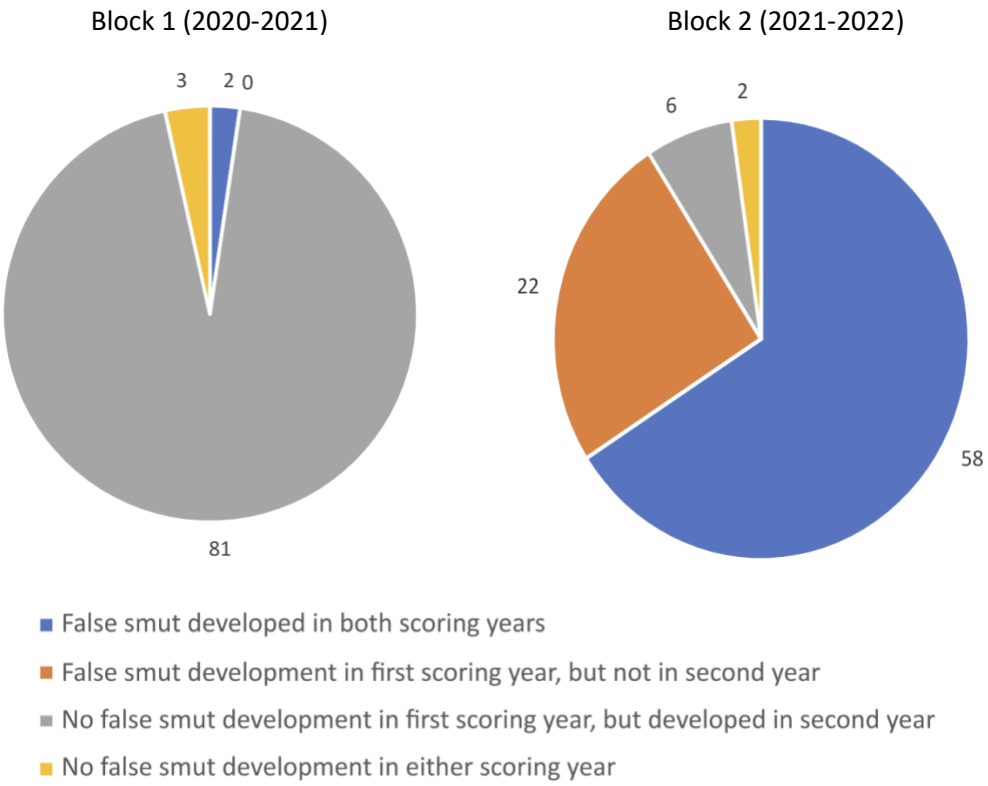
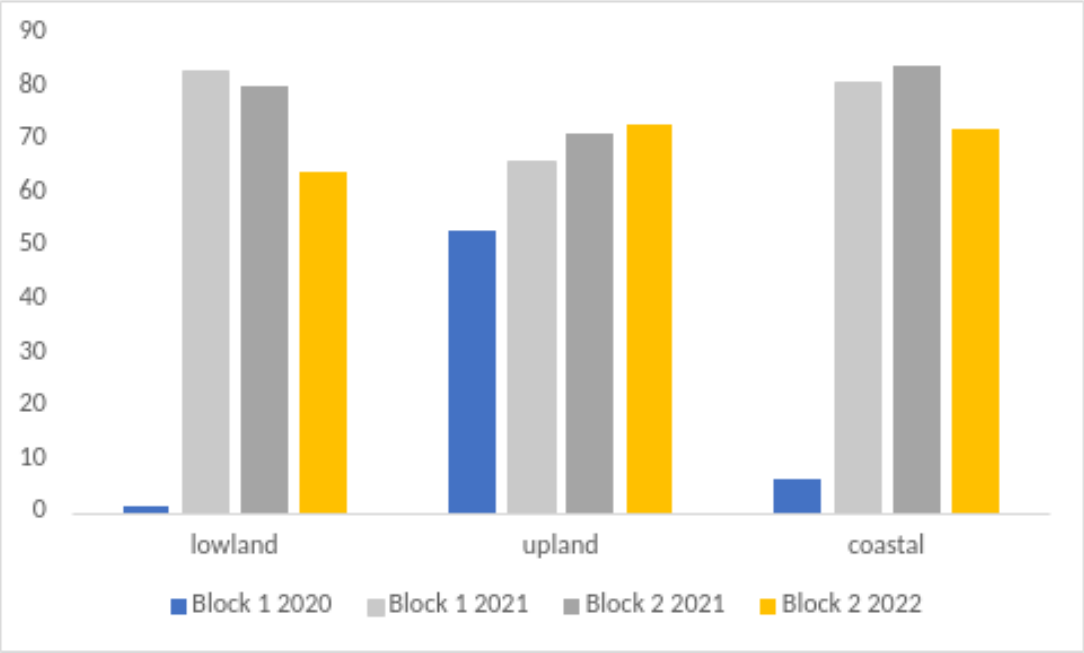


Figure 2.8. Frequency distribution of false smut presence in the CBI-Watkinsville diversity panel for Block 1 and Block 2 separated by ecotype (lowland, upland, and coastal).



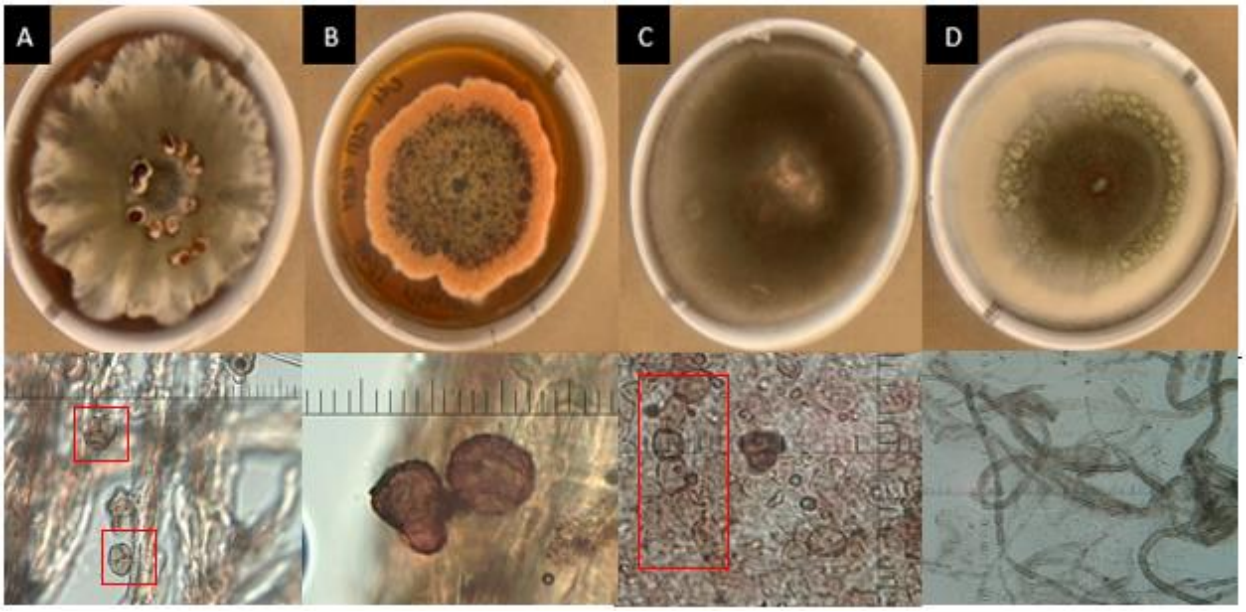


Figure 2.9. Morphology of the four *Epicoccum* species grown for 14-days on PDA plates with chloramphenicol at 25 °C under dark conditions (top) and mycelium and spores (if present) under the microscope (40X). *E. andropogonis* (A), *E. nigrum* (B), *E. sorghinum* (C), and *E. spegazzinii* (D). Red squares highlight spores in image.

