

**Characterization of *Fusarium* species and
Alternaria alternata, and their effects on
switchgrass health and chemical components**

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DEDICATION

I dedicate my thesis to my brother, James M. Collins.

I finally made it.

I wish you could be here to celebrate with me.

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I would like to acknowledge my friends, family, and colleagues for their support throughout this experience. I couldn't have done it without you. I would like to express my deepest appreciation to my committee chair, Dr. B.H. Ownley, for taking this wildlife biologist under her wing, and showing me the endless possibilities of scientific research. Without her guidance and persistence, this thesis would not be possible. I would like to thank my committee members, Dr. K.D. Gwinn, Dr. N. Labbé, and Dr. R. Trigiano, for their assistance and insight during the course of this project. I would like to give a special thanks to my lab mates A. Bruce, T. Collins, M. Dee, K. Mantooth, and S. Yerukala for pushing me to be the best researcher possible. I could not have asked for a better group of bright and talented individuals to work with. In addition, I would like to thank the Southeastern Partnership for Integrated Biomass Supply Systems, United States Department of Agriculture, and the University of Tennessee for funding this research.

ABSTRACT

Panicum virgatum L., readily referred to as Switchgrass, is a perennial warm-season bunch grass, used as an alternative energy source for biofuel production. There is insufficient research on switchgrass pathogens, and is expected that an increase in disease pressure will result as more land is reserved for this perennial crop. The purpose of this research was to identify and characterize pathogenic *Alternaria alternata* and *Fusarium* species on Switchgrass (*Panicum virgatum* L.), and to evaluate their impact on stand establishment and plant health. Molecular identifications of fungal isolates obtained from infected commercial switchgrass seed yielded eight species (*A. alternata*, *F. acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. tricinctum*, and *Phoma herbarum*). Pathogenicity and virulence of identified isolates were determined with detached leaf assays. Except for *F. oxysporum*, all species were found to be pathogenic on the detached switchgrass leaves, with virulence varying between species and isolates within species. *Fusarium graminearum* isolates were the most virulent. Seed inoculation and whole plant inoculation assays were conducted to determine stand establishment and plant health. Overall, isolates of *F. graminearum* and *F. armeniacum* had the greatest impact on switchgrass stand and health and should be targeted for disease resistance development. *Alternaria alternata* isolates were found to be less virulent when compared to *Fusarium* isolates, only impacting moisture content of infected plants. Virulent isolate TBW107 was selected to determine the

susceptibility of four switchgrass cultivars ('Blade 1101', 'Blade 1102', 'Alamo', and 'Whippet') to *F. graminearum* infection. 'Blade 1102' and 'Alamo' were found to be susceptible and 'Whippet' highly susceptible to *F. graminearum* infection. 'Blade 1101' was moderately resistant to *F. graminearum* infection and should be further studied to identify the source(s) responsible for resistance for cultivar development. The chemical analysis of switchgrass infected with virulent *F. graminearum*, *F. armeniacum*, and *Bipolaris oryzae* resulted in a significant increase in ash for *B. oryzae* infected biomass, and a decrease in carbohydrate content for *B. oryzae* and *F. armeniacum* infected biomass, reducing biomass fitness for biofuel conversion.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Continual uptake of non-renewable resources, rising prices of oil, and emissions of greenhouse gases have led to an expanded interest in renewable energy. A considerable amount of research is being conducted on potential biological materials, or biomass. Biomass is non-fossilized material from photosynthetic organisms (Roddy 2012) adapted as a renewable energy source. Biomass is processed into bioenergy that are oxygenated, clean, and can exist as solids (bio-char, carbon fiber, bioplastics, and nanotubes), liquids (bioethanol, biodiesel, bio-oil, biobutanol, and biokerosene), or as a gas (biomethanol, biopropane, biosyngas, and biohydrogen) (Demirbas 2009; Chen et al. 2016; Jain et al. 2014; Daggett et al. 2007; Lewis et al. 2015; Kim et al. 2014a; Kim et al. 2014b; Filson et al. 2007). Bioenergy have potential uses in fuel transportation, electricity, heat, manufacturing, and medical application (Roddy 2012; Filson et al. 2011; Daggett et al. 2007). An increase in biofuel consumption would ease dependence on foreign oil and natural gas, and could cut greenhouse gas emissions by 86% (Balat 2009; Kumar et al. 2009).

Bioethanol and biodiesel are the most commonly produced biofuels and obtained through direct conversion of biomass. Bioethanol, or ethanol, is commonly used as a gasoline fuel additive that functions as an octane-booster, and is aimed to reduce petroleum consumption and air pollution (Chu and Lee 2007). To reduce gasoline consumption in the United States, 36 billion gallons of bioethanol are produced annually. Of the 36 billion gallons, 16 billion are made from nonfood-

plant materials, or lignocellulosic feedstocks (Robertson et al. 2008; Voith 2009). Lignocellulosic feedstocks, like switchgrass, are perennials that can be harvested several times a year, require limited cultivation costs, and do not compete with food supplies. As technology advances and oil prices continue to decline, new uses for lignocellulosic feedstocks have emerged. Carbon fiber derived from lignin, is widely used in the automotive and aerospace industry as well as insulation (Lewis et al. 2015; Filson et al. 2011). Lignin pellets are being used to restore nutrients to poor soil and lignin nanotubes as a vehicle to for gene deliver into human cells (Kim et al. 2014; Ten et al. 2014).

Switchgrass

Switchgrass (*Panicum virgatum* L.), a native perennial, C4 grass is widely distributed throughout North America. Switchgrass consists of two main ecotypes, upland and lowland. Upland varieties are shorter and better adapted to cooler, harsh environments then the taller, warm thriving lowland varieties. Currently, switchgrass is projected to encompass a significant portion of the renewable fuel market due to its high biomass yield, its ability to adapt and thrive on marginal land under stress, and its high net energy stability (Bouton 2008; Yuan et al. 2008). Research shows that carbon emissions from switchgrass are approximately 160% less than corn (McLaughlin 2002), produce 540% more renewable energy than nonrenewable energy consumed, with greenhouse gas emissions 94% lower than that of gasoline (Schmer et al. 2008), and switchgrass can produce 55% more ethanol energy per hectare than corn (McLaughlin 1997).

Ecological and agricultural benefits of switchgrass include reduced soil erosion, carbon sequestration, nitrogen cycling, water quality improvement, feed-based agriculture, and its potential to be grown on the 35 million acres (14.2 million hectares) of marginal land currently not used by farmers under the terms of USDA's Conservation Reserve Program (Yuan et al. 2008; Keshwani 2009; Bouton 2008). The agricultural benefits of switchgrass have promoted targeted breeding of lowland and highland varieties, resulting in significant biomass yield gains over the past several decades (Casler 2012). However, improvement of genetic resistance of switchgrass to disease has been limited (Serba et al. 2015).

Switchgrass Pathogens

Research has identified a number of abiotic and biotic factors that influence switchgrass stand establishment, biomass quality and yield, and stand longevity in monoculture. Fungal genera, such as *Bipolaris*, *Puccinia*, *Alternaria*, *Fusarium*, and *Tilletia*, impact switchgrass stand establishment and biomass yield (Farr and Rossman 2016; Vu 2011; Fajolu 2012). According to the USDA Fungal Database, 152 fungal isolates have been recovered from switchgrass, but confirmation of pathogenicity with Koch's postulates has not been demonstrated for most of these (Ghmire et al. 2011; Gravert and Munkvold 2002). In 2011, a study was conducted at the University of Tennessee to identify potential seedborne pathogens of switchgrass (Vu et al. 2011a). Over 9,000 switchgrass seeds from 30 commercial seed lots were examined for fungal infection. More than 2,000 fungi were isolated and infection rates ranged from <1% to 87%

among the seed lots (Vu et al. 2011a). Fungal genera isolated from seed included *Aspergillus*, *Bipolaris*, *Curvularia*, *Eurotium*, *Fusarium*, *Penicillium*, *Phoma*, *Pithomyces*, and *Xylaria*. The most common fungal species isolated from seed were *Alternaria alternata*, *Bipolaris oryzae*, and *Fusarium graminearum*. In 2011, fungal pathogens were collected from symptomatic switchgrass from growers' fields in Tennessee (Vu 2011), and included *Alternaria alternata* (Vu et al. 2012), *Bipolaris oryzae* (Vu et al. 2013), *B. sorokiniana* (Vu et al. 2011b), *B. spicifera* (Vu et al. 2011c), *B. victoriae* (Fajolu et al. unpublished), *Curvularia lunata* var. *aeria* (Fajolu et al. 2012), *Fusarium acuminatum*, *F. equiseti*, *F. graminearum*, *F. pseudograminearum*, *Pithomyces chartarum* (Vu 2011) and *Sclerotinia homoeocarpa* (Vu et al. 2011d). The four *Bipolaris* spp. from the symptomatic tissue were further evaluated for pathogenicity and virulence towards switchgrass (Fajolu 2012). All four species proved to be pathogenic on 'Alamo' switchgrass at low inoculum concentrations.

Fusarium Species

Ten species of *Fusarium* have been identified on switchgrass, these include *F. acuminatum*, *F. equiseti*, *F. moniliforme*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. pseudograminearum*, *F. scirpi*, *F. solani*, and *F. subglutinans* (Farr and Rossman 2016). *Fusarium* spp. (teleomorphs: *Gibberella* spp., *Nectria* spp., *Calonectria* spp., and *Micronectria* spp.) are a diverse assemblage of filamentous, mitosporic fungi. Most species are endophytic or nonpathogenic, making up an abundant portion of the microbial community. A few of the

endophytic *Fusarium* spp. have been used as biocontrols, and are added to soil to protect plants against fungal pathogens, including pathogenic species of *Fusarium* (Kavroulakis et al. 2007). Some species are phytopathogenic on a variety of plants under favorable environmental conditions (Booth 1971; Doohan et al. 2003). Pathogenic fusaria have the ability to reduce biomass yield and contaminate grain stores. They produce harmful mycotoxins that affect both human and animal health. The primary mycotoxins produced by *Fusarium* include fumonisins and trichothecenes. Economically important diseases caused by *Fusarium* spp. include Fusarium head blight of wheat and ear rot of corn (Parry et al. 1995; Sutton 1982). Between 1991 and 1996, *Fusarium* seedling blight and root rot caused an estimated 3 billion U.S. dollars in barley and wheat losses (Summerell et al. 2010). Currently, over a thousand species have been identified, making morphological identification difficult (Leslie and Summerell 2006). Aspects of morphological identification include colony structure and color, media pigmentation, and conidial characteristics (length, width, number of septa, shape of apical and basal cell, and presence of microconidia). Species confirmation is often achieved through molecular identification of the translation elongation factor 1- α sequence (O'Donnell et al. 1998) and internal transcribed spacer region of the 18S ribosomal subunit (White et al. 1990).

Alternaria Species

Alternaria alternata, *A. panici*, and other *Alternaria* spp. have been reported on switchgrass (Farr and Rossman 2016) *Alternaria* species are major plant pathogens, with wide host ranges. *Alternaria* spp. are also common allergens in humans, due to the amount of airborne conidia they produce. Conidia cling to the respiratory tract, disrupting respiration. Some *Alternaria* spp. produce an array of toxic compounds and cause alternariosis and alternariotoxicosis in humans. There are approximately 300 *Alternaria* species (Lawrence et al. 2012) that occur worldwide, many of which produce club-shaped conidia. Identification of *Alternaria* spp. via conidial morphology can be difficult due to conidial plasticity and low genetic variability among species (Misaghi et al. 1978; Kusaba and Tsunge 1995; Pryor and Gilbertson 2000). Molecular identification of the internal transcribed spacer region (Kusaba and Tsunge 1995) is used for species identification. *Alternaria* spp. are prolific pathogens causing approximately 20% of agricultural spoilage (Lawrence et al. 2013). Some *Alternaria* spp. show promise as biocontrol agents against invasive plant species such as *Amaranthus retroflexus* (Ghorbani et al. 2000).

Phoma Species

Phoma herbarum, *P. sorghina*, and *Phoma* sp. have been found on switchgrass. Currently, approximately 200 *Phoma* species have been identified, and divided into two groups: plurivorous (saprophytic or weakly parasitic) fungi found mainly

in temperate regions of Eurasia, and pathogens of cultivated plants (Van der Aa et al. 1990). Phylogenetic studies have distinguished six distinct clades (Aveskamp et al. 2010). Conidia are colorless and unicellular, with black to brown pycnidia (Boerema et al. 2004). The most important plant pathogenic species is *Phoma betae*, causes heart rot and blight of beets and *P. batata* produces dry rot in sweet potato (Boerema et al. 2004). Several species of *Phoma* cause disease on grasses, such as wild rice (Nyvall and Percich 1999). *Phoma herbarum* is an infectious fish pathogen in salmonoids, invading the air bladder and digestive track of fry and fingerlings, leading to gut obstruction, peritonitis, and visceral necrosis (Ross et al. 2011). Molecular identification of *Phoma* spp. is obtained through polymerase chain reaction of the internal transcribed spacer, β -tubulin, and 18S regions (de Gruyter et al. 2009; Aveskamp et al. 2010).

Research Objectives and Justifications

This research has the following five main objectives:

Objective 1: Complete Koch's postulates and write first reports for seedborne pathogens not previously reported on switchgrass including *F. armeniacum*, *F. tricinctum*, and *Phoma herbarum*.

Justification: Identification of new seedborne switchgrass pathogens is essential for prevention and treatment of infected switchgrass seed. As switchgrass is

increasingly cultivated and seed transported, efficient disease management and rapid detection will be necessary to reduce disease spread.

Objective 2: Assess *Fusarium* species morphology, virulence diversity and impact on switchgrass plant health.

Justification: Little information has been published in regards to virulence of *Fusarium* on switchgrass. There is no report documenting the variation in virulence of *Fusarium* species on switchgrass, which will be necessary in resistant cultivar development and sustainable agricultural practices for disease management.

Objective 3: Assess *A. alternata* morphology, virulence diversity, and impact on switchgrass plant health.

Justification: Known to cause disease on switchgrass, *A. alternata* is a prime candidate for virulence diversity evaluation. To develop *Alternaria* resistant switchgrass cultivars, more information is needed on *Alternaria* pathogenicity and virulence.

Objective 4: Evaluate the disease reaction of four switchgrass cultivars to *F. graminearum*.

Justification: Economic losses can be reduced through genetic resistance of switchgrass to fungal infections. Currently, there are no reports of disease

resistance of switchgrass cultivars against *F. graminearum*. Identifying resistant cultivars could act as an effective control for *Fusarium* infection and disease.

Objective 5: Assess virulent *F. armeniacum*, *F. graminearum*, and *Bipolaris oryzae* on switchgrass biomass chemical components.

Justification: Biomass converted into biofuels from infected switchgrass may impact biofuel processing. Alteration of chemical components caused by *Fusarium armeniacum*, *F. graminearum*, and *Bipolaris oryzae* infection could promote economic losses in biofuel industries.

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CHAPTER TWO
IDENTIFICATION OF FUNGAL PATHOGENS OBTAINED FROM
COMMERCIAL SWITCHGRASS SEED

A. Vu, O.L. Fajolu, K. Mantooth, M.M. Dee, and A. Bruce assisted in fungal collection, isolation, and morphological identification of *Fusarium* species and *Alternaria alternata*, and determination of pathogenicity. N. Labbé, R. Trigiano, K.D. Gwinn, and B.H. Ownley provided guidance and funding for the studies.

Abstract

A warm-season perennial, switchgrass is currently the prime candidate for cellulosic ethanol production. However, little research has been conducted to identify seedborne pathogens of this economic crop, and their impact on plant health. About 9,000 seed from 30 commercial seed lots were tested for fungal infection in a previous study. Infection rates among seed lots ranged from less than 1% to 87% with over 2,300 fungal isolations made. Koch's postulates were conducted to determine the pathogenicity of the recovered isolates. Although new pathogens were discovered many of the recovered isolates were only identified to genus. For this study, *Fusarium* and *Phoma* isolates were identified based on conidial morphology, colony characteristics, and comparison of translation elongation factor 1-alpha protein and internal transcribed spacer (ITS) sequences of ribosomal DNA with published nucleotide sequences in the National Center for Biotechnology Information (NCBI) GenBank and *Fusarium* ID databases. Whole plant and seed inoculation assays were conducted in growth chambers to confirm pathogenicity and satisfy Koch's postulate. *Fusarium*

armeniacum, *F. tricinctum*, and *Phoma herbarum*, previously not described on switchgrass, were pathogenic.

Introduction

Switchgrass (*Panicum virgatum* L.) is the current front-runner in cellulosic ethanol production. This native perennial plant is a prime candidate due to its low cost of production, high-energy output, and reduced greenhouse emissions.

Switchgrass ethanol production was estimated to produce 94% less greenhouse emissions than gasoline (Schmer et al.2008). Switchgrass is also a valuable biomass feedstock because of its ability to thrive on marginal soils. This characteristic could allow switchgrass to be grown on the more than 35 million acres (14.2 million hectares) of marginal land on which farmers are currently paid not to plant, under the terms of the United States Department of Agriculture (USDA) Conservation Reserve Program.

The potential of switchgrass as a bioenergy producer has prompted breeding programs resulting in significant gains in switchgrass biomass yields. Breeding programs and other research have also classified a number of abiotic and biotic stresses that can influence stand establishment, biomass, and stand health, with little research conducted on seed- and soilborne pathogens. The potential for these pathogens to be a major limiting factor in switchgrass cultivation may be heightened as this bioenergy crop is grown in monoculture over large acreages of land.

According to the online USDA Fungal Database, there have been 150 fungal species reported from switchgrass, including over 406 records, with approximately 75 tested for pathogenicity (Farr and Rossman 2016). Prior to this study, two projects were conducted at the University of Tennessee to identify potential new pathogens of switchgrass. In 2011, nine pathogenic species were identified from diseased switchgrass samples collected from several locations in East Tennessee (Vu 2011). Six fungi species identified had been previously reported as pathogens of switchgrass: *Alternaria alternata* (Gravert and Munkvold 2002), *Bipolaris oryzae*, *B. sorokiniana*, *Fusarium acuminatum*, *F. equiseti*, and *F. pseudograminearum* (Farr and Rossman 2016). In addition, three pathogens not previously reported on switchgrass were described: *B. spicifera* (Vu et al. 2011c), *Pithomyces chartarum* (Vu et al. 2013), and *Sclerotinia homoeocarpa* (Vu et al. 2011d). To assess the presence of seedborne pathogens, commercial seed of seven cultivars were tested from eleven seed production companies located in the United States (Vu 2011). Fungal infection rates among the 30 sampled seed lots ranged from less than 1% to 87% (Vu et al. 2011b). Most frequently observed pathogens were *B. oryzae*, *A. alternata*, and *F. graminearum* / *pseudograminearum* (Vu et al. 2011b). In a second study, the impact of four *Bipolaris* spp. on switchgrass biomass yield was determined (Fajolu 2012). Morphology and virulence varied among *Bipolaris* spp. when applied to switchgrass. Inoculum density played an important role in biomass reductions. At low and high inoculum levels, isolates of *B. oryzae*, *B. sorokiniana*,

and *B. victoriae* reduced biomass from 70% to 88% and stand by 52% to 74%, respectively (Fajolu 2012). *Bipolaris spicifera* isolates reduced biomass yields by 5% and plant stand by 38% at the low and high inoculum levels, respectively. The purpose of this research was to advance information on pathogenicity and virulence of unidentified species of seed- and soil-borne fungi that were isolated in the study on pathogens of commercial switchgrass seed, and to determine their impact on crop yield and quality of switchgrass.

Materials and Methods

Methods to isolate pathogens from commercial switchgrass seed were outlined in Vu (2011a) and are briefly described for each specific fungus below. Fungi were cultured from samples of 30 switchgrass seed lots provided by 11 U.S. companies, and included 7 cultivars (Table 1.1). Resultant fungal colonies were transferred to PDA and stored at 4°C until identification could be performed.

Initial identifications were based on morphological features, such as colony morphology, pigmentation on culture medium, and conidial characteristics. To confirm identifications, ribosomal DNA from the internal transcribed spacer (ITS) region, and translation elongation factor 1- α (TEF1- α) region were amplified with polymerase chain reaction (PCR) with primers ITS-1 forward, and ITS-4 reverse (White et al., 1990), and TEF1- α forward and TEF2- α reverse (O'Donnell et al., 1998), respectively. The resulting PCR amplicons were sequenced and checked

for identity against nucleotide sequences in the GenBank database with BLASTn (NCBI), and in Fusarium ID.

For pathogenicity tests, seed were surface-sterilized with 10% Clorox® for 20 min, rinsed with sterile water, and dried. All seed were subjected to cold stratification, during which they were soaked for 1 h in sterile distilled (DI) water, and placed in Petri dishes fitted with sterile filter paper that was saturated with sterile DI water. Plates were kept for 12 h at 4.4°C.

Whole plant and seed pathogenicity assays were carried out to complete Koch's postulates. To harvest conidia, culture plates were flooded with 5 ml of sterile DI water containing 10- μ l Tween-20 (Fisher Scientific, Dubuque, Iowa) and then gently rubbed with a sterile rubber policeman. For seed inoculation assays, 100 surface sterilized 'Alamo' switchgrass seed were placed in a Petri dish with 5 ml of 1×10^5 conidia/ ml sterile DI water; 50 seed were placed in a Petri dish with sterile DI water, to serve as a control. Seed were planted in 9×9-cm² pots containing 50% ProMix Potting and Seeding Mix (Premier Tech Horticulture, Rivière-du-Loup, Québec, Canada) and 50% Turface ProLeague (Profile Products, Buffalo Grove, IL) (vol/vol). Each pot received 20 seeds, with five replicate pots per fungal isolate. Pots were covered with clear plastic, and secured with a rubber band for 5 days. Switchgrass plants were kept in growth chambers at 28°C with a 12-h photoperiod. At 6 weeks old, diseased tissue was excised from infected plants, surface sterilized, and plated onto PDA. The reisolated fungi were confirmed to be the original isolate based on morphological

and molecular identification techniques. During whole plant pathogenicity assays, sterilized seed were sown in 9×9-cm² pots. A 1×10⁵ conidial spore suspension (1×10⁵ conidia/ml water) was sprayed on five replicates of 6-week-old switchgrass seedlings in a growth chamber at 28°C with a 12-h photoperiod. Plants were kept at high humidity for 7 days. Four weeks post inoculation, symptomatic plant tissue was collected and the fungi re-isolated for identification, thus fulfilling Koch's postulates.

Fusarium armeniacum

In 2009, *Fusarium armeniacum* was isolated from commercial 'Alamo' switchgrass seed. In 2014, the stored culture was transferred to PDA and carnation leaf agar amended with 3.45 mg fenpropathrin/liter and 10 mg/liter rifampicin for morphological identification. Genomic DNA was extracted from mycelia grown on full-strength PDA using a Direct Pure PCR Kit (ThermoFisher Scientific, Waltham, MA). Polymerase chain reaction amplicons were obtained for translation elongation factor 1- α (TEF1- α) sequences with primers TEF1- α forward and TEF2- α reverse (O'Donnell et al., 1998).

To satisfy Koch's postulates, pathogenicity studies were conducted. For seed assays, 200 'Alamo' switchgrass seeds were sterilized (10% commercial bleach for 20 min). Half of the sterilized seed were inoculated with 5 ml of a 10⁵ conidial suspension of *F. armeniacum*, the other half with sterile water to serve as controls, and the seed placed on a shaker overnight. Conidia were harvested by

flooding PDA plates with 5 ml of sterile water and 5 µl of Tween-20, and then concentrated to 10^5 conidia/ml sterile water using a hemocytometer. The seed were planted in potting mix, and grown in a growth chamber at 28°C, with a 12-h photoperiod for 6 weeks. For whole plant assays, 200 'Alamo' switchgrass seeds were sterilized (10% commercial bleach for 20 min). Seed were planted in potting mix and grown in a growth chamber at 28°C with a 12-h photoperiod. Six-week-old switchgrass plants were wounded by trimming the tops to a height of approximately 5 cm. Five pots were sprayed with a conidial spore suspension of 10^5 spores/ml sterile water and subjected to high humidity by enclosure in a plastic bag for 7 days. The controls were sprayed with sterile water and subjected to the same conditions. The experiments were set-up in a completely randomized design, with five replicate pots (20 seeds each) for both controls and inoculated seed.

Fusarium tricinctum

An isolate of *Fusarium tricinctum* was recovered from surface-sterilized (95% ethanol, 1 min; 20% commercial bleach, 3 min; 95% ethanol, 1 min) 'Kanlow' switchgrass seed that was plated on 2% water agar amended with 3.45 mg fenpropathrin/liter and 10 mg/liter rifampicin. The fungus was transferred to full strength potato dextrose agar and carnation leaf agar (CLA) amended with 3.45 mg fenpropathrin/liter (Danitol 2.4 EC, Valent Chemical, Walnut Creek, CA) and 10 mg/liter rifampicin for morphological identification.

A Direct Phire PCR Kit was used to extract genomic DNA from mycelia grown in full-strength PDA. Translation elongation factor 1- α (TEF1- α) sequences with primers TEF1- α forward and TEF2- α reverse (O'Donnell et al., 1998) were used to acquire polymerase chain reaction amplicons.

Pathogenicity studies were conducted with 200 surface-sterilized (10% Clorox® bleach for 20 min) 'Alamo' switchgrass seeds. Cultures were transferred to CLA to induce conidial production. Conidia were harvested by flooding plates with 5 ml of sterile water and 5 μ l Tween-20, and concentrated to 10^5 conidia/ml sterile water using a hemocytometer. One hundred sterilized seed were inoculated with 5 ml of the *F. tricinctum* 10^5 conidial suspension. The other 100 were inoculated with sterile water to serve as controls. Inoculated and uninoculated 'Alamo' switchgrass seed, consisting of five replicate pots (20 seeds per pot); each were planted in potting mix, and grown in a growth chamber at 28°C with a 12-h photoperiod for 6 weeks. A species of *Fusarium* that caused leaf wilting, streaking, and root necrosis was observed.

Phoma herbarum

A 2009 commercial seed study of 'Blackwell' switchgrass (*Panicum virgatum* L.) yielded mitosporic fungi growing from some of the seed. Seed were surface-sterilized (95% ethanol for 1 min, 20% commercial bleach for 3 min, and 95% ethanol for 1 min), and plated on 2% water agar amended with 5 μ l/liter miticide (2.4 EC Danitol, Valent Chemical, Walnut Creek, CA) and 10 mg/liter rifampicin.

Plates were incubated at 26°C for 48 h. An aerial, asexual fungus was isolated and transferred to potato dextrose agar (PDA) for morphological identification.

A pathogenicity study was conducted with 6-week-old 'Alamo' switchgrass plants grown from surface-sterilized seed to satisfy Koch's postulates. Ten pots with twenty plants each were prepared. Plants were wounded by trimming the tops. Five of the replicate pots were sprayed with a conidial suspension concentrated to 10^5 conidia/ml sterile water and the other half were sprayed with sterile water. For 7 days both inoculated plants and controls were subject to high humidity by enclosing the pots in plastic bags. Plants were maintained in a growth chamber at 28°C, with a 12-h photoperiod for 4 weeks. White to green foliar leaf spots, leaf streaking, and general chlorosis was observed 2 to 4 weeks post-inoculation for all replicated pots inoculated with *P. herbarum*.

The internal transcribed spacer (ITS) regions from the original isolate and reisolate, recovered from the pathogenicity assay, were amplified using a Direct Phire Kit, with primer pairs ITS 1 forward and ITS 4 reverse (White et al., 1990). Polymerase chain reaction amplicons were sequenced and submitted to GenBank. A BLAST search (BLASTn, NCBI) was run against GenBank isolates.

Results and Discussion

Fusarium armeniacum

Switchgrass infected with *F. armeniacum* exhibited brown to red leaf spots, root necrosis, biomass yield loss, and seedling mortality. Identification of the original isolate and the re-isolate from necrotic leaf tissues was confirmed with comparison of TEF1- α PCR amplicons with those in Fusarium ID. The 570-bp sequence for TEF1- α had 99.5% identity to *F. armeniacum* isolate FD_01843_EF-1a in Fusarium ID, and had 100% nucleotide identity to multiple *F. armeniacum* isolates in GenBank (Accession Nos. KT339753.1, KJ737376.1, and HM744692.1). The TEF1- α sequence was deposited in GenBank as Accession No. KU685394. Based on morphology and sequence analysis, the re-isolate was identified as *F. armeniacum*.