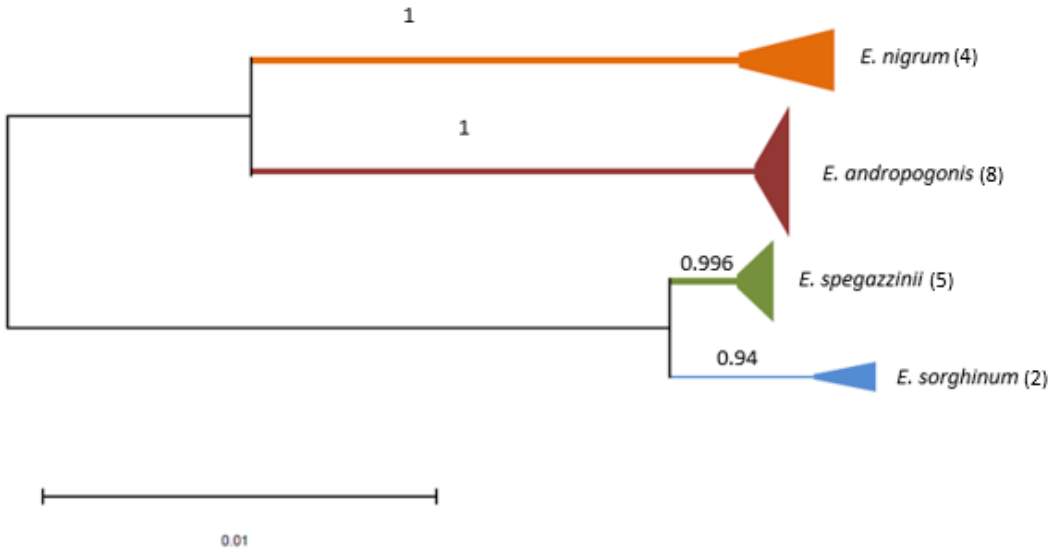


Figure 2.10. Maximum Likelihood phylogeny of the 19 *Epicoccum* isolates based on the combined sequenced regions (1,377 bp) of ITS region (503 bp), β-tubulin gene (287 bp), and elongation factor 1-alpha gene (587 bp). Bootstrap values are present on the branches. No *Epicoccum* reference sequences were included due to no single isolate having all 3 genes published on the NCBI database.

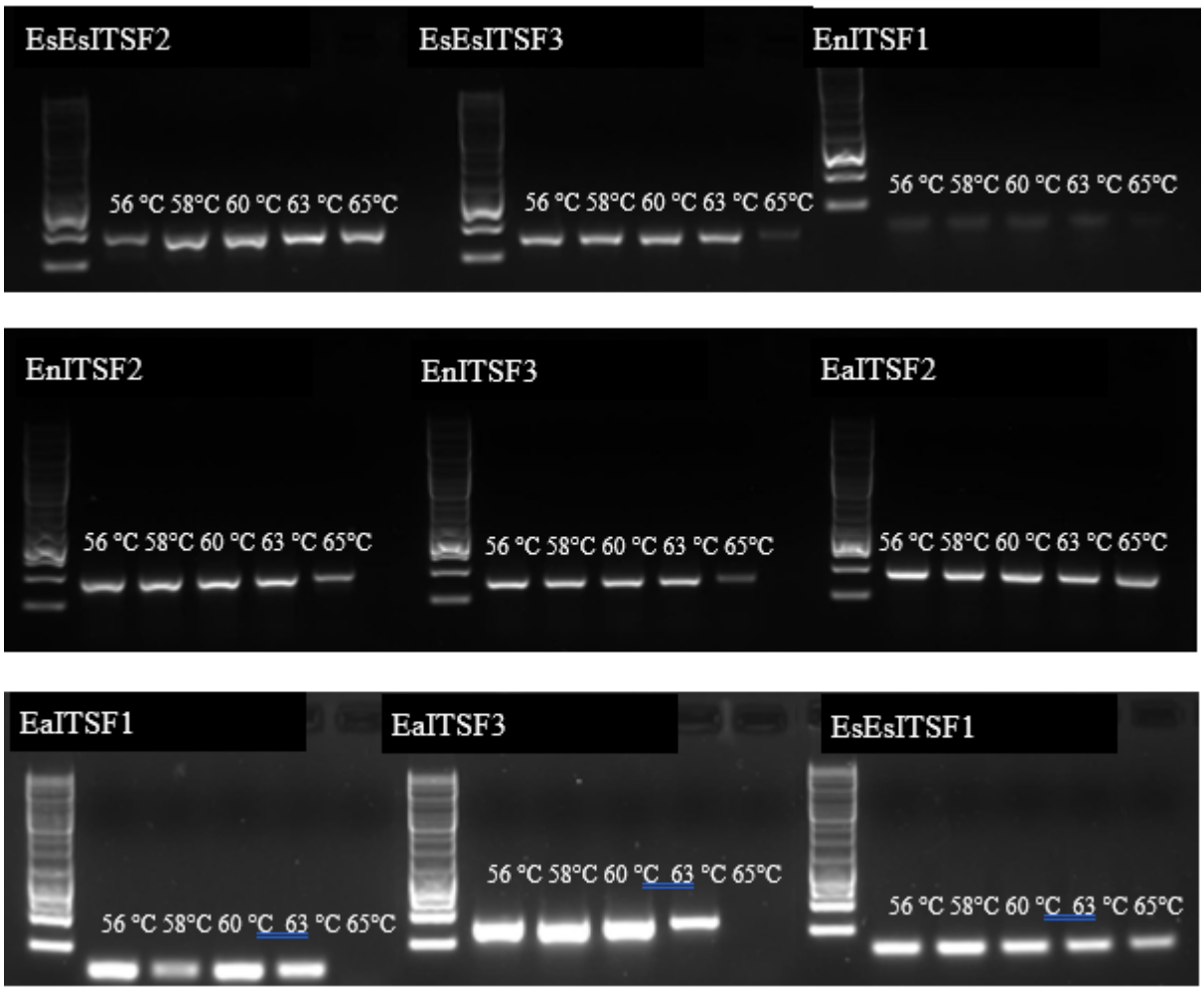


Figure 2.11. Optimal annealing temperature determined by temperature gradient from 56 °C to 65 °C for *Epicoccum* primer sets. *E. andropogonis* isolate M-CBI-W-EA (EaITSF1, EaITSF2 and EaITSF3), *E. nigrum* isolate J4-CBI-W-En (EnITSF1, EnITSF2 and EnITSF3), and *E. sorghinum* isolate E-BESC-W-ESO (EsEsITSF1, EsEsITSF2, and EsEsITSF3).

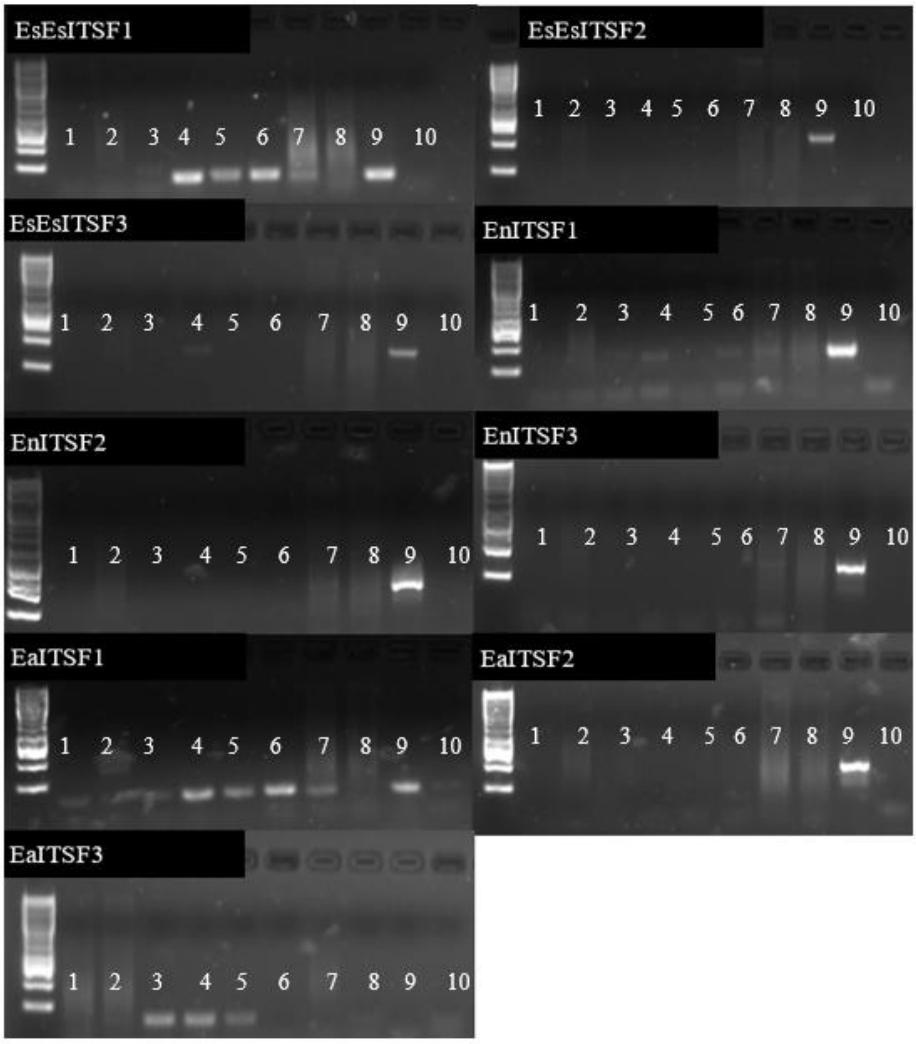


Figure 2.12. Specificity tests of primer sets with annealing temperature of 58 °C against non-target species *Fusarium oxysporum* isolate M1-BESC-W-F (1), *Coniochaeta* isolate M3-CBI-T-Co (2), *Nigrospora* sp. isolate M4-CBI-T-Ni (3), *Alternaria* sp. isolate M10-CBI-W-Al (4), *Curvularia* sp. isolate M11-CBI-W-Cu (5), *Bipolaris* sp. isolate M12- CBI-W-Bi (6), Summer leaf DNA (7), Alamo leaf DNA (8), and target *Epicoccum* DNA as a positive control (9), and water control (10). *E. andropogonis* isolate M-CBI-W-EA (EaITSF1, EaITSF2 and EaITSF3), *E. nigrum* isolate J4-CBI-W-En (EnITSF1, EnITSF2 and EnITSF3), and *E. sorghinum* isolate E-BESC-W-ESO (EsEsITSF1, EsEsITSF2, and EsEsITSF3).

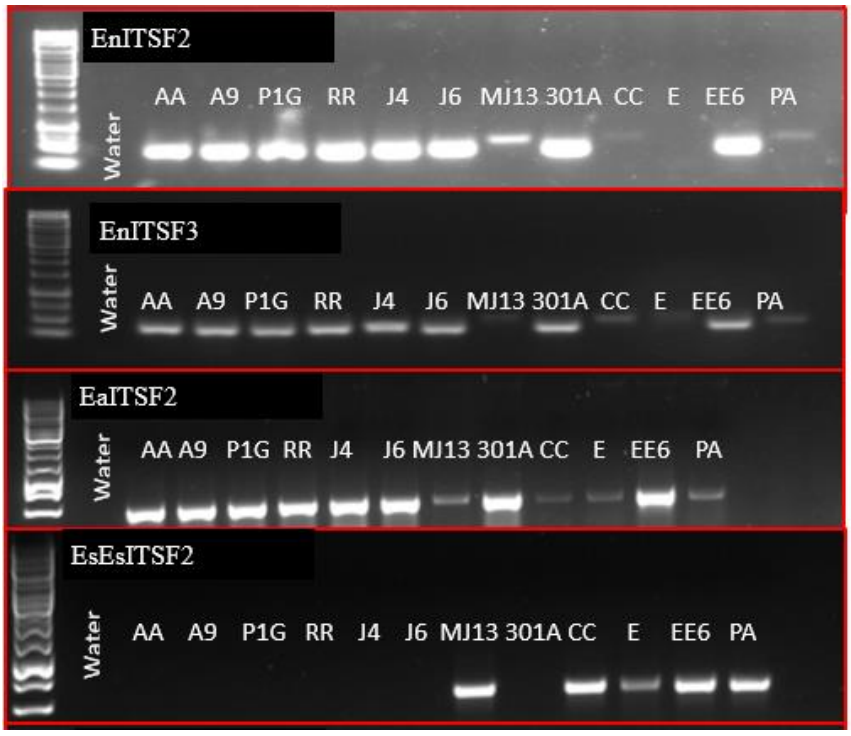


Figure 2.13. *Epicoccum* species-specific primer test against 4 *E. andropogonis* (AA-CBI-W-EA, A9-CBI-W-EA, P1G-BESC-W-EA, and RR-CBI-W-EA), 4 *E. nigrum* (J4-CBI-W-EN, J6- CBI-W-EN, MJ13-CBI-T-EN, and 301A-CBI-T-EN), 2 *E. sorghinum* (E-CBI-T-ESO and PA-CBI-W-ESO), and 2 *E,spegazzinii* (CC-CBI-T-ESP and EE6-CBI-W-ESP).

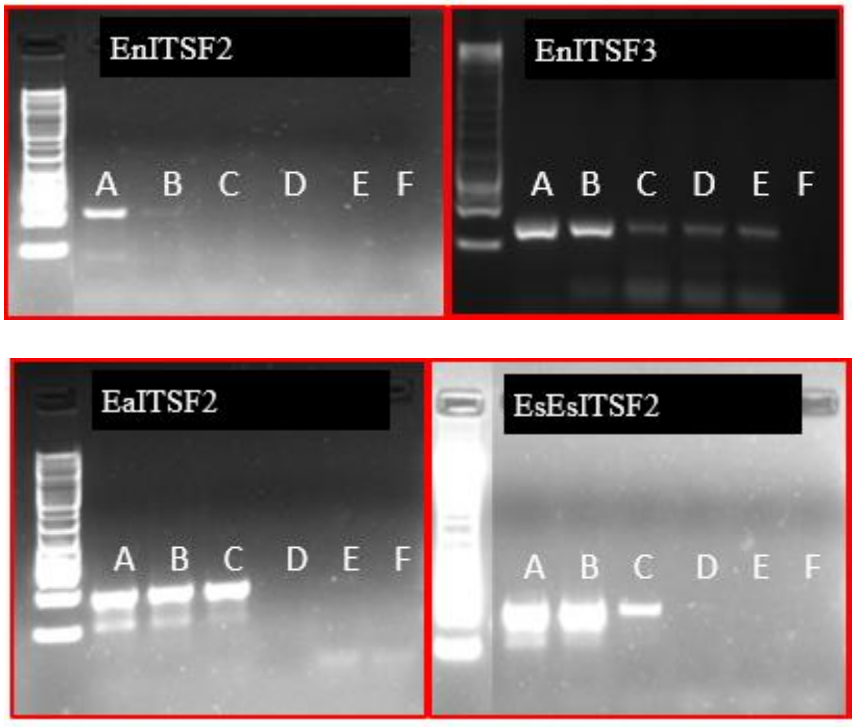


Figure 2.14. Sensitivity tests on species-specific primer sets at 50 ng/µL (A), 5 ng/µL (B), 0.5 ng/µL (C), 0.05 ng/µL (D), 0.005 ng/µL (E) and water (F). *E. andropogonis* isolate M-CBI-W-EA (EaITSF2), *E. nigrum* isolate J4-CBI-W-En (EnITSF1, EnITSF2 and EnITSF3), and *E. sorghinum* isolate E-BESC-W-ESO (EsEsITSF2, and EsEsITSF3).