Mycelia were white and abundant, with pale tan pigmentation produced in the agar. Macroconidia were 3 to 5 septate, curved, with a long tapered apical cell and a distinctly foot-shaped basal cell. Macroconidia ranged from 23 to 30 μm in length and were formed singly, as described for *F. armeniacum* (Leslie and Summerell, 2006) (Fig. 2.1). Only 60% of inoculated 'Alamo' switchgrass seed germinated, compared with 70% germination in controls. After six weeks, control switchgrass plants showed no disease symptoms, while inoculated plants exhibited brown to red leaf spots, root necrosis, and seedling death (Fig 2.1). To

our knowledge, this is the first report of *F. armeniacum* causing leaf spot of switchgrass in North America.

Fusarium tricinctum

Mycelia were white and dense, with pale to dark orange pigmentation produced in the agar. Macroconidia were slender, falcate, 3 septate, and 34 to 44 µm in length, with a long and tapered apical cell, and a well-developed foot-shaped basal cell (Leslie and Summerell, 2006) (Fig. 2.2).

The sequence for TEF1-α had a 99.0% match to sequences of multiple isolates of *F. tricinctum* in GenBank (KJ623252.1 and KF939493.1), and 99.0% identical match to *F. tricinctum* isolate FD_01315_EF-1a in Fusarium ID. The 412-bp sequence was deposited in GenBank as Accession No. KU886242. Fifty percent of inoculated 'Alamo' switchgrass seed germinated, compared with 70% germination in controls. After six weeks, control switchgrass plants showed no disease symptoms, whereas inoculated plants had visible leaf necrosis and wilting. Lesions were excised, surface sterilized, and plated on PDA (Fig. 2.2). The original isolate and the re-isolate were identified as *F. tricinctum* based on morphological and molecular characters.

To our knowledge, this is the first report of *F. tricinctum* causing leaf spot of switchgrass in North America, extending the known host range of the pathogen (Farr and Rossman 2016).

Phoma herbarum

Mycelia were white to red to gray in color, with red to black pigmentation when grown on PDA. Conidia produced were small, cylindrical, aseptate, yellow to brown in color, and ranged in size from 7 to 11×2 to $4 \mu\text{m}$, consistent with characteristics described previously for *Phoma herbarum* (Wehmeyer, 1946) (Fig. 2.3).

White to green foliar leaf spots, leaf streaking, and general chlorosis were observed 2 to 4 weeks post-inoculation for all replicated pots inoculated with *P. herbarum*. Control plants showed no disease symptoms. Lesions were excised, surface sterilized, plated on PDA, and identified morphologically and molecularly (Fig. 2.3).

The ITS region sequences were 509 bp and had 99% identity to multiple isolates of *P. herbarum* (Accession Nos. KP942899.1, KJ767078.1, and AB369456.1).

The sequence was deposited in GenBank as Accession No. KU710251. Based on morphological and molecular characters, the isolate and re-isolate were identified as *P. herbarum*. To our knowledge, this is the first confirmed report of switchgrass as a natural host for *P. herbarum*.

Conclusion

Results from this study indicate that fungal pathogens are present in commercial switchgrass seed. Many of the identified pathogens cause disease in other economically important crops including Bermuda grass, ryegrass, various turfgrasses, wheat, corn, and rice. Management practices for switchgrass are needed to prevent disease outbreaks and spread through distribution of infected seed. Management changes would be paramount in optimizing crop yields. As switchgrass is increasingly grown and cultivated as a biofuels crop, clean seed practices will be needed to reduce seedborne transmitted diseases. Rapid disease identification procedures and breeding of more resistant cultivars will also be necessary.

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Vu, A.L., Gwinn, K.D., and Ownley, B.H. 2011d. First report of *Sclerotinia homoeocarpa* causing dollar spot on switchgrass in the United States. *Plant Disease* 95:1585.

Vu, A.L., Gwinn, K.D., and Ownley, B.H. 2013. First Report of Leaf Spot on Switchgrass Caused by *Pithomyces chartarum* in the United States. *Plant Disease* 97: 1655.

Appendix

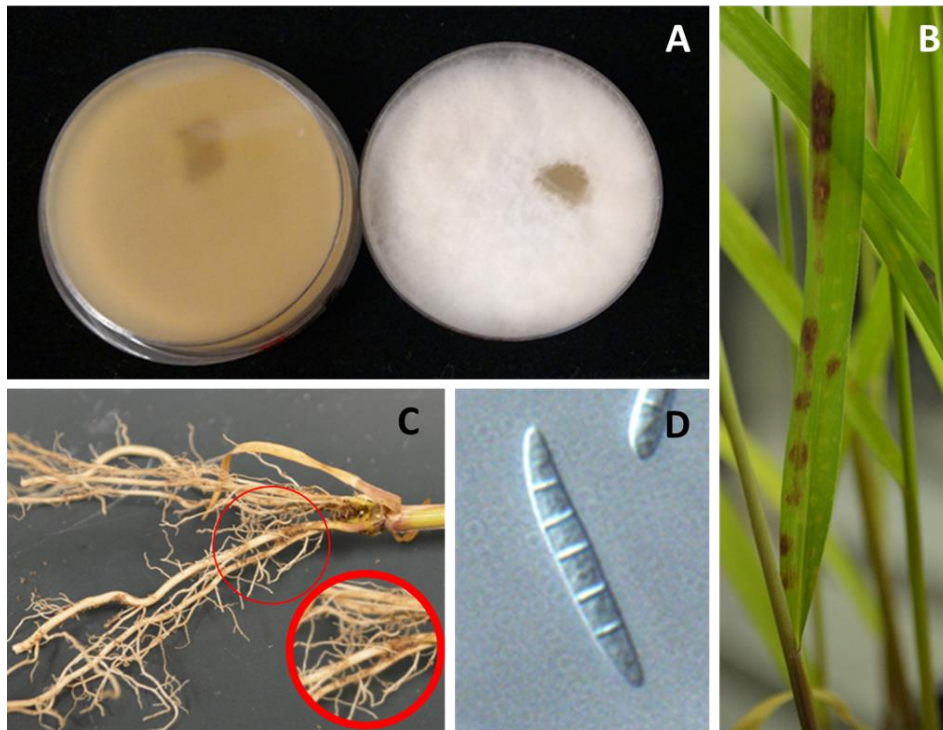


Figure 2.1 *Fusarium armeniacum*. A. *Fusarium armeniacum* (left) pigmentation in PDA (right) mycelium; B. Leaf spot symptoms on 'Alamo' switchgrass at 6 weeks-old; C. Root necrosis on 'Alamo' switchgrass at 6 weeks; D. *Fusarium armeniacum* macroconidium

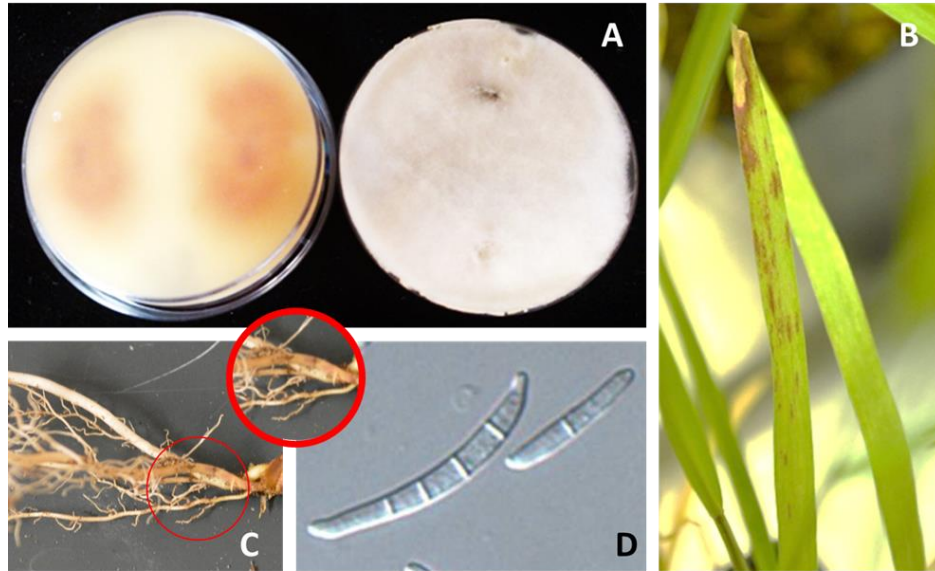


Figure 2.2 *Fusarium tricinctum* A. *Fusarium tricinctum* (left) pigmentation in PDA (right) mycelium; B. Leaf spot symptoms on 'Alamo' switchgrass at 6 weeks; C. Root necrosis on 'Alamo' switchgrass at 6 weeks; D. *Fusarium tricinctum* (left) macroconidium (right) microconidium

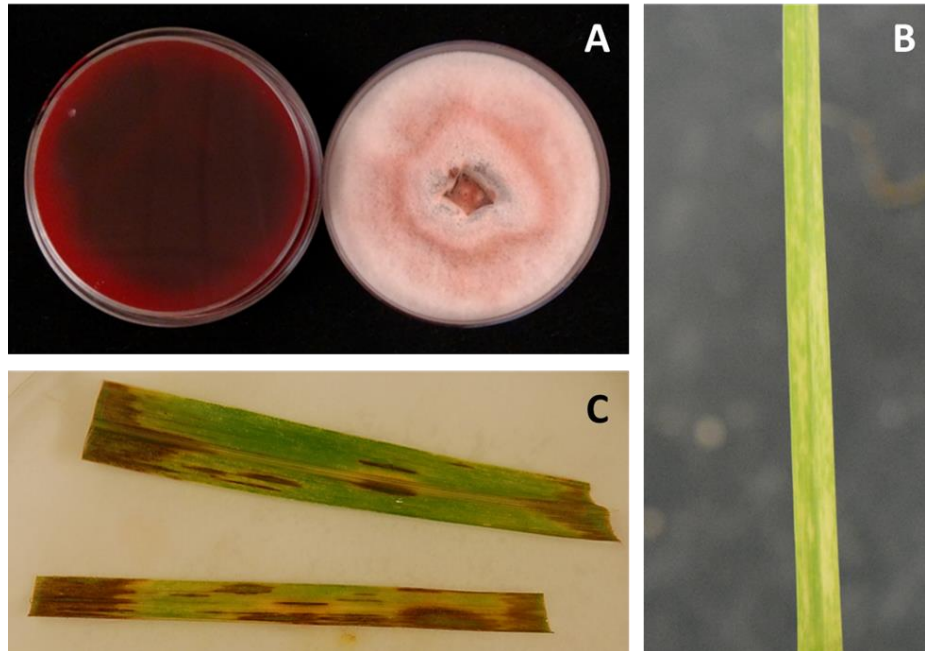


Figure 2.3 *Phoma herbarum* A. *Phoma herbarum* (left) pigmentation in PDA (right) mycelium; B. Leaf spot symptoms on 'Alamo' switchgrass at 10 weeks; C. Leaf spot symptoms on 3-cm cut leaf sections of 'Alamo' switchgrass inoculated with 25 μ l of 10^5 conidia/ml sterile water

Table 2.1: Cultivars and source of switchgrass seed

Cultivar	Company	Company Location	Seed Source Location
‘Alamo’	Company 1	CO	TX
	Company 2	NM	TX
	Company 3	PA	MO
	Company 5	OK	OK
	Company 6	OK	OK
	Company 7	KY	TN
	Company 8	KS / CO	OK
	Company 9	TX	TX
	Company 10	OK	OK
	Company 1	CO	OK
‘Blackwell’	Company 2	NM	SD
	Company 3	PA	PA
	Company 5	OK	SD
	Company 7	KY	TN
	Company 8	KS / CO	KS
	Company 9	TX	TX
	Company 10	OK	TX
	Company 1	CO	IL
	Company 3	PA	PA
	Company 7	KY	KY
‘Cave-in-Rock’	Company 8	KS / CO	KS
	Company 11	IA	IA
	Company 1	CO	OK
	Company 3	PA	IA
	Company 5	OK	OK
‘Kanlow’	Company 7	KY	MO
	Company 8	KS / CO	MO
	Company 4	WI	CO
	Company 5	OK	OK
	Company 6	OK	OK
‘Forestburg’			
‘Cimarron’			
‘Whippet’			

CHAPTER THREE

ASSESSMENT OF FUSARIUM SPECIES MORPHOLOGY, VIRULENCE DIVERSITY, AND IMPACT ON SWITCHGRASS HEALTH

A. Vu, O.L. Fajolu, K. Mantooth, M.M. Dee, and A. Bruce assisted in fungal collection, isolation, and morphological identification of *Fusarium* species and determination of pathogenicity. N. Labbé, R. Trigiano, K.D. Gwinn, and B.H. Ownley provided guidance and funding for the studies.

Abstract

Fusarium species can cause disease in switchgrass (*Panicum virgatum* L.). Based on detached leaf assays, *F. acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, and *F. tricinctum* were pathogenic on switchgrass. The aims of this study were to determine the impact of virulent isolates of five *Fusarium* spp. on plant stand, growth, and health. Seed and whole plant inoculations were conducted to determine mean disease response of ‘Alamo’ switchgrass to *Fusarium* infection. Percent germination, plant height, disease severity, and plant yield (fresh and oven dry weight after 7 days at 45°C) were evaluated. During seed inoculation assays, decreases in seed germination were prevalent across all five species tested, with the greatest variation in virulence seen among isolates of *F. graminearum*. Infection of established plants yielded reductions in height, increased foliar disease, and reduced fresh weight of plants when compared to the control. Replicated trials were arranged in a completely randomized design and data were analyzed with Proc Glimmix (PC-SAS 9.3). For whole plants treated with *Fusarium* isolates, there were significant reductions in fresh weight, and increased foliar disease compared with untreated controls, especially with *F. graminearum* and *F. armeniacum* isolates. This suggests that

Fusarium spp. can reduce switchgrass stand establishment and affect plant health.

Introduction

In 2007, the Energy Independence and Security Act mandated that by 2022, at least 36 billion gallons of transportation fuel in the U.S. must originate from renewable resources (110th 3 United States Congress, 2007), with a large proportion of biofuels produced from cellulosic crops, such as switchgrass (*Panicum virgatum* L.). Switchgrass is a promising biofuel alternative to corn due to its status as a native perennial species, ability to thrive on a wide range of soil types, adaptability to climate, and high biomass yields (Bouton 2008; Yuan et al. 2008). Switchgrass also has a high net energy balance, designating it as a viable candidate for the United States Department of Energy Biofuel Feedstock Development Program. As research intensifies on this new alternative biofuel crop, more data is needed on the biotic and abiotic stresses that influence switchgrass establishment, biomass, and longevity. Currently, over 152 fungal isolations have been made from switchgrass, including 61 different species (Farr and Rossman 2016), with only half tested for disease response. Research on the impacts of these pathogens on switchgrass production is lacking.