CHAPTER III

EFFECT OF EPICOCCUM SPECIES ASSOCIATED WITH FALSE SMUT ON SWITCHGRASS SEEDLING SURVIVAL AND PHENOTYPIC TRAITS AT MATURITY

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ABSTRACT

False smut is a fungal disease of flowers that was observed on switchgrass (*Panicum virgatum)* for the first time in Georgia and Tennessee in 2019. Four *Epicoccum* species, *E. andropogonis, E. nigrum, E. sorghinum* and *E. spegazzinii* were associated with false smut symptoms on switchgrass florets. The four *Epicoccum* isolates were inoculated independently on accessions Summer, Alamo and Blackwell seedlings to determine their pathogenic or endophytic status. *E. sorghinum* and *E. spegazzinii* significantly ($p<0.01$) lowered the survival rate of the Summer and Blackwell switchgrass seedlings by 13% compared to the non-inoculated control but did not affect Alamo seedlings survival rate. *E. andropogonis* and *E. nigrum* did not affect the survival rate of all three accessions and exhibited a non-pathogenic status. Seedling inoculations with each of the four *Epicoccum* spp. were utilized on Summer and Alamo switchgrass to determine potential effects on sixteen adult plant phenotypes at plant maturity through two experiments. Overall, the results showed *Epicoccum* species × accession × experiment effect on at least four of the sixteen traits measured. The non-pathogenic *Epicoccum* species tested showed distinct behavior in Summer and Alamo accessions with potential beneficial effects on upland vs. lowland ecotypes. *Epicoccum andropogonis* and *E. nigrum* significantly ($p<0.001$) increased 8 traits related to biomass or had no effect depending on the experiment for the Summer accession. However, a decrease ($p<0.001$) was observed in 6 and 7 traits out of the sixteen traits related to biomass in the Alamo accession inoculated with *E. andropogonis* and *E. nigrum*, respectively.

INTRODUCTION

Panicum virgatum (switchgrass) was selected in the U.S. as the model herbaceous crop to produce biofuel (Wright, 2007). Switchgrass has beneficial effects on soil, water, and wildlife habitat and the amount and quality of energy obtained in the form of cellulosic ethanol are high (Mclaughlin, 1998). Switchgrass requires low nutrient inputs that allow it to be grown on marginal lands that can no longer support the growth of conventional crops. There are two distinct ecotypes of switchgrass: lowland ecotypes are found in the southern U.S. and upland ecotypes are grown in southern Canada and the northern U.S. (Hultquist, 1996; Lewandowski, 2003). The upland and lowland ecotypes exhibit different phenotypic traits. The lowland ecotype is adapted to humid, warm environments, has a longer growth period, later flowering, thick stems, and grows taller than the upland which is adapted to cold semi-arid environments, and exhibits opposite growth behavior (Porter, 1966). Several *Epicoccum* spp. have been shown to be beneficial endophytes that improve plant development. Endophytes are microorganisms that form a symbiotic relationship or a neutral interaction with a host plant for part of the host life cycle. They can improve the growth and overall health of the host plant by means of osmotic balance, regulation of stomata, root morphology modification, mineral uptake enhancement, and alteration of metabolism (Anyasi, 2019).

Exploration of the endophytes in switchgrass is required to understand the potential benefits that could be provided by these fungi to biomass production. Endophytes from 18 different taxonomic groups were isolated from a population of upland switchgrass in Oklahoma including species of *Alternaria*, *Codinaeopsis*, *Fusarium*, *Gibberella*, *Hypocrea* and *Periconia* (Ghimire, 2011). Endophytes constituted 50% and 58% of the shoot and root fungal

communities, respectively (Ghimire, 2011). *Epicoccum nigrum* increased total shoot and root biomass production by 25-33% of infected upland switchgrass (Kleczewski et al. 2012). In a similar study, 86% of 1,339 fungal endophytes (*Sordariomycetes*, *Hypocreales*, and *Fusarium* spp.) isolated from a monoculture switchgrass field in Kentucky and reinoculated in Alamo switchgrass increased plant height (Xia, 2018).

Epicoccum spp. have also potential as biocontrol agents. Application of *E. nigrum* 7 days before inoculation of *Claviceps africana,* and an additional application of *E. nigrum* three days after *C. africana*, significantly reduced the severity of ergot disease on sorghum bicolor in greenhouse trials (Bhuiyan, 2003). However, some *Epicoccum* species were also considered as weak pathogens and have been reported on at least 46 host plants (Taguiam, 2021). *E. nigrum* inoculation resulted in disease on both sugar beet (*Beta vulgaris ssp. vulgaris*) and red clover (*Trifolium pratense*) (Ogórek, 2020). *E. andropogonis* has been reported as an epiphyte on the fungus *C. purpurea* and this association resulted in false smut development in sugarcane (Singh, 1976). False smut of switchgrass, presumably caused by *Epicoccum* spp. colonizing ergot of *Claviceps* in flowers, has also recently been reported (Bahri, unpublished; Chapter 2). However, it is unknown if *Epicoccum* spp. associated with false smut of switchgrass are endophytic or pathogenic to the host.

The objectives of this study were to determine the effect of four *Epicoccum* species associated with false smut on switchgrass seedling survival and adult phenotypic traits after inoculations to confirm their endophytic or pathogenic status (Figure 3.1).

MATERIALS AND METHODS

Fungal cultures

Epicoccum nigrum (isolate J4-CBI-W-EN), *E. andropogonis* (isolate M-CBI-W-EA), *E. sorghinum* (isolate D-CBI-T-ESO), and *E. spegazzinii* (isolate E-CBI-T-ESP) (Table 3.1) were isolated from switchgrass florets presenting false smut symptoms (Chapter 2) and stored on PDA plates at 4° C in 24-hour dark. Working cultures of each fungus were obtained by culturing on PDA amended with 1 µg ml^{-1} chloramphenicol for 14 days at 25° C in a 24-hour dark cycle incubator (Isotemp 637F Incubator Oven; Fisher Scientific Pittsburgh, PA).

Seedling inoculations with *Epicoccum* species

Seeds of the upland switchgrass ecotypes, Summer and Blackwell, and seeds of the lowland ecotype, Alamo, were propagated by Applewood Seed Co. in Arvada, Colorado and ordered through U.S. National Plant Germplasm System (https://npgsweb.ars-grin.gov/gringlobal/accessiondetail?id=1098746). Seeds were stored at 23° C in 24-hour dark upon arrival at UGA Griffin campus. Prior to all assays, seeds were surface sterilized for 3 min in 3% sodium hypochlorite, 70% ethanol for 3 min, and rinsed with sterile water 3 times. Surface sterilized seeds (25) were then placed on moistened, sterilized 9 cm filter paper (VWR, Radnor, PN) in sterile 9 cm petri dishes (VWR, Radnor, PN) and petri plates were sealed with parafilm (Sigma-Aldrich, St. Louis, MO) and were incubated at 23° C for 14 days on a benchtop. Individual seedlings were then transferred to a plastic magenta GA-7 plant culture box (PlantMedia) with sterilized 9 cm filter paper (VWR, Radnor, PN) moistened with 10 ml of sterile, distilled water. Mycelial plugs (1 cm × 1 cm) from a 14-day culture of *Epicoccum* spp. were placed beside the seedlings in the box and incubated for 7 days to allow for the mycelium to infect seedlings. The seedlings in the control group were mock-inoculated with a 1 cm × 1 cm plug of PDA amended with 1 µg ml^{-1} chloramphenicol (Fisher Bioreagents, Pittsburgh, PA) (Kleczewski et al., 2012).

Effect of *Epicoccum* spp. on seedling survival

Individual seedlings (12 germinated seedlings with emerging leaf of 1 cm) from Summer, Alamo, and Blackwell accessions were inoculated as described above with 24 mycelium plugs for a 2:1 plug: seedling ratio. Seedlings were incubated in magenta boxes for 7 days and then transplanted into cone-tainers (Greenhouse Megastore, Danville, IL) filled with Sun Gro Professional growing mix (Agawam, MA) and placed on a greenhouse bench (average daytime and nighttime temperature of 26° C and 22° C, respectively). After 7 days, the establishment of switchgrass seedlings was accessed to determine survival rate (%). Z distribution was used to compare the percentage of survival of each *Epicoccum* treatment group to the non-inoculated control group in R studio (Rstudio Team, 2020). The experiment was conducted twice for each switchgrass accession with 5 treatment groups (4 different inoculated treatments corresponding to each of the *Epicoccum* species and one non-inoculated control group). Each treatment group was represented by 12 seedlings. A single switchgrass seedling was one experimental unit. Z distribution was performed on the data codified on a binary system (0 for survived seedling and 1 for dead seedling) to determine the experiment effect on survival data (Rstudio Team, 2020).