A 2011 survey was conducted at the University of Tennessee to identify seedborne pathogens of switchgrass isolated from commercial seed produced across the U.S. (Vu et al. 2011). The most common genera recovered from seed

were *Alternaria*, *Bipolaris*, and *Fusarium*. Many *Fusarium* spp. are well known seed and soilborne pathogens that have the ability to persist in soil without a host plant for extended periods, making disease management difficult (Leslie and Summerell 2006). Several *Fusarium* spp. isolated from the seed have the ability to negatively impact switchgrass production (Vu 2011). *Fusarium acuminatum*, is a known switchgrass pathogen, causing brown leaf spots, root necrosis, stem spots, and stem streaking (Vu 2011). *Fusarium acuminatum* is generally a plant saprophyte or secondary pathogen but can be pathogenic on legumes and cereal crops (Leslie and Summerell 2006). *Fusarium equiseti*, responsible for brown leaf spots, general chlorosis, and stunting of switchgrass, has a wide host range encompassing everything from cereal and fruit crops to pine and pecan (Xue et al. 2006; Ocamp and Juzwik 1995; Lazarotto et al. 2014). The most economically important *Fusarium* species, *F. graminearum*, has a global distribution, causing crippling yield losses in corn, rye, barley, and wheat (Leslie and Summerell 2006). *Fusarium graminearum* causes brown spots and streaking on leaves and stems of switchgrass (Vu 2011). Other *Fusarium* spp. isolated from infected seed include, *F. armeniacum*, *F. oxysporum*, and *F. tricinctum*. Currently, these species have not been tested for pathogenicity on switchgrass.

Although several *Fusarium* spp. are known pathogens of switchgrass, no research has been conducted to determine virulence variation among species and among isolates on switchgrass, as well as the impact of *Fusarium* spp. on stand establishment, yields, and stand health. The potential impact of *Fusarium*

on switchgrass production requires the evaluation of plant yields, providing information that may be used in agricultural and disease management. To assess diversity in morphology, pathology, and virulence of *F. acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, and *F. tricinctum* on switchgrass, detached leaf, seed inoculation, and whole plant inoculation assays were conducted.

Materials and Methods

Fusarium species

Fusarium isolates were recovered from infected switchgrass seed, obtained from commercial seed companies in the U.S, included cultivars of Alamo, Blackwell, Cave-n-Rock, and Kanlow (Vu 2011). Seed were surface-sterilized with 1% NaOCl (Clorox®, Oakland, CA) amended with 10 µl Tween-20 (U.S. Biochemical Corp., Cleveland, OH) for 1 min, rinsed three times with sterile deionized (DI) water, and dried on sterile filter paper (Mathur and Kongsdal 2003). Seed were placed onto 9-cm diameter potato dextrose agar (PDA) amended with 100 mg chloramphenicol/l (Sigma-Aldrich, St. Louis, MO). Hyphal colonies observed were transferred to individual PDA plates for identification.

Isolate identification

Morphology of each isolate was evaluated based on colonial and conidial properties. Each isolate was grown for 7 days at 25°C on PDA amended with 3.45 mg fenpropathrin/l (Danitol 2.4 EC, Valent Chemical, Walnut Creek, CA) and 10 mg/l rifampicin (Sigma-Aldrich, St. Louis, MO). Colony morphology was characterized by mycelial color, media pigmentation, and mycelial growth pattern. Conidia were assessed based on size, shape, features of the apical and basal cells, number of septa, and presence or absence of microconidia.

A Direct Phire PCR Kit (ThermoFisher Scientific, Waltham, MA) was used to extract genomic DNA from aerial mycelium of each isolate. Polymerase chain reaction (PCR) of the ribosomal DNA was carried out with translation elongation factor primers TEF1- α and TEF2- α , which target sequences for translation elongation factor 1- α (TEF1- α) protein (O'Donnell, Kistler et al. 1998). PCR amplicons were sequenced and checked against published nucleotide sequences in the GenBank database using BLASTn (National Center for Biotechnology Information) and Fusarium ID.

Seed sterilization and cold stratification

Seed were surface-sterilized for 20 min in 10% Clorox® on a magnetic stirrer, and rinsed with sterile deionized (DI) water. During cold stratification, seed were soaked for 1 h in sterile DI water then placed in Petri dishes fitted with sterile filter paper moistened with sterile DI water. Plates were kept for 12 h at 4.4°C.

Detached leaf assay

Isolates of *F. acuminatum* (n = 3), *F. armeniacum* (n = 7), *F. equiseti* (n = 6), *F. graminearum* (n = 9), *F. oxysporum* (n = 3), and *F. tricinctum* (n = 1) were prescreened for pathogenicity and virulence on detached switchgrass leaves.

Assays were arranged in a randomized block design (RBD), with blocks separated by replicate. Five-cm leaf sections were harvested from 8-week-old switchgrass, grown from surface-sterilized and cold stratified seed. Detached leaves were surface-sterilized in 95% ethanol for 30 s, 10% Clorox® for 1 min, 95% ethanol for 30 s, and rinsed in sterile DI water. Three surface-sterilized leaf blades were placed in each sterile Petri dish, which had been fitted with a 9-cm diameter sterile filter paper disk, moistened with sterile DI water.

Inoculum for the detached leaf assay was prepared by flooding fungal colonies with 5 ml of sterile DI water containing 10- μ l Tween-20 (Fisher Scientific, Dubuque, Iowa) and gently scraping with a sterile rubber policeman to release conidia. Tween-20 was added as a surfactant to promoting conidial dispersal. Conidia were concentrated to 1×10^4 per ml, and leaf sections were inoculated with five 10- μ l droplets of conidial suspension. For controls, leaf sections were inoculated with five 10- μ l droplets of sterile DI water. Petri dishes were incubated in moist chambers, to increase relative humidity. Assays consisted of four replicate dishes per isolate and control.

Detached leaves were visually inspected for 7-days post inoculation (dpi) for disease symptoms. Leaves were photographed at 7-dpi with a Nikon mounted to

a photo stand, to assess pathogenicity and virulence. Percent disease area of each leaf section was measured using Assess 2.0 image analysis software for disease quantification (Lamari 2008). Isolate aggressiveness was determined by the average percent lesion area. Lesion areas less than 6% were considered non-aggressive, 7% to 25% moderately aggressive, and 26% to 100% highly aggressive.

Seed inoculation and virulence assessment

Virulent isolates *F. acuminatum* (n=2), *F. armeniacum* (n=2), *F. equiseti* (n=3), *F. graminearum* (n=5), and *F. tricinctum* (n=1) from the detached leaf assay were selected for seed inoculation assays to satisfy Koch's postulates.

For each isolate, 100 'Alamo' switchgrass seeds were surface-sterilized (10% Clorox® for 20 min on a magnetic stirrer). Seed were subjected to cold stratification to induce germination. Seed were placed on a shaker overnight, with 50 surface sterilized seed placed in a Petri dish with 5 ml of a 1×10^5 conidia/ ml sterile DI water, and the remaining 50 seed placed in a Petri dish with 5 ml sterile DI water to serve as controls. Conidia were harvested as described previously. Seed were planted in $9 \times 9\text{-cm}^2$ pots in 50% ProMix Potting and Seeding Mix (Premier Tech Horticulture, Rivière-du-Loup, Québec, Canada) and 50% Turface ProLeague (Profile Products, Buffalo Grove, IL) (vol/vol). Plastic bags were placed over the pots and secured with a rubber band for five days to increase relative humidity and enhance seed germination. Switchgrass plants were placed

in growth chambers and kept at 28°C with a 12-h photoperiod for 6 weeks. Experiments were set-up in a completely randomized design, with five replicate pots (20 seeds each) per isolate. To evaluation virulence of isolates, the following parameters were measured: percent seed germination, necrotic lesions and root rot ratings plant height from crown to leaf tip, and yields (fresh and oven dry weight after 7 days at 45°C) per pot. Switchgrass were graded on a disease rating scale from 0 to 5 (Ahmed et al. 2009; Iftikhar et al. 2012), with 0 = no symptoms; 1 = 1% to 5% disease symptoms on plants or roots within a pot; 2 = 6% to 25% disease symptoms; 3 = 26% to 50% disease symptoms; 4 = 51% to 80% disease symptoms; and 5 = 81% to 100% disease symptoms or complete plant death.

Whole plant inoculation and virulence assessment

Whole plant inoculations were conducted for all isolates evaluated in this study, including *F. acuminatum* (n = 2), *F. armeniacum* (n = 2), *F. equiseti* (n = 3), *F. graminearum* (n = 5), and *F. tricinctum* (n = 1). For each isolate, approximately 1150 'Alamo' switchgrass seed were surface-sterilized, cold stratified, and planted in 9 x 9-cm² pots containing potting mix (30 per pot). Pots were covered with plastic for five days to promote seed germination. Plants were grown in growth chambers at 28°C with a 12-h photoperiod. Pots of 4-week-old switchgrass were thinned to 20 plants per pot, allowing for germination discrepancies, i.e. in some treatments, less than 20 plants germinated. Six-week-

old switchgrass plants were wounded by trimming plants to approximately 5 cm in height.

Trimmed plants were sprayed with a conidial spore suspension of 10^5 conidia/ml sterile DI water (7 ml per pot) and covered with a plastic bag for 7 days to stimulate conidial germination. Controls were inoculated with sterile DI water and subjected to the same conditions. Virulence was evaluated 28 dpi, based on necrotic lesions and root rot ratings, yields (fresh and oven dry weight after 7 days at 45°C) per pot, plant height from crown to leaf tip, and stand count. Assays were set-up in a completely randomized design, with five replicate pots (20 seeds each) per isolate. Root rot and necrotic lesions were rated using the same parameters as described for seed infected switchgrass (Ahmed et al. 2009; Iftikhar et al. 2012).

Isolate recovery from infected plant tissue

Fusarium isolates were re-isolated from symptomatic leaf and root tissues excised from infected plants. Infected tissues were surface-sterilized (1 min 95% ethanol, 30 sec 30% Clorox, 1 min 95% ethanol, and rinsed in sterile DI water), air-dried, and plated on 2% water agar amended with 10 mg/l rifampicin and 8 µl/l 2,4 EC Danitol miticide (Valent, Walnut Creek, California). Hyphae observed growing from the infected tissues were sub-cultured onto PDA and identification confirmed through morphological characteristics and identity of TEF1-α PCR amplicons.

Statistical analysis

A mixed model procedure ANOVA (SAS 9.4 Inc., Cary NC) was used to analyze data. A random block design (RBD) was used for detached leaf assays, with replicates separated as blocks and each leaf section as samples. To determine pathogen aggressiveness, least square means were calculated and significant effects were separated with least significant difference. For whole plant and seed inoculations, ANOVA was used to determine the effects of species, and isolate on switchgrass. A completely randomized design (CRD) with factorial sampling was used for virulence assays, with mean separation calculated to determine differences in isolate virulence. A significance probability value of 0.05 was used in all analyses.

Results

Morphological characteristics

There was wide morphological variation among *Fusarium* spp. included in this study (Fig. 3.1.) *Fusarium acuminatum* displayed white to pale orange floccose mycelium, with a tan to pale orange pigmentation produced in PDA (Fig. 3.1 a-b). Macroconidia were 45 to 88 μm long, curved, with 3 to 5 septa. The basal cell of macroconidia was foot-shaped, and the apical cell was tapered and curved (Fig. 3.1 c). *Fusarium armeniacum* produced white, abundant mycelium, with a pale tan pigmentation in PDA (Fig. 3.1 d-e). Macroconidia were curved, with 3 to 5 septa. The apical cell of conidia was long and tapered, and the basal cell

distinctly foot-shaped. Macroconidia were 23 to 30 μm in length (Fig. 3.1 f).

Abundant mycelia, ranging in color from white, to pale orange, to tan were seen in isolates of *Fusarium equiseti*. Pigmentation in PDA fluctuated from a pale to dark orange (Fig. 3.1 g-h). Macroconidia were abundant with 5 to 7 septa, and were 27.5 to 88 μm long. Conidia were slender, with a tapered and whip-like apical cell. The basal cell was elongated and foot-shaped (Fig. 3.1 i). Aerial mycelia of *Fusarium graminearum* were white, to pale orange, to pink. Pink to red pigmentation were formed in PDA (Fig. 3.1 j-k). Macroconidia were sparse with a foot-shaped, basal cell and tapered apical cell. Macroconidia were slender, thick-walled, moderately curved, with 5 to 6 septa, and were 35 to 87 μm in length (Fig. 3.1 l). Isolates of *Fusarium oxysporum* exhibited flat to floccose mycelia that varied from white to pale purple to dark purple, with a pale purple to dark purple pigmentation produced in PDA. (Fig. 3.1 m-n) Macroconidia and microconidia were observed on PDA. Macroconidia were slender and curved, with a curved and tapered apical cell. Conidia were 42 to 55 μm long, three-septate, with a foot-shaped or pointed basal cell. Microconidia were ovoid- or kidney-shaped with 0 to 1 septa (Fig. 3.1 o-p). *Fusarium tricinctum* isolates yielded mycelia that were white and dense, with pale to dark orange pigments in PDA (Fig. 3.1 q-r). Macroconidia were slender, falcate, three-septate, and 34 to 44 μm in length. The apical cell was curved and tapered, and the basal cell had a well-developed foot shape. Microconidia were 0 to 1 septate, napiform and pyriform. Microconidia were 15 to 25 μm long (Fig. 3.1 s-t).

Detached leaf assay

Isolates of *F. acuminatum* (n = 1), *F. armeniacum* (n = 2), *F. equiseti* (n = 1), *F. graminearum* (n = 5), and *F. tricinctum* (n = 1) were pathogenic on switchgrass, causing necrotic lesions on detached leaf blade at 7 dpi (Fig. 3.2). Based on percent diseased leaf area, significant differences were observed in pathogen aggressiveness, at both the species and isolate level ($P = 0.05$) (Fig 3.3).

Fusarium graminearum isolates were the most aggressive, *F. acuminatum*, *F. armeniacum*, *F. equiseti*, and *F. tricinctum* were moderately aggressive, and *F. oxysporum* were non-aggressive. *Fusarium graminearum* isolate TBW107 was the most aggressive with a mean disease leaf area of 42.5% at 7 dpi. Moderately aggressive isolates (AK109, JCIM15, CA12, EA61, AK58, JCIM7, and RSA117) ranged from 10.2% to 23% disease leaf area 7 dpi. Excluding isolate JCIM 15, *F. equiseti* isolates showed limited virulence on switchgrass, with lesion areas up to 6%. Isolates with a mean of less than 10% disease leaf area were considered non-aggressive. *Fusarium oxysporum* isolates had mean disease leaf areas between 0% and 4%, suggesting that *F. oxysporum* may be a benign or potentially beneficial endophyte in switchgrass.