Effect of *Epicoccum* spp. on switchgrass growth phenotypes at maturity

Seedlings of Summer (220) and Alamo (220) were inoculated with 1 cm × 1 cm fungal plugs of *E. nigrum*, *E. andropogonis*, *E. sorghinum*, or *E. spegazzinii*, respectively, and mock-inoculated with plug of PDA chloramphenicol as a control at a ratio of 1:1 (plug: seedling) as described above. After 7 days, switchgrass seedlings were planted in cone-tainers filled with Sun Gro Professional growing mix and placed on a greenhouse bench (average daytime and nighttime temperature of 26° C and 22° C, respectively) at the University of Georgia Griffin campus, Griffin, GA until the majority of plants had flowered and set seed (Summer, 6 months;

Alamo, 10 months). Plants were watered as needed. After 81 days, the switchgrass cone-tainers were placed in shallow bins (15 cone-tainers per bin) filled with water. After 90 days, plants were fertilized monthly with 20-20-20 General Purpose fertilizer (J. R. Peters, Inc; Allentown, PA 18106). Once the majority of plants had matured in the control treatment, 16 phenotypic traits were measured including plant height (cm), panicle length (cm), number of tillers per plant, tiller diameter (mm), percentage of flowering tillers per plant (%), percentage of flowering plants per treatment group (%), fresh shoot biomass per plant (g), dried shoot biomass per plant (g), fresh root biomass per plant (g), and dry root biomass per plant (g). A main tiller, defined as the tallest flowering tiller, was assigned to each flowering plant. Main tiller height (cm), panicle length, (cm) diameter (mm), flower count, and reproductive development stage were separated and analyzed.

Plant height was measured from the crown node at the soil surface for each tiller of the plant. Flowering tillers were measured to the top of the panicle while nonflowering tillers were measured to the tallest node. Tillers taller than 10 cm were averaged to determine the overall plant height for a single plant. Tillers shorter than 10 cm were excluded. Panicle length was measured from the flag leaf node to the top of the panicle and averaged across a plant. Individual tillers of a plant were measured with a digital caliber (General Ultratech, New York, NY) between the first and second node. Single tillers with less than two nodes were removed from data set. Peduncles were removed from the panicle of flowering tillers and counted to determine flower count for the main tiller of the plant. Flowers and seeds from each flowering tiller were counted and then assigned a reproductive developmental stage (Figure 3.2). The score scale, as described by Moore (1991), consisted of the boot stage (R0), panicle emergence (R1), pendula elongation (R2), emergence of reproductive structures (R3), fertilization/ presence of caryopsis

(R4), soft dough (S1), hard dough (S2), and mature seed (S3) (Fig 3.2). The reproductive developmental stages were converted to a 1-8 scale (R0=1, S3=8). The number of flowering tillers were divided by the total number of tillers to determine the percentage of flowering tillers per plant. Only Plants that flowered were used to determine an average for percentage of flowering tillers per plant for each treatment group. The average percentage of flowering tillers per plant for each treatment group was compared to the non-inoculated control group.

The percentage of flowering plants was found by dividing the flowering plants by the total number of plants in the respective treatment group. Fresh shoot biomass was measured after cutting each tiller (including panicle with peduncle removed) from the crown node at the soil's surface. Tillers were stored in a brown paper bag and dried for 48 hours at 60 °C (BlueM laboratory oven model number LO-850-p, New Columbia, PA) and dry shot biomass determined. Roots were removed from cone-tainers, gently washed to remove potting mix, weighed to obtained root biomass then stored in a brown paper bag and dried for 48-hours at 60 °C to obtain dry root biomass.

The experiment was conducted twice (started 2 weeks apart) and had 5 treatment groups (four *Epicoccum* species and one non-inoculated control group). Each treatment group had 15 single plant replicates. A single switchgrass plant was one experimental unit grown in a single cone-tainer. Analysis of variation (ANOVA) and a post-hoc honest significant difference test (post-hoc Tukey's HSD) was performed on the plant height, panicle length, diameter, flower count, reproduction stage, fresh shoot biomass (fresh and dry), and root biomass (fresh and dry) data for all pairwise comparisons between treatment groups. A z distribution was performed on percentage of flowering tillers per plant and percent flowering plants by treatment group. All statistical tests were performed in R studio (Rstudio Team, 2020) to determine significant

differences between treatment groups for all phenotypic traits (10 whole plant traits; 5 main tiller traits) at $\alpha < 0.05$.

RESULTS

Effect of *Epicoccum* species on seedling survival

Summer, Alamo, and Blackwell seedlings were inoculated with *E. nigrum, E. andropogonis, E. spegazzinii* or *E. sorghinum.* This experiment was conducted twice and z distribution test revealed no treatment × experiment interaction so data from both experiments were combined. *E. andropogonis,* and *E. nigrum* inoculated Summer, Alamo, and Blackwell seedlings resulted in pink and yellowish orange root coloration, respectively, after the 7-day incubation period (Figure 3.3). When inoculated with *E. nigrum* Summer and Blackwell seedlings were observed to have a decrease ($p<0.0005$) in survival rate. Seedlings inoculated with *E. sorghinum* and *E. spegazzinii* displayed root necrosis on the three switchgrass accessions (Figure 3.3). Summer and Blackwell seedlings inoculated with *E. sorghinum* had a significant ($p<0.00001$) reduction in survival (29% and 4%, respectively) compared to the control group. Inoculation with *E. spegazzinii* also reduced survival rate ($p<0.00001$) in Summer (17%) and Blackwell (0%) accessions compared to control group (Table 3.2). No significant difference was observed on the Alamo accession when inoculated with *E. sorghinum* and *E. spegazzinii.* No significant decrease in survival was observed for *E. andropogonis* on the three accessions.

Effect of *Epicoccum* inoculations on sixteen switchgrass phenotypic traits at maturity

Data from the two experiments were tested to determine if there was a treatment × experiment × ecotype interaction and found that at least 9 of the 16 traits had a significant interaction so all traits were separated by accession and by experiment.

Effect of *E. spegazzinii*

Inoculation of Summer seedlings with *E. spegazzinii* was associated with significantly ($p<0.001$) lower dry shoot biomass and a decrease in percentage of flowering plants per treatment group compared to the non-inoculated control plants in the first experiment (Table 3.3). No other differences were observed on the other traits. In the second experiment, inoculation of Summer seedlings with *E. spegazzinii* was associated with significant ($p<0.001$) increases in ten phenotypic traits (plant height, panicle length, flowering time, tiller diameter, percentage of flowering tillers per plant, percentage of flowering plants per treatment group, fresh shoot and root biomass, and dry shoot and root biomass) (Table 3.4). Inoculation of Alamo seedlings with *E. spegazzinii* was associated with significantly lower tiller diameter ($p<0.001$), percentage of flowering tiller per plant ($p<0.001$) and percentage of flowering plants per treatment group ($p<0.0001$) compared to the non-treated control plants in the first experiment (Table 3.5). Inoculation with *E. spegazzinii* resulted in significant ($p<0.001$) increases in plant height, panicle length, tiller diameter, fresh shoot biomass and percentage of flowering tillers per plant in Alamo (Table 3.6).

Effect of *E. sorghinum*

Inoculation of Summer seedlings with *E. sorghinum* decreased the percentage of flowering tillers per plant, but did not affect any other adult plant phenotypes compared to the non-inoculated control plants in the first experiment (Table 3.3). In the second experiment, *E. sorghinum* treatment was associated with significantly ($p<0.05$) greater dry shoot biomass (Table 3.4). Inoculation of Alamo seedlings with *E. sorghinum* significantly ($p<0.001$) decreased tiller diameter in the first experiment (Table 3.5) but increased tiller diameter in the second experiment (Table 3.6).

Effect of *E. andropogonis*

Summer plants inoculated with *E. andropogonis* had significant ($p<0.001$) increase number of tillers per plant, percentage of flowering tillers per plant and percentage of flowering plants per treatment group than the non-inoculated control plants in the first experiment (Table 3.3). However, Summer plants inoculated with *E. andropogonis* only showed increased ($p<0.001$) percentage of flowering tillers per plant in the second experiment (Table 3.4). Seedling inoculation of Alamo switchgrass with *E. andropogonis* was associated with significantly ($p<0.001$) shorter plant height compared to the non-inoculated control in the first and second experiment (Table 3.5). In the second experiment, *E. andropogonis* treatment also resulted in significant ($p<0.001$) decreases in number of tillers per plant, fresh shoot biomass, percentage of flowering tillers and percentage of flowing plants per treatment group in the second experiment (Table 3.6).

Effect of *E. nigrum*

Inoculation with *E. nigrum* showed a decrease in percentage of flower tillers per plant compared to the non-inoculated control in the first experiment (Table 3.3). In the second experiment, significant ($p<0.001$) increases were observed in nine adult phenotypes of Summer including plant height, tiller diameter, flowering time, percentage of flowering tillers per plant, percentage of flowering plants per treatment group, shoot and root biomasses (fresh and dry) (Table 3.4). Seedling inoculation of Alamo switchgrass with *E. nigrum* resulted a significant ($p<0.001$) increase in plant height and increased tiller diameter compared to the non-inoculated control plants in the first experiment (Table 3.5). However, *E. nigrum* treatment was associated with significant ($p<0.001$) decrease in plant height, number of tillers per plant, tiller diameter, fresh shoot biomass, percentage of flowering tillers per plant and percentage of flowering plants by treatment group in the second experiment (Table 3.6).

DISCUSSION

False smut of switchgrass is presumed to be caused by *Epicoccum* spp. colonizing ergot of *Claviceps* in switchgrass flowers (Chapter 2). *Epicoccum* spp. can have endophytic or pathogenic life strategies depending on the host plant. Exploration of endophytes in switchgrass is needed. The objectives of the present study were to determine if four *Epicoccum* species isolated from switchgrass florets presenting false smut symptoms affected seedling survival and adult plant phenotypes of upland and lowland switchgrass ecotypes.

Switchgrass ecotypes were differentially affected at maturity by the *Epicoccum* species in the present study. In this study, *E. nigrum* was shown to increase the fresh shoot biomass of the Summer accession roots that grew to maturity in the second inoculation experiment. *E. nigrum* was also reported as an endophyte in sugarcane and increased roots biomass compared to non-inoculated control plants (Fávaro, 2012). However, *E. nigrum* lowered the fresh shoot biomass production in the Alamo accession in the second experiment of the study. The same trend was observed with *E. andropogonis* increasing tiller count in Summer accession, but lowering multiple phenotypic traits related to biomass in the Alamo accession.

Two of the species, *E. andropogonis* and *E. nigrum*, changed root color morphology to pinkish-red and yellowing orange, respectively. *E. andropogonis* did not cause seedling mortality (on all three switchgrass accessions) suggesting this species is not pathogenic on switchgrass. However, *E. nigrum* lowered the survival rate of Summer and Blackwell seedlings by 31% and 52%, respectively. A gradient of seeling to fungal plug ratio could reveal that an increase in exposure level of all the *Epicoccum* species result in a decrease in survival rate. Other plant endophyte species have been shown to modify root morphology. *Neotyphodium coenophialum*

caused an increase in root hair and diameter of lateral roots compared to non-infected tall fescue plants (Malinowski, 1999).

Seedling inoculations with *E. spegazzinii* and *E. sorghinum* resulted in necrotic root morphology and increased mortality of upland ecotype (Summer and Blackwell accessions) compared to non-inoculated seedlings. However, on the lowland ecotype tested (Alamo accession), *E. sorghinum* and *E. spegazzinii* were not pathogenic. *Epicoccum* species have been reported to act as pathogens on 46 host plants (Taguiam, 2021). Specifically, *E. sorghinum* has been reported to cause the disease leaf spot and leaf sheath on maize and disease of dragon fruit (Chen, 2021; Taguiam, 2020). *E. sorghinum* and *E. spegaazinii* were shown to be more genetically similar than *E. andropogonis* and *E. nigrum* which suggests the two species may have a similar biological impact on host plants (Chapter 2). Reduced germination due to *Epicoccum* spp. (or other pathogens) could pose a problem when establishing switchgrass fields. In the United States, there are some potential issues in the development of switchgrass fields including cost, and space (Soldavini, 2018). The establishment year presented the greatest impact value, in factors related to greenhouse gas emissions, when accessing a 4-year growth cycle of switchgrass in the Mediterranean region of Spain (Escobar et al., 2017). The establishment year is also the costliest year for farmers because switchgrass energy is primarily focused on the growth of the root system (Hultquist, 1996; Lewandowski, 2003). Little research has been conducted on the potential negative impact seed-borne disease may pose on switchgrass seedlings and the economic loss when establishing switchgrass fields. Switchgrass seedlings are exposed to disease that persist in the field soil.

Based on results from the seedling mortality study, we assumed that *E. sorghinum* and *E. spegazzinii* could negatively affect adult plant phenotypes of upland ecotype. However, clear

effects were not observed. One explanation could be that the seedling inoculation assay did not result in colonization of the switchgrass plants through maturation. Increased specificity and sensitivity of the primers designed in Chapter 2 would allow testing of different plant tissues for the presence or absence of *Epicoccum* spp. A second explanation could be that the negative effects of *E. sorghinum* and *E. spegazzinii* selected for switchgrass seedlings that were either resistant or had increased fitness compared to the seedlings that died in the assay. The relationship between inoculation dose (i.e. the amount of mycelium exposure for each seedling) and survival is unknown. Colonization of the roots at a level that does not kill them could allow for *E. sorghinum* or *E. spegazzinii* to then affect adult plant phenotypes. This needs further investigation.

The results of this study concurred with previous findings that *E. nigrum* has the potential benefits on the upland ecotype but could have a negative impact on the lowland ecotype. In this study, switchgrass infected with *E. andropogonis* or *E. nigrum* showed similar morphological changes on seedling roots. Further evaluation of *Epicoccum* spp. impact on switchgrass growth and development is required to determine its potential beneficial effects on biomass traits.

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Table 3.1. *Epicoccum* spp. used for switchgrass (Summer and Alamo) inoculations. Species, isolate ID, panel and field location and collection year are listed.

***Epicoccum* species**	**Isolate ID**	**Field location**	**Collection year**
E. andropogonis	M-CBI-W-EA	CBI-Watkinsville	2021
E. nigrum	J4-CBI-W-EN	CBI-Watkinsville	2021
E. sorghinum	D-CBI-T-ESO	CBI-Tifton	2020
E. spegazzinii	E-CBI-T-ESP	CBI-Tifton	2020

Table 3.2. Percent survival rate (p-value from Z distribution test compared to non-inoculated control group) of switchgrass upland (Blackwell and Summer) and lowland (Alamo) inoculated with *Epicoccum* spp. as well as in the non-inoculated control. Survival rates were evaluated across 2 independent experiments and 12 experimental units (seedling) within experiment.

Ecotypes	**Non-inoculated**	***E. andropogonis***	***E. nigrum***	***E. sorghinum***	***E. spegazzinii***
Alamo	80	85 (NS)	80 (0.0003)	55 (0.00001)	71 (0.00001)
Blackwell	88	72 (NS)	36 (0.0002)	4 (0.00001)	0 (0.00001)
Summer	100	96 (NS)	69 (NS)	29 (NS)	17 (NS)

Table 3.3. Means of the sixteen phenotypic traits assessed for *Epicoccum* spp. inoculated and non-inoculated Summer switchgrass after 6 months (Experiment 1).