Seed Germination

Seed germination was reduced by inoculation with *Fusarium* isolates at 10^5 conidia/ml inoculum density. There were significant differences among *Fusarium* species and isolates within species for seed germination. At the species level, reduction in seed germination ranged from 15.4% to 42.6% (Fig. 3.4 A).

Fusarium graminearum was the most virulent causing the greatest impact on seed germination. *Fusarium tricinctum* was the least virulent and caused the least impact on seed germination ($P < 0.05$). Virulence of isolates within species varied greatly. Within *F. graminearum*, isolate ECNR21 was the most virulent with 22.8% germination and TBW107 was the least virulent with a 54% germination. Only two isolates of *F. acuminatum* were tested, with isolate WA3 being the most virulent at 30% germination and AK109 the least at 65% germination.

Germination rates of seeds treated with *F. armeniacum* and *F. equiseti* isolates were not significantly different and ranged from 40% to 44% and 33% to 33.8%, respectively.

Only a single isolate of *F. tricinctum* (AK58) was available, making virulence evaluation difficult. Across all isolates, *F. graminearum* ECNR 21 was the most virulent and *F. acuminatum* AK109 was the least virulent.

Disease severity

Symptoms observed on seedlings of seed inoculated with 10^5 conidia/ml included leaf spot, stem necrosis, seedling blight, and root necrosis (Fig 3.5). All five species caused foliar symptoms. *Fusarium graminearum* and *F. armeniacum* caused stem necrosis and root rot. Diseased plant area was significant for all species tested (Fig. 3.4 B). *Fusarium graminearum* was the most virulent with diseased plant area greater than the other four species ($P < 0.05$). *Fusarium acuminatum* was the least virulent species. Significant differences were found in percent diseased area for isolates within *F. graminearum* and *F. equiseti*. *Fusarium graminearum* isolate ECNR21 and *F. equiseti* isolate ABW1 had the least diseased plant area and *F. graminearum* TBW107 and *F. equiseti* JCIM15 the most.

Seedlings inoculated with 10^5 conidia/ml exhibited leaf spots, stem necrosis, and seedling blight. All *Fusarium* species caused foliar symptoms, with *F. graminearum* and *F. armeniacum* isolates being the most virulent. Foliar disease was significant for all species tested (Fig. 3.6 A). With many of the seedlings exhibiting leaf spot and leaf necrosis. *Fusarium graminearum* isolates had the greatest percent disease area and *F. equiseti* the least. For *F. graminearum* and *F. armeniacum*, significant differences were found between isolates.

Plant height

In the whole plant inoculations study, *F. graminearum* and *F. armeniacum* significantly reduced plant height when compared to the control (Fig. 3.6 B). *Fusarium graminearum* isolate TBW107 and *F. armeniacum* isolate RSA117 reduced plant height by an average of 5.77 and 5.61 cm, respectively.

Plant Yield

Fresh weight yields of switchgrass infected with *Fusarium* spp. were significantly reduced. For seed inoculation, differences in fresh weight among species and isolates were significant (Fig. 3.4 C). Compared to the control, isolates of *F. armeniacum*, *F. equiseti*, and *F. graminearum* had significantly reduced yields. Isolates RSA117, JCIM15, and EA61 had the greatest average fresh weight reductions per pot at 1.69, 1.77, and 2.33 g, respectively. Significant differences in plant fresh weight reductions were also found between *F. equiseti* and *F. graminearum* isolates. No significant differences were observed in 45°C oven dry weights after 7 days.

Similarly, in seedling inoculations, *F. armeniacum*, *F. equiseti*, and *F. graminearum* caused significant reductions in fresh weight when compared to the control (Fig. 3.6 C). *Fusarium acuminatum* isolate AK109 significantly reduced yield when compared to the control. *Fusarium graminearum* isolate TBW107 had the highest yield reduction at an average of 2.11 g per pot. Significant differences in virulence were found between isolates within a species, including, *F.*

acuminatum, *F. armeniacum*, *F. equiseti*, and *F. graminearum*. With only one isolate, virulence of *F. tricinctum* could not be determined. Oven dry weights yielded no significant differences.

Discussion

Fusarium isolates within and between species varied in colonial and conidial morphology. Historically, *Fusarium* species have been identified based on the morphological species concept under Linnaean definitions, under the criteria that morphological consistency remains within a species and that there are consistent differences between species (Mayer 1963). While morphological species concepts allow for rapid identification, there are few detectable characteristics that can be used in *Fusarium* identification including colony growth, pattern, and color; culture pigmentation on media, presence or absence of microconidia, and morphology of macroconidia. Many of the physical characteristics used to identify *Fusarium* vary based on the environment in which they are produced (Burgess 1981). *Fusarium* must be grown on specific media and under particular incubation condition for correct identification (Gerlach and Nirenberg 1982; Nelson et al. 1983). *Fusarium* isolates used in this study varied in mycelial and conidial morphology within and among species, making identification difficult (Fig 3.1). Morphological identifications were used to rapidly identify and group *Fusarium* isolates. Phylogenetic species concepts were used to accurately identify *Fusarium* isolates to species.

This study uncovered variations in pathogenicity and virulence among *Fusarium* spp., and isolates within species. Pathogenicity was defined as the capability of a pathogen to infect and cause disease in switchgrass, and virulence defined as the amount of damage produced (Sacristán and García-Arenal, 2008). *Fusarium graminearum* was the most virulent species, causing significant disease on detached switchgrass leaves 7 dpi. Highly virulent pathogens can rapidly infect host plant tissues, are easily transmitted, and can overcome plant disease resistance (Sacristán and García-Arenal 2008; Fisher et al. 2012). Determining pathogenic and virulent isolates of *Fusarium* could help promote disease resistance through switchgrass breeding programs, development of superior quality certified seed and disease management strategies, thus reducing the impact of *Fusarium* on switchgrass. Further research will be needed to determine virulence factors associated with highly infectious isolates.

One of the major problems in switchgrass cultivation is stand establishment (Parrish and Fike 2005). During seed inoculation assays, *F. graminearum* isolates significantly reduced germination. It is likely that seedborne pathogens like *Fusarium* play a significant role in low seed germination. Moreover, the height and vigor of established switchgrass plants were greatly impacted by *Fusarium*, reducing fresh weight. Reduction of plant height of *Fusarium graminearum* and *F. armeniacum* were similar when applied to whole plants.

All *Fusarium* species, except *F. tricinctum*, reduced switchgrass fresh weight when applied to seed or whole plants. *Fusarium graminearum* had the greatest

impact on fresh weight of infected seed and whole plants with a mean reduction of 1.77 and 2.11 g per pot. *Tilletia maclaganii*, the causal agent of smut on switchgrass, reduced fresh weight by 38 to 82%, and biomass by 40% (Thomsen et al. 2008). *Bipolaris*, *Alternaria*, *Xylaria*, and *Phaeosphaeria* species can cause up to 61% total dry mass reduction in switchgrass biomass (Kleczewski et al. 2012). High biomass yield combined with limited fertilizer application makes switchgrass a suitable bioenergy crop (Bouton, 2008). Pathogen threats to switchgrass biomass are of economic concern, as biomass yields must be maximized to increase net energy yields.

Cost-effective disease management is needed to reduce economic losses.

Fusarium spp. have been isolated from seed of different cultivars from different parts of the U.S., leaving cause for concern and *F. graminearum* was the most frequently isolated and widely distributed species (Vu, 2011). *Fusarium armeniacum* was the second most virulent species on 'Alamo', and had been an unknown pathogen of switchgrass until this study. Because only one isolate was tested for *F. tricinctum*, it is possible that isolates that are more virulent will emerge on switchgrass. *Fusarium tricinctum* isolate AK58 was a moderately aggressive pathogen on 'Alamo'.

Of the *Fusarium* species, *F. acuminatum*, *F. armeniacum*, *F. equiseti*, and *F. tricinctum* have only been reported on switchgrass in Tennessee, whereas *F. graminearum* has been reported from different states in the U.S. and has been

isolated from important grain crops and grasses such as wheat, corn, barley, oat, rye, rice, sorghum, and bermudagrass (Farr and Rossman, 2016).

Throughout the seed and whole plant studies, *F. graminearum* isolates were consistently the most virulent. Inoculation with *F. graminearum* isolates resulted in the lowest seed germination rates, most stunted growth, highest disease severity, and largest fresh weight reductions of plants. Greater impacts on fresh weight were found for whole plants inoculated with *Fusarium*, whereas stand reduction was observed only from the seed inoculation experiments. Planting of infected seeds and subsequent infection of an established stand is problematic to switchgrass production. Survival of *Fusarium* species on seeds may be major source of inoculum. As commercial seed is increasingly sold and distributed, reports of switchgrass pathogens will rise. Seed certification practices and cost-effective seed treatment will be paramount to reduce pathogen spread.

Based on the results of the virulence assays, *Fusarium* isolates can be classified into the following three virulence groups: highly virulent (26% to 100%), moderately virulent (7% to 25%), and non-aggressive (<6%). Most of the isolates were moderately virulent. The information learned from pathogenicity and virulence variability in *Fusarium* will promote disease management strategies, leading to reduced impacts of fungal pathogens on switchgrass, and provide valuable information to develop resistant cultivars in switchgrass breeding programs.

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Appendix

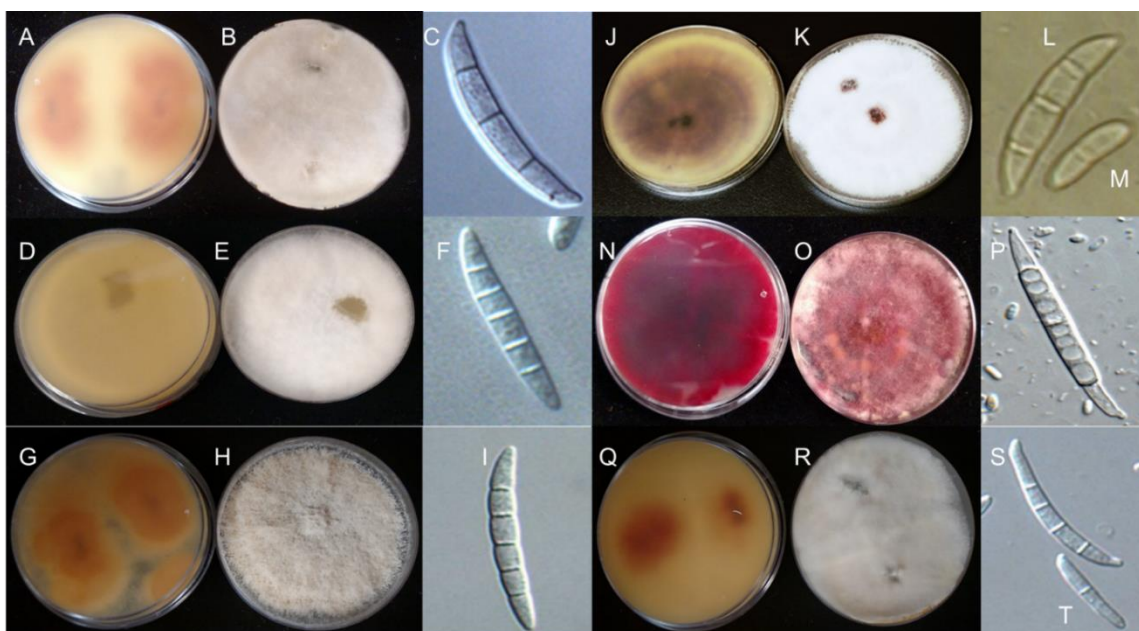


Figure 3.1 Morphological characteristics of six *Fusarium* species on potato dextrose agar (PDA). *Fusarium acuminatum* (A) reverse, (B) top, (C) macroconidium; *Fusarium armeniacum* (D) reverse, (E) top, (F) macroconidium; *Fusarium equiseti* (G) reverse, (H) top, (I) macroconidium; *Fusarium oxysporum* (J) reverse, (K) top, (L) macroconidium, (M) microconidium; *Fusarium graminearum* (N) reverse, (O) top, (P) macroconidium; *Fusarium tricinctum* (Q) reverse, (R) top, (S) macroconidium, (T) microconidium.

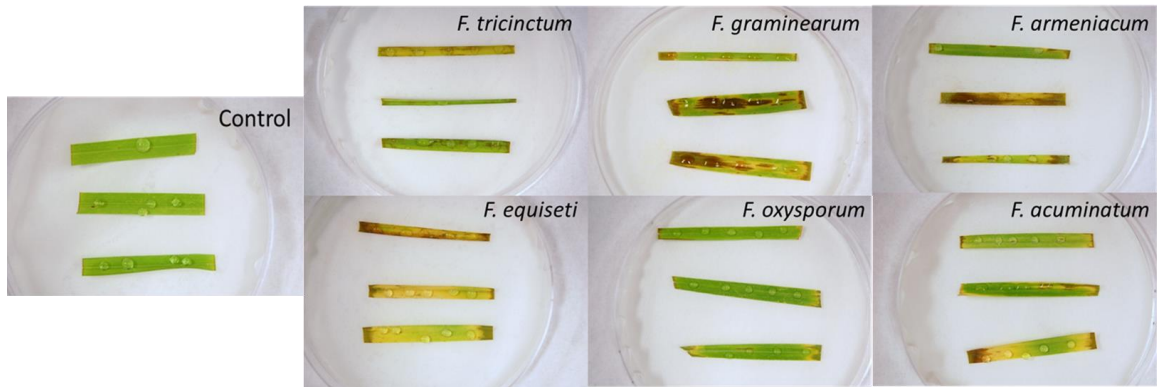


Figure 3.2 Disease symptoms caused by *Fusarium* spp. on detached switchgrass leaf blades 7 days post-inoculation.

Figure 3.3 Effect of Fusarium isolates recovered from commercial seed on percent lesion area of switchgrass in detached leaf assays^a

^aData reported are mean values of eight replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P = 0.05$.

^b*Fusarium* species used: *Fusarium acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, and *F. tricinctum*.