Phenotypic traits	Non-inoculated		*E. andropogonis*		*E. nigrum*		*E. spegazzinii*		*E. sorghinum*		P-value[a]
Plant height (cm)	40.8 (3.7)	abc	54.7 (3.9)	a	37.7 (3.6)	bc	26.5 (3.2)	c	54.6 (4.8)	ab	<0.001
Panicle length (cm)	38.6 (6.1)	a	39.3 (1.9)	a	28.3 (1.2)	a	NA	NA	40.4 (3.0)	a	NS
Tiller count	5.4 (0.4)	bc	8.9 (1.4)	a	4.9 (0.7)	bc	3.0 (0.5)	c	6.9 (0.9)	ab	<0.001
Tiller diameter (mm)	1.19 (0.08)	ab	1.33 (0.06)	a	1.12 (0.07)	ab	0.94 (0.10)	a	1.33 (0.07)	ab	<0.05
Flowering time (days)	97.7 (6.4)	a	109.8 (5.6)	a	93.0 (13.0)	a	NA	NA	104.1 (3.8)	a	NS
Percentage of flowering tillers (%)	9.90	b	33.8	a	3.90	b	0	b	25.2	a	<0.001
Percentage of flower plant per treatment (%)	40.0	b	80.0	a	13.3	c	0	c	60.0	b	<0.001
Fresh shoot biomass (g)	7.31 (0.5)	abc	13.8 (1.4)	a	5.50 (0.3)	bc	2.42 (0.2)	ab	10.5 (1.0)	c	<0.001
Fresh root biomass (g)	28.1 (5.4)	abc	40.6 (7.3)	a	17.7 (2.6)	bc	3.48 (1.0)	c	40.3 (7.9)	ab	<0.001
Dry shoot biomass (g)	2.96 (1.1)	ab	5.73 (3.3)	a	2.09 (0.7)	bc	0.88 (0.5)	c	4.27 (2.3)	a	<0.001
Dry root biomass (g)	3.79 (0.6)	abc	6.34 (1.3)	a	2.60 (0.6)	bc	0.76 (0.2)	c	6.20 (1.4)	ab	<0.001
Main tiller height (cm)	89.1 (15.7)	a	116.5 (11.4)	a	76.8 (2.8)	a	NA	NA	124.9 (7.5)	a	NS
Main tiller panicle length (cm)	31.5 (5.4)	a	44.1 (5.4)	a	27.8 (1.8)	a	NA	NA	46.2 (4.7)	a	NS
Main tiller diameter (mm)	1.8 (0.22)	a	2.0 (0.16)	a	1.7 (0.21)	a	NA	NA	2.1 (0.19)	a	NS
Main tiller flower count	45.3 (16.4)	a	74.8 (10.7)	a	54.5 (46.8)	a	NA	NA	118.1 (18.1)	a	NS

Main tiller reproductive score	6.3 (0.24)	a	6.3 (0.44)	a	7.3 (0.25)	a	NA	NA	7.2 (0.24)	a	NS

[a] NS = not significant

NA = Not available (traits not observed in treatment group)

Table 3.4. Means of the sixteen phenotypic traits assessed for *Epicoccum* spp. inoculated and non-inoculated Summer switchgrass after 6 months (Experiment 2).

Phenotypic traits	**Non-inoculated**		***E. andropogonis***		***E. nigrum***		***E. spegazzinii***		***E. sorghinum***		**P-value[a]**
Plant height (cm)	41.7 (3.6)	c	59.0 (4.6)	c	84.6 (5.0)	b	109.7 (7.2)	a	47.6 (4.3)	c	<0.001
Panicle length (cm)	33.8 (4.9)	b	47.4 (3.1)	ab	47.2 (2.9)	ab	54.8 (3.2)	a	44.4 (3.7)	ab	<0.05
Tiller count	6.0 (0.8)	a	6.4 (0.8)	a	5.7 (0.5)	a	5.1 (0.9)	a	6.3 (0.5)	a	NS
Tiller diameter (mm)	1.07 (0.06)	c	1.28 (0.08)	c	1.66 (0.06)	ab	1.82 (0.12)	a	1.37 (0.08)	bc	<0.001
Flowering time (days)	111.6 (6.4)	a	98.4 (2.6)	ab	82.9 (3.5)	b	81.8 (5.5)	b	97.8 (3.2)	ab	<0.001
Percentage of flowering tillers (%)	12.2	b	30	a	60.5	a	72.5	a	13.1	b	<0.001
Percentage of flower plant per treatment (%)	47.0	b	40.0	b	100	a	100	a	33.0	b	<0.001
Fresh shoot biomass (g)	7.28 (0.9)	a	11.2 (2.7)	ab	15.3 (0.9)	bc	21.5 (2.0)	bc	8.62 (1.7)	a	<0.001
Fresh root biomass (g)	17.0 (2.3)	c	25.2 (6.7)	bc	41.7 (2.6)	ab	43.9 (5.2)	a	22.7 (4.7)	bc	<0.001
Dry shoot biomass (g)	2.93 (0.4)	c	4.15 (1.2)	bc	6.16 (0.39)	ab	8.39 (0.83)	bc	3.31 (0.78)	a	<0.05
Dry root biomass (g)	2.22 (0.36)	c	3.6 (1.1)	bc	6.75 (0.33)	a	6.71 (0.8)	a	3.36 (0.8)	bc	<0.001
Main tiller height (cm)	117.7 (11.3)	ab	107.9 (13.9)	b	130.0 (8.6)	a	161.1 (5.7)	a	103.7 (10.8)	b	NS
Main tiller panicle length (cm)	40.1 (6.5)	a	57.3 (5.3)	a	52.7 (5.9)	a	68.8 (4.2)	a	40.3 (6.0)	a	NS
Main tiller diameter (mm)	1.9 (0.11)	a	1.8 (0.22)	a	2.2 (0.14)	a	2.3 (0.23)	a	2.1 (0.45)	a	NS
Main tiller flower count	51.2 (18.1)	b	122.2 (17.7)	ab	125.2 (33.0)	ab	230.1 (49.0)	a	61.0 (19.7)	ab	<0.01
Main tiller reproductive score	6.9 (0.66)	a	5.5 (0.80)	a	7.0 (0.26)	a	5.6 (0.99)	a	5.5 (0.52)	a	NS

[a] NS = not significant

Table 3.5. Means of the fourteen phenotypic traits assessed for *Epicoccum* spp. inoculated and non-inoculated Alamo switchgrass after 10 months (Experiment 1).

Phenotypic traits	**Non-inoculated**		***E. andropogonis***		***E. nigrum***		***E. spegazzinii***		***E. sorghinum***		**P-value[a]**
Plant height (cm)	77.5 (4.7)	bc	62.2 (3.3)	d	105.5 (4.6)	a	63.1 (2.9)	cd	88.3 (3.9)	b	<0.001
Panicle length (cm)	40.0 (5.8)	a	51.0 (11.1)	a	61.3 (5.8)	a	NA	NA	44.4 (6.9)	a	NS
Tiller count	4.9 (0.35)	a	7.5 (1.01)	a	5.8 (0.49)	a	5.1 (0.88)	a	6.0 (0.73)	a	NS
Tiller diameter (mm)	2.05 (0.08)	b	2.07 (0.06)	b	2.49 (0.06)	a	1.36 (0.06)	c	1.58 (0.06)	c	<0.001
Flowering time (days)	140.0 (5.0)	a	139.3 (12.0)	a	145.9 (3.2)	a	NA	NA	157.5 (13.5)	a	NS
Percentage of flowering tillers (%)	9.0	b	3.7	b	20.0	a	0	c	10.0	b	<0.001
Percentage of flower plant per treatment (%)	33.0	b	27.0	b	53.0	a	0	c	31.0	b	<0.001
Fresh shoot biomass (g)	14.2 (1.5)	ab	16.3 (2.3)	ab	22.2 (2.1)	a	11.3 (2.0)	b	20.9 (3.3)	a	<0.005
Dry shoot biomass (g)	7.7 (0.8)	ab	9.2 (1.3)	ab	12.0 (1.2)	a	5.7 (1.1)	b	10.6 (1.7)	ab	NS
Main tiller height (cm)	168.0 (6.8)	ab	123.4 (14.1)	b	170.0 (9.9)	a	NA	NA	140.5 (21.5)	ab	<0.05
Main tiller panicle length (cm)	44.5 (11.1)	a	51.0 (11.1)	a	63.4 (7.6)	a	NA	NA	38.6 (8.6)	a	NS
Main tiller diameter (mm)	2.77 (0.39)	a	2.90 (0.18)	a	3.08 (0.23)	a	NA	NA	2.52 (0.21)	a	NS
Main tiller flower count	180.3 (115.9)	a	96.8 (26.6)	a	87.6 (29.8)	a	NA	NA	39.0 (10.6)	a	NS

Main tiller reproductive score	5.5 (1.50)	a	5.6 (0.29)	a	6.4 (0.32)	a	NA	NA	5.3 (0.53)	a	NS

[a] NS = not significant

Fresh root biomass and dry root biomass were not measured for Alamo due to tangled root overgrowth.