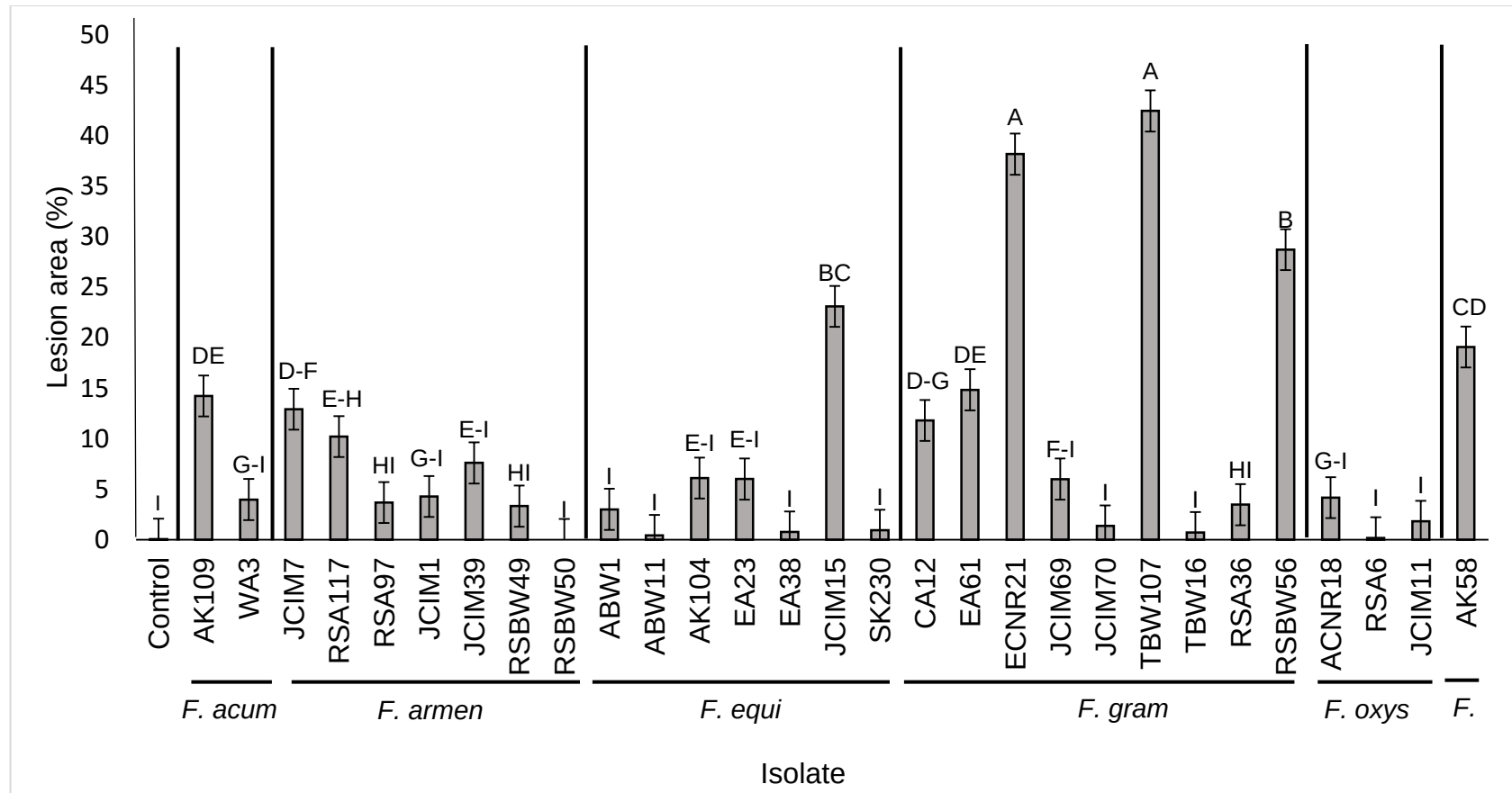


Figure 3.3 Continued

Figure 3.4 Impact of Fusarium species on switchgrass following seed inoculation. Main effect of Fusarium on (A) seed germination, (B) disease severity, and (C) fresh weight^a

^aData reported are mean values of four to nine replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P = 0.05$.

^b*Fusarium* species used: *Fusarium acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, and *F. tricinctum*

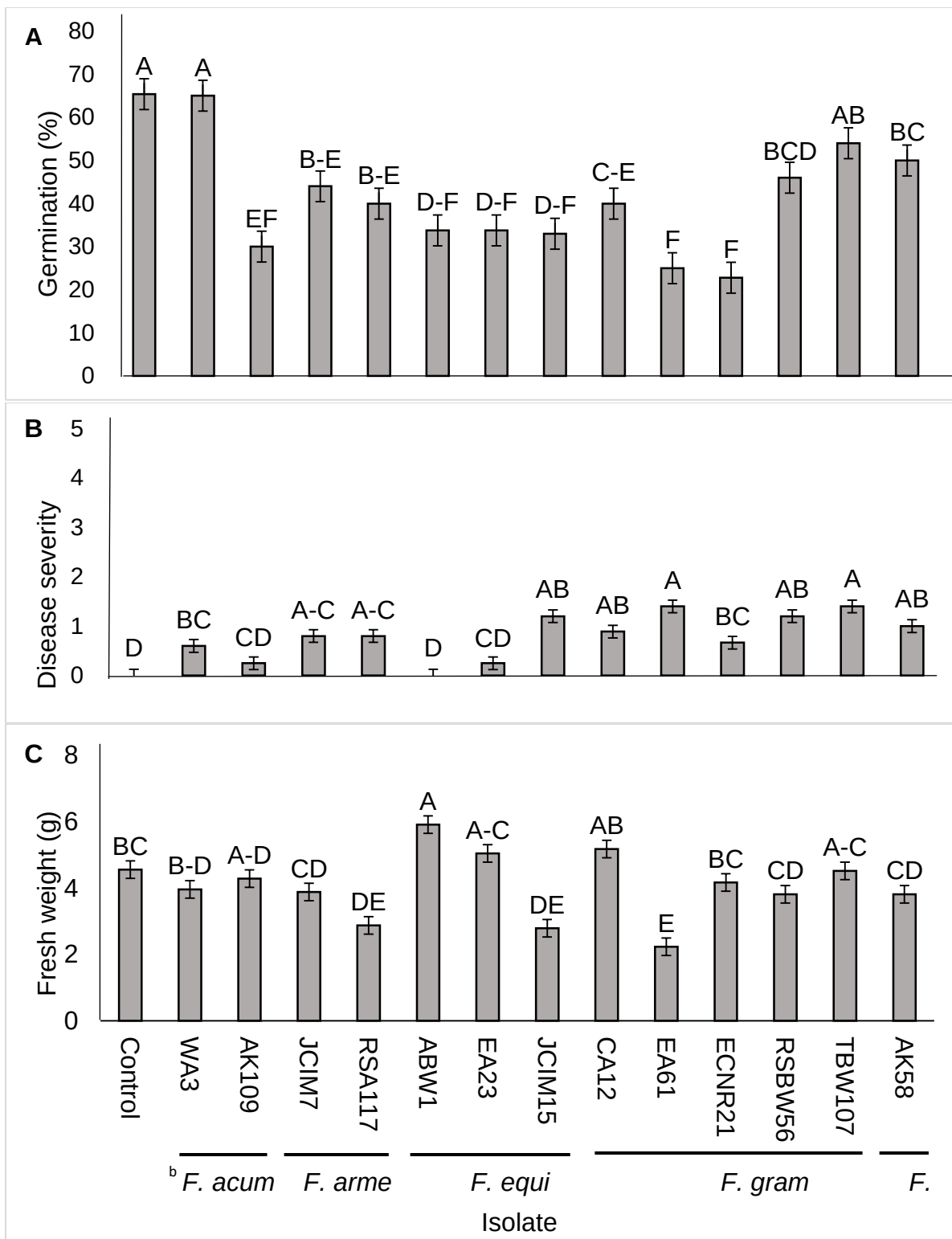


Figure 3.4 Continued

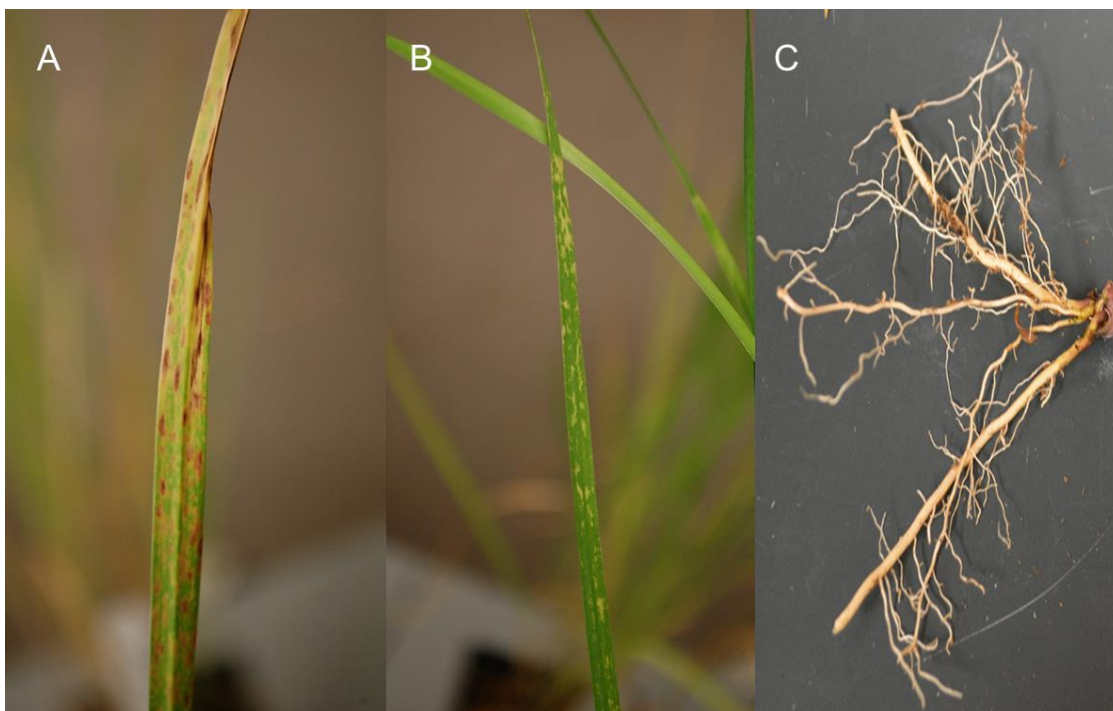


*Figure 3.5 Disease symptoms on emerged seedlings from inoculated seeds. (A) Leaf spot on plant grown from seed inoculated with 10^5 conidia of *Fusarium graminearum*, (B) leaf spot on plant grown from seed inoculated with 10^5 conidia of *F. armeniacum*, and (C) root necrosis induced by 10^5 conidia/ml of *F. graminearum*.*

Figure 3.6 Impact of Fusarium species on switchgrass following whole plant inoculation. Main effect of Fusarium on (A) disease severity, (B) plant height, and (C) fresh weight^a

^aData reported are mean values of four to nine replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P = 0.05$.

^b*Fusarium* species used: *Fusarium acuminatum*, *F. armeniacum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, and *F. tricinctum*.

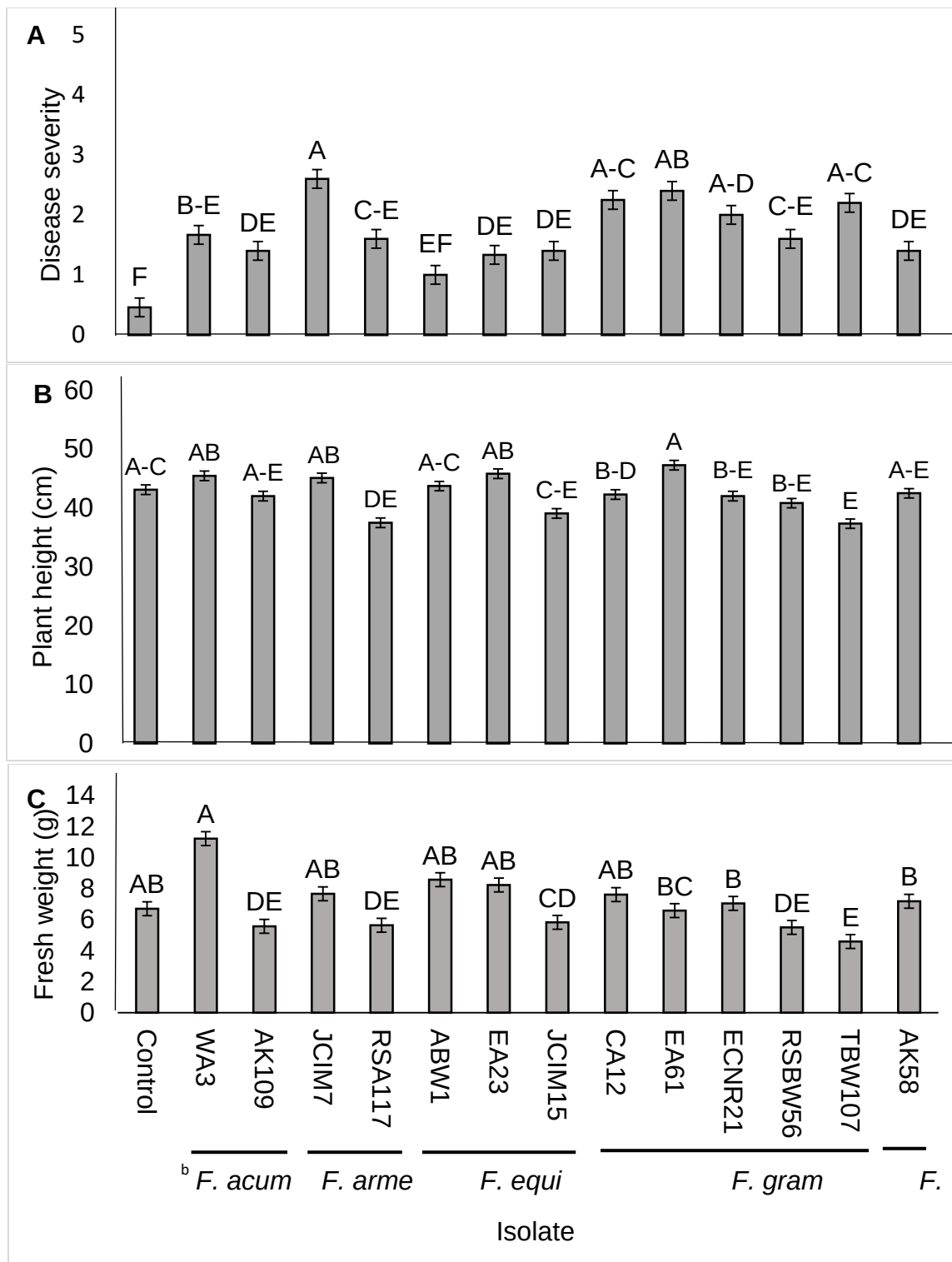


Figure 3.6 Continued

CHAPTER FOUR

**ASSESSMENT OF *ALTERNARIA ALTERNATA* MORPHOLOGY,
VIRULENCE DIVERSITY, AND IMPACT ON SWITCHGRASS
PLANT HEALTH**

Abstract

Alternaria alternata causes foliar disease in switchgrass (*Panicum virgatum* L.), but the extent of its impact on this host is unknown. Six isolates of *A. alternata* were tested for pathogenicity and virulence, and impact on switchgrass germination, plant health, and biomass. Isolates of *A. alternata* were recovered from infected commercial seed. Tests were arranged in a completely randomized design and data analyzed with mixed model ANOVA and F-LSD (SAS 9.4). Six isolates were screened for pathogenicity with a detached leaf assay using 'Alamo' switchgrass. All *A. alternata* isolates were pathogenic on detached leaves ($P < 0.05$), with lesion areas ranging from 8.8% to 23.3%. Seed and whole plant inoculations were conducted to determine *A. alternata* impact on plant health. Foliar disease ratings of plants from seed inoculated with all isolates were significantly greater than in untreated controls. In whole plant inoculations, all isolates caused greater foliar disease severity than untreated controls. Four isolates significantly reduced shoot fresh weight in whole plant inoculations. Isolates did not reduce plant stand, suggesting that *A. alternata* may cause less economic losses than other fungal pathogens of switchgrass, such as *Bipolaris* and *Fusarium* species.

Introduction

Native perennial switchgrass (*Panicum virgatum* L.) has become the dedicated cellulosic biofuel production crop (Bouton 2008; Bouton 2007; McLaughlin 2005). Over the past several decades, targeted breeding has given rise to significant gains in biomass yield and improved feedstock conversion properties and synthesis of co-products (Casler 2012; Bouton 2007; McLaughlin 2005), but there has been little improvement in genetic resistance to diseases in switchgrass. In order to control disease, integrated agronomic management strategies and the development of resistant cultivars will be needed (Stewart and Chromey 2011).

Seed and soilborne diseases of switchgrass have been largely neglected. Seedborne diseases studied, such as *Bipolaris* spp., have been found to impact seedling established, biomass quality, and yield (Fajolu 2012). Several fungal pathogens have been recorded on switchgrass (Farr et al. 1989; Tiffany et al. 1990; and Gravert and Munkvold 2002), but with limited knowledge on their virulence and impact on stand and yield. To assess seedborne pathogens of switchgrass, a study was conducted at the University of Tennessee. During the 2011 study, commercial seed lots from across the U.S. were tested for fungal infection. Over 2,000 fungal isolates were recovered, consisting primarily of *Alternaria*, *Bipolaris*, and *Fusarium* (Vu et al. 2011). Several species of *Alternaria*, *Bipolaris*, and *Fusarium* are known pathogens of switchgrass and other economically important crops (Vu et. al 2011; Farr and Rossman 2016).

Alternaria alternata, *Alternaria panici*, and *Alternaria sp.* have all been reported to cause disease on switchgrass, but little research has been conducted to identify the extent of damage these pathogens can ensue on switchgrass establishment and biomass (Farr and Rossman 2016). *Alternaria* species are common plant pathogens with wide host ranges. *Alternaria* spp. cause approximately 20% of agricultural spoilage in the U.S. (Lawrence et al. 2013) and have the potential to negatively impact switchgrass germination, health, and growth. Thus, the objectives of this study were to identify diversity in morphology, pathology, and virulence amongst *Alternaria alternata* isolates, and determine the impacts of *A. alternata* on stand establishment and biomass yield.

Materials and Methods

Alternaria alternata

Alternaria alternata isolates (n = 6) were recovered from surface-sterilized commercial switchgrass seed from 'Alamo', 'Blackwell', and 'Cave-n-Rock' seed, and plated on potato dextrose agar (PDA) amended with 100 mg chloramphenicol/liter (Sigma-Aldrich, St. Louis, MO) (Vu 2011). Seed were surface-sterilized with 1% NaOCl (Clorox®, Oakland, CA) amended with 10 µl Tween-20 (U.S. Biochemical Corp., Cleveland, OH) for 1 min, rinsed three times with sterile deionized (DI) water, and dried on sterile filter paper (Mathur and

Kongsdal 2003). Hyphal colonies observed growing from seed were transferred to PDA plates for identification.

Isolate identification

Each isolate was grown *Alternaria* sporulation media (ASM) amended with 3.45 mg fenpropathrin/liter (Danitol 2.4 EC, Valent Chemical, Walnut Creek, CA) and 10 mg/liter rifampicin (Sigma-Aldrich, St. Louis, MO) for 7 days at 25°C. Colony morphology was characterized by mycelial color, media pigmentation, and growth pattern. Conidia were assessed based on size, number and type of septa, and shape.

Genomic DNA was extracted from aerial mycelium of each isolate with a Direct Phire PCR Kit (ThermoFisher Scientific, Waltham, MA). Ribosomal DNA from the internal transcribed spacer (ITS) region of the 18s subunit was amplified through polymerase chain reaction (PCR) with primers ITS1 and ITS4 (White et al. 1990). The DNA fragments were sequenced and checked against the GenBank database with BLASTn (National Center for Biotechnology Information).

Seed sterilization and cold stratification

For each assay, seed were surface-sterilized in 10% Clorox® for 20 min on a magnetic stirrer, and rinsed with sterile deionized (DI) water. Cold stratification was used to induce seed germination. Seed were soaked in sterile DI water for 1 hr, and then water was drained and the seed placed in Petri dishes on sterile filter paper moistened with sterile DI water. Seeds were kept at 4.4°C for 12 h.

Detached leaf assay

Alternaria alternata (n = 6) isolates were prescreened for pathogenicity and virulence on detached switchgrass leaves. The test was arranged in a randomized complete block design with three samples per replicate and four replicates of each treatment and control. Plant material was harvested from 8-week-old switchgrass, grown from surface-sterilized and cold stratified seed. Five-cm blades were surface-sterilized in 95% ethanol for 30 s, 10% Clorox® for 1 min, 95% ethanol for 30 s, and rinsed in sterile DI water. For each plate, three surface-sterilized leaf blades were placed in a sterile Petri dish, on a 9-cm diameter sterile filter paper disk moistened with sterile DI water.

For inoculum production, conidia were harvested by flooding the surface of fungal plates with 5 ml sterile DI water containing 10- μ l Tween-20 (Fisher Scientific, Dubuque, Iowa) and gently scraping the fungal colony with a sterile rubber policeman. Tween-20 was added to act as a surfactant, enhancing the dispersal of conidia. Each leaf segment was inoculated with five 10- μ l droplets containing 1×10^4 conidia/ml. Control leaf sections were inoculated with five 10- μ l droplets of sterile DI water. The assay consisted of four replicate Petri dishes for each isolate and the control. Petri dishes of the same replicate were incubated at room temperature in a moist chamber, to increase relative humidity.

Detached leaves were visually inspected daily for disease symptoms. Leaf sections, at 7 days post inoculation (dpi), were photographed with a 4100 Nikon mounted to a photo stand. To assess pathogenicity and virulence, percent

disease area of each leaf section was measured using Assess 2.0 image analysis software for disease quantification (Lamari 2008).

Seed inoculation and virulence assessment

Alternaria alternata (n = 6) isolates from the detached leaf assay were used in seed inoculation assays. Assays were set-up in a completely randomized design, with five replicate pots (20 seeds each) per isolate. For each isolate, 100 'Alamo' switchgrass seeds were surface-sterilized and subjected to cold stratification. Half of the surface sterilized seed were inoculated with 5 ml of a 1×10^5 conidial suspension, the other half with 5 ml sterile DI water to serve as controls, and the seed placed on a shaker overnight. Conidia were harvested by flooding ASM plates with 5 ml of sterile water and 10 μ l tween, and concentrated to 1×10^5 conidia/ml sterile DI water using a hemocytometer. Seed were planted in 9×9 -cm² pots in potting mix and covered with a plastic bag for 5 days. Bags were secured with rubber bands to increase relative humidity and enhance seed germination. Plants were grown in growth chambers at 28°C, with a 12-h photoperiod for 6 weeks. Virulence evaluation included necrotic lesions and root rot ratings, percent seed germination, plant height from crown to leaf tip, and biomass yield (fresh and oven dry weight after 7 days at 45°C) per pot. Percent disease and root rot were graded on a disease rating scale from 0 to 5 (Ahmed et al. 2009; Iftikhar et al. 2012), with 0 = no symptoms; 1 = 1% to 5% disease symptoms; 2 = 6% to 25% disease symptoms; 3 = 26% to 50% disease

symptoms; 4 = 51% to 80% disease symptoms; and 5 = 81% to 100% disease symptoms or complete plant death.

Whole plant inoculation and virulence assessment

Six isolates of *A. alternata* were tested in whole plant inoculations to assess impact of disease when inoculated after stand establishment. Experiments were designed as a completely randomized design, with five replicate pots (20 seeds each) per isolate.

Approximately 1150 'Alamo' switchgrass seeds were surface-sterilized and cold stratified. Seed were planted in potting mix (30 per pot), and pots covered with plastic bags for 5 days. Pots were kept in growth chambers at 28°C with a 12-h photoperiod. At 4 weeks after planting, pots were thinned to 20 stems per pot to allow for germination differences. Six-week-old switchgrass plants were wounded by trimming the tops to a height of approximately 5 cm. Pots were sprayed with a conidial spore suspension of 1×10^5 spores/ml sterile DI water (7 ml per pot) and subjected to high humidity by covering the pots with plastic bags for 7 days.

Controls were sprayed with sterile DI water and subjected to the same conditions. At 28 dpi, virulence of each isolate was evaluated based plant height from crown to leaf tip, yield (fresh and oven dry weight after 7 days at 45°C) per pot, and stand count. Percent disease and root rot were graded on a using the same disease rating scale as in seed inoculations (Ahmed et al. 2009; Iftikhar et al. 2012).

Isolate recovery from infected plant tissue

Alternaria alternata isolates were re-isolated from symptomatic leaf and root tissues of infected plants. Infected tissue was excised and surface-sterilized (1 min 95% ethanol, 30 sec 30% Clorox®, 1 min 95% ethanol, and rinsed in sterile DI water). Samples were air-dried and plated on 2% water agar amended with 10 mg/l rifampicin and 8 µl/l 2,4 EC Danitol miticide (Valent, Walnut Creek, CA). Plates were incubated and resulting hyphae sub-cultured on ASM. Identification was confirmed through morphology and sequence identity of ITS PCR amplicons.

Statistical analysis

Data were analyzed with ANOVA using a mixed model procedure (SAS 9.4 Inc., Cary NC). Least Square Means were calculated and significant effects were further analyzed with least significant difference ($P = 0.05$) to determine pathogen aggressiveness.

Results

Six *Alternaria alternata* isolates were screened for pathogenicity and virulence on 'Alamo' switchgrass detached leaf segments, seed, and 4-week-old seedlings. Seed germination, post-emergence seedling death, percent diseased plant area, plant height, and plant weight were measured to determine the virulence and impact of *A. alternata* on switchgrass grown from inoculated seed, or receiving

inoculum on older plants. Differences in seed germination, post-emergence seedling death, and plant height were not significant at $P = 0.05$.

Morphological Characteristics

All isolates were identified as *Alternaria alternata* through morphological and molecular identification. Isolates were grown in *Alternaria* sporulation media (ASM) and exhibited dense mycelia ranging from white to gray, to black in color. Pigments produced on ASM were white to gray to black. The conidia were often beaked, with longitudinal and transverse septa. Conidia were dark and borne singly or in chains and with an average size of $42.5 \times 12.5 \mu\text{m}$ (Fig. 4.1).

Morphological features and growth were consistent with characteristics described previously for *Alternaria alternata* (Andersen and Roberts 2001; Farr and Rossman 2016).

Detached leaf assay

All six *A. alternata* isolates tested were pathogenic on detached switchgrass leaves at 7 dpi (Fig. 4.2). Percent disease leaf area of inoculated switchgrass leaf segments was significant when compared to the control, and ranged from 8.8% to 23.3% at 7 dpi ($P = 0.05$) (Fig. 4.3). Isolate TBW131 had the highest percent disease leaf area with 23.3%. No significant differences in virulence were found between isolates.

Disease Severity

Leaf spot and leaf streaking were common symptoms of infected switchgrass plants inoculated at the seed and seedling stage (Fig. 4.5). All six isolates caused foliar symptoms. Isolates SCNR82 and TBW131 had the highest percent diseased plant area in seed inoculation assays ($P < 0.05$) (Fig. 4.4). Mean percent diseased area for inoculated seed ranged from 0% to 25%. In whole plant inoculations, all isolates caused significantly greater foliar disease severity than untreated controls, but there were no significant differences between isolates (Fig. 4.6 A).

Plant Yield

Whole plant inoculations resulted in significant differences in fresh weight, but not oven dry weight. Four of the six isolates tested significantly reduced fresh weight when compared to the control ($P = 0.05$) (Fig. 4.6 B). When compared with the control, mean yield reductions ranged from 0.38 to 0.68 g per pot.

Discussion

Conidial plasticity makes it difficult to identify *Alternaria* to species. Specific media (*Alternaria* sporulation media and dichloran rose bengal yeast extract sucrose agar) can be used to aid in identification (Andersen and Roberts 2001). Polymerase chain reaction of the internal transcribed spacer region of the 18S subunit was used to confirm morphological identifications. A detached leaf assay was conducted to determine whether *A. alternata* caused disease on switchgrass

(pathogenicity), how much damaged was produced (virulence), and if virulence variation occurred among isolates (Sacristán and García-Arenal, 2008). All isolates were found to be pathogenic on switchgrass 7 dpi. There were no significant differences in virulence between isolates. During whole plant and seed inoculation assays, percent diseased area ranged from 0% to 25%. While significantly different from the control, disease severity was relatively low, indicating that *Alternaria alternata* is not as virulent on switchgrass as other known pathogens of this host. No significant differences were found for seed germination, post-emergence damping off, or plant height. This data suggests *A. alternata* does not have a significant impact on stand establishment. Inoculation with isolates SBW74 and TBW 131 resulted in significantly reduced fresh biomass when compared to the control in whole plant inoculation assays. Although *A. alternata* has the ability to reduce fresh weight of established plants, it yields relatively low disease levels, suggesting that it is likely a weak or secondary pathogen of switchgrass.