Table 3.6. Means of the sixteen phenotypic traits assessed for *Epicoccum* spp. inoculated and non-inoculated Alamo switchgrass after 10 months. (Experiment 2).

Phenotypic traits	**Non-inoculated**		***E. andropogonis***		***E. nigrum***		***E. spegazzinii***		***E. sorghinum***		**P-value[a]**
Plant height (cm)	112.5 (5.1)	b	86.2 (5.5)	c	84.4 (3.6)	c	157.2 (7.3)	a	122.5 (7.3)	b	<0.001
Panicle length (cm)	64.0 (2.8)	bc	59.3 (4.2)	bc	48.7 (5.1)	c	79.9 (3.2)	a	79.4 (3.6)	ab	<0.001
Tiller count	10.0 (1.3)	a	4.8 (0.8)	b	5.9 (0.6)	b	13.7 (1.6)	a	9.4 (2.0)	ab	<0.001
Tiller diameter (mm)	2.21 (0.06)	b	2.18 (0.06)	b	1.72 (0.07)	c	2.58 (0.08)	a	2.72 (0.11)	a	<0.001
Flowering time (days)	144.5 (3.4)	ab	146.0 (10.5)	ab	162.0 (8.6)	a	136.8 (1.8)	ab	131.3 (7.9)	b	<0.05
Percentage of flowering tillers (%)	43.3	b	18.0	c	13.5	c	63.4	a	38.3	b	<0.001
Percentage of flower plant per treatment (%)	100	a	20.0	b	47.0	b	100	a	50.0	b	<0.001
Fresh shoot biomass (g)	42.1 (2.9)	b	14.0 (2.8)	c	18.7 (1.4)	c	68.2 (5.5)	a	42.2 (11.1)	b	<0.001
Dry shoot biomass (g)	25.2 (1.7)	b	8.2 (1.7)	c	11.2 (0.9)	c	52.0 (3.2)	a	27.1 (8.0)	b	<0.001
Main tiller height (cm)	112.5 (10.0)	b	86.2 (34.2)	c	84.4 (8.8)	c	157.2 (12.7)	a	122.5 (3.8)	b	<0.001
Main tiller panicle length (cm)	73.2 (5.4)	ab	53.8 (14.9)	ab	50.5 (8.6)	b	92.7 (8.5)	a	89.1 (8.9)	ab	NS
Main tiller diameter (mm)	2.71 (0.14)	a	2.79 (0.49)	a	2.64 (0.25)	a	3.01 (0.26)	a	2.96 (0.23)	a	NS
Main tiller flower count	205 (32.3)	b	200 (135.7)	b	82.0 (19.1)	b	491 (173.2)	a	213 (65.6)	ab	<0.05

Main tiller reproductive score	6.8 (0.29)	a	6.8 (0.73)	a	8.0 (0.0)	a	7.3 (0.75)	a	7.3 (0.44)	a	NS

[a] NS = not significant

Fresh root biomass and dry root biomass were not measured for Alamo due to tangled root overgrowth.

Figure 3.1. False smut symptoms in the CBI-Watkinsville field (A), false smut brain-like structure in switchgrass spikelet (B), and 14-day-old colonies of *E. andropogonis* (C), *E. nigrum* (D), *E. sorghinum* (E) and *E. spegazzinii* (F) on PDA media after 14 days of incubation at 25°C in the dark.

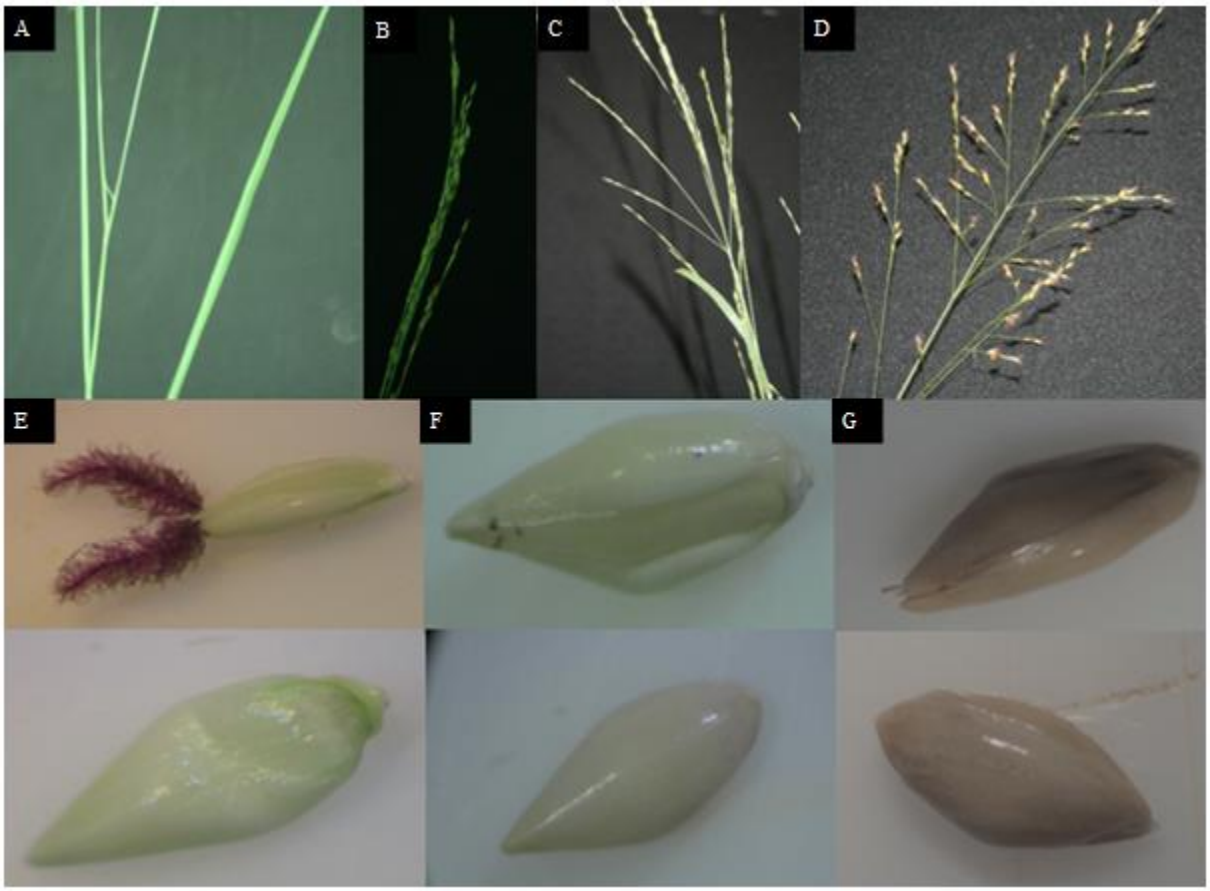

Figure 3.2. Stages of switchgrass flowering development as described by Hardin (2013) and Moore (1991): boot stage designated R0 (A), panicle emergence designated R1 (B), peduncle elongation designated R2 (C) and emergence of reproductive structures designated R3 (D); stages of seed development: soft dough designated S1 (E), hard dough designated S2 (F), and mature seed designated S3 (G).

Figure 3.3. Root morphology of Summer seedlings 7-days after inoculation with *Epicoccum nigrum* or *E. andropogonis* (potential endophytes on Summer accession) or *E. spegazzinii* or *E. sorghinum* (potential pathogens on Summer accession) and PDA control (non-inoculated). Similar root morphology color changes were observed on Blackwell seedlings (not shown in figure).