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Appendix

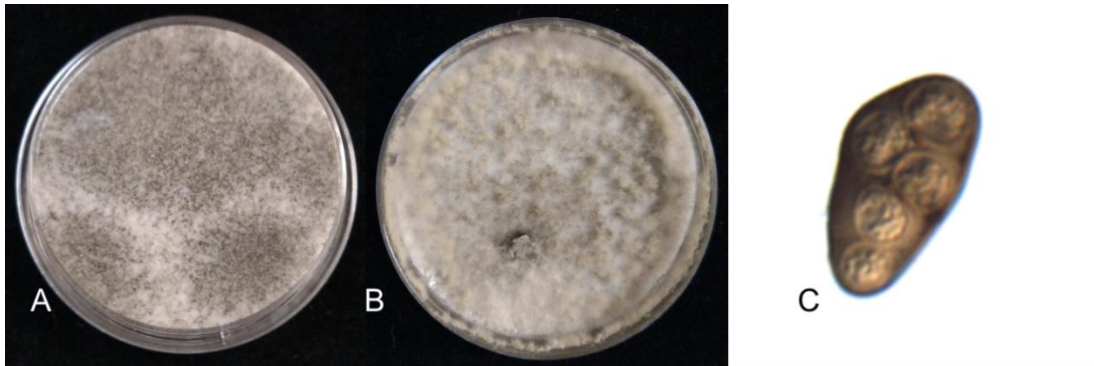


Figure 4.1 Morphological characteristics of *Alternaria alternata* on *Alternaria* sporulation medium. *Alternaria alternata* (A) reverse, (B) top, (C) macroconidia.

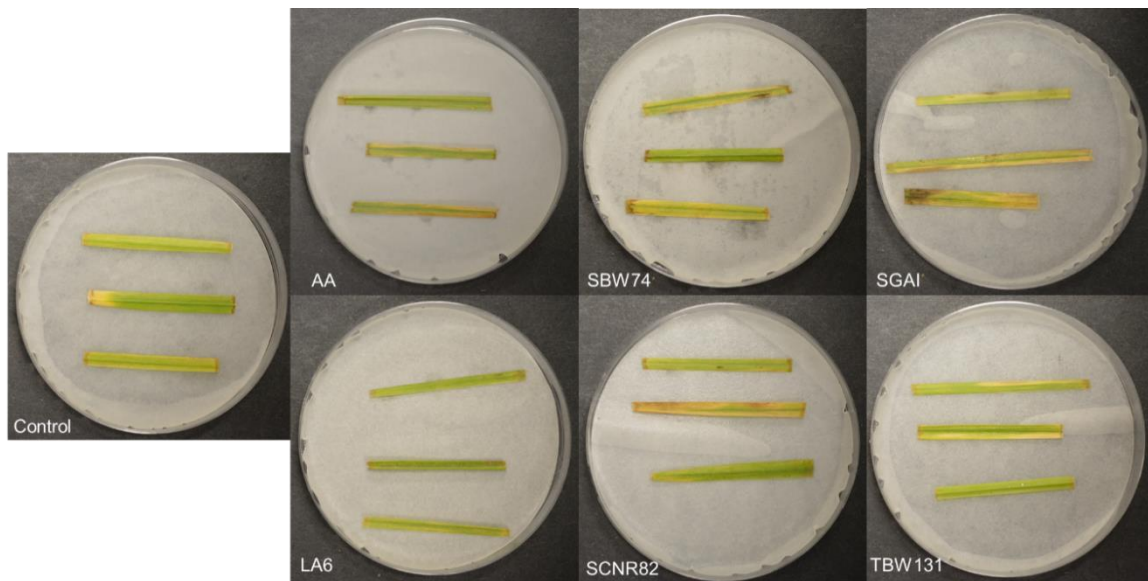
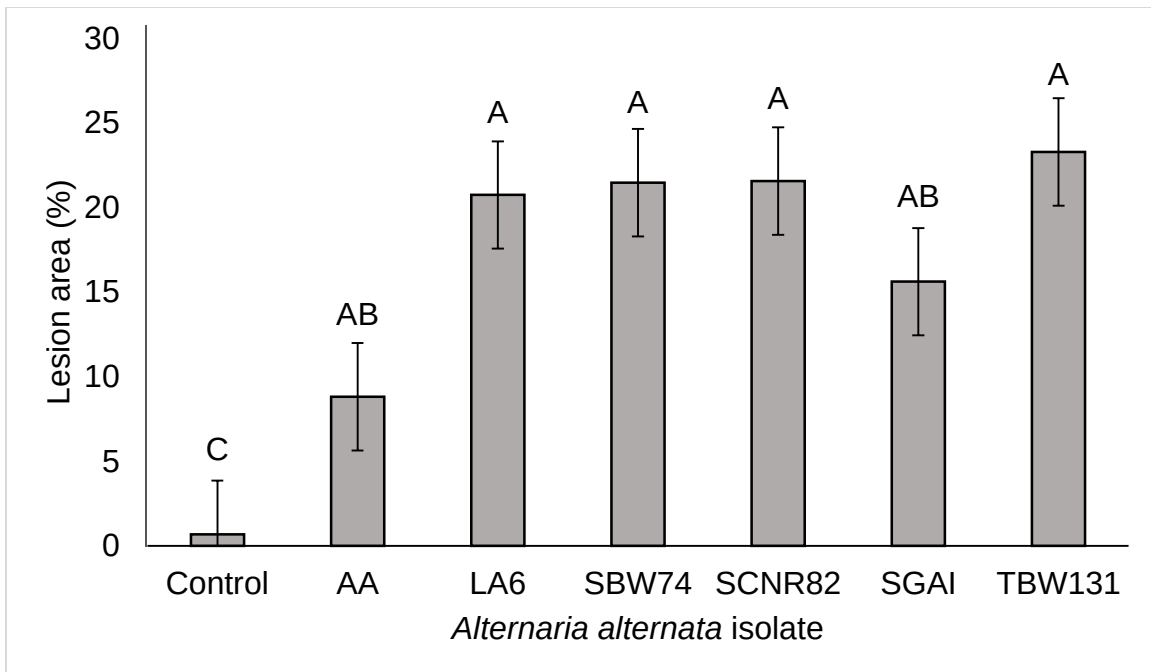
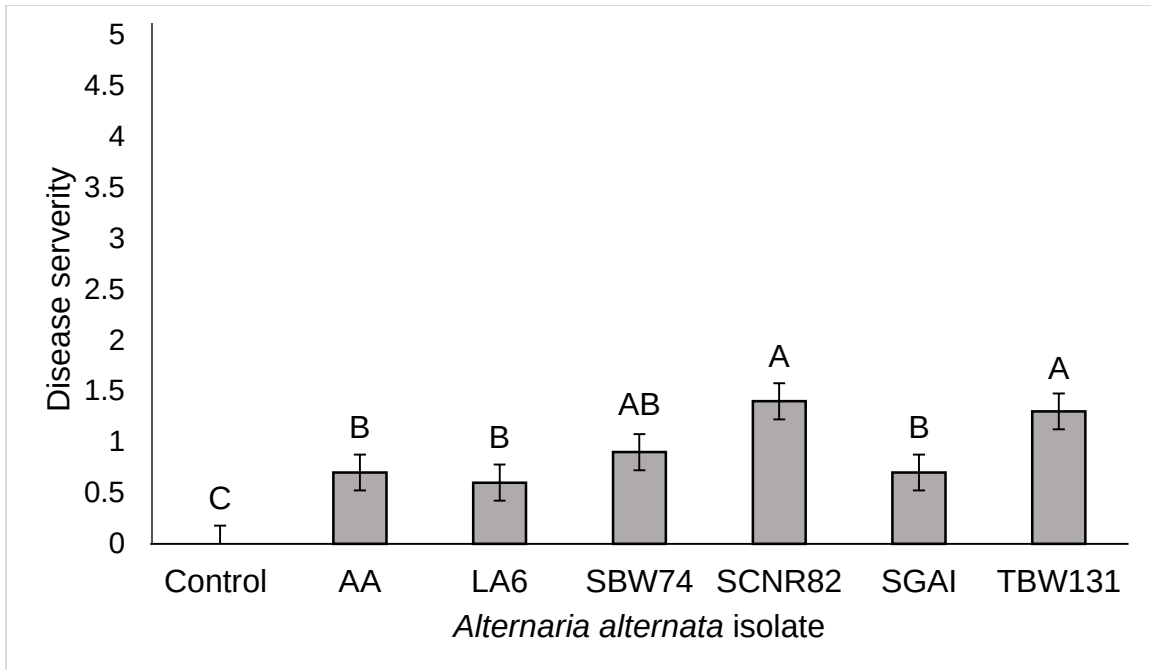


Figure 4.2 Disease symptoms caused by six isolates of *Alternaria alternata* on detached switchgrass leaf blades 7 days post-inoculation



^aData reported are mean values of eight replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P = 0.05$

Figure 4.3 Effect of *Alternaria alternata* isolates from commercial seed on percent lesion area of switchgrass in detached leaf assays^a

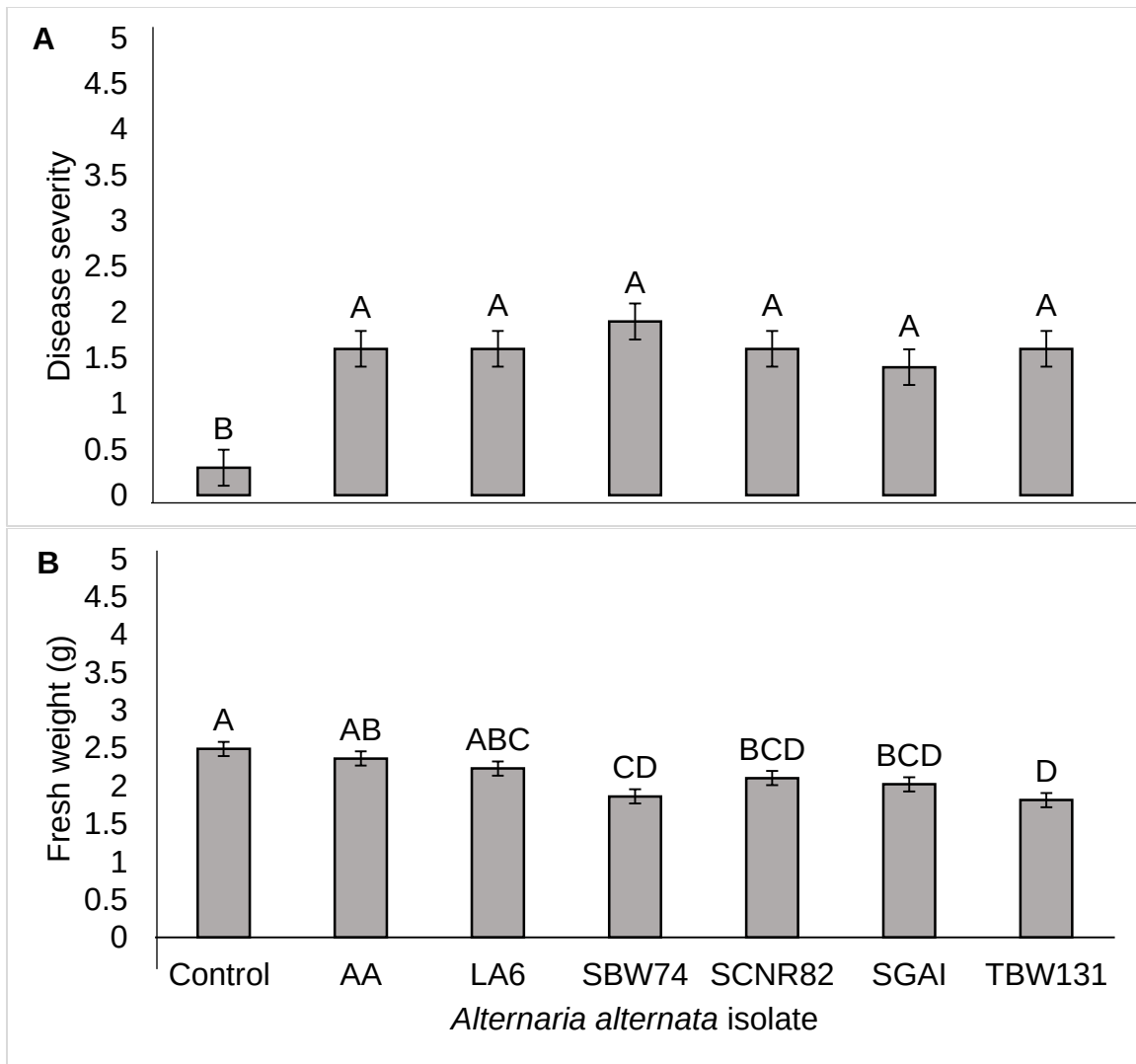


^aData reported are mean values of ten replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P < 0.0001$

Figure 4.4 Impact of *Alternaria alternata* on switchgrass following seed inoculation. Main effect of *A. alternata* on disease severity^a



Figure 4.5 Disease symptoms on emerged seedlings from inoculated seeds. (A) Leaf spot on plant grown from seed inoculated with 10^5 conidia/ml of *A. alternata* and (B) root necrosis induced by 10^5 conidia/ml of *A. alternata*.



^aData reported are mean values of ten replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P=0.05$, respectively.

Figure 4.6 Impact of Alternaria alternata on switchgrass following whole plant inoculation. Main effect of A. alternata on (A) disease severity and (B) fresh biomass yield^a

CHAPTER FIVE
SUSCEPTIBILITY OF FOUR SWITCHGRASS CULTIVARS TO
FUSARIUM GRAMINEARUM

A. Vu, O.L. Fajolu, K. Mantooth, M.M. Dee, and A. Bruce assisted in fungal collection, isolation and morphological identification of *Fusarium graminearum* isolate TBW107. N. Labbé, R. Trigiano, K.D. Gwinn, and B.H. Ownley provided guidance and funding for the study.

Abstract

Fusarium graminearum is an important pathogen of switchgrass causing leaf spot, stem streaking, seedling blight, rot root, stunting, and biomass reduction. To identify potential pathogen resistance, four switchgrass cultivars ('Blade1101', 'Blade1102', 'Alamo', and 'Whippet') were screened against a virulent isolate of *Fusarium graminearum*, TBW107. Cultivars Blade1102 and Alamo were susceptible and 'Whippet' was highly susceptible. Cultivar 'Blade1101' was found to be moderately resistant against isolate TBW107. Additional cultivars and *F. graminearum* isolates should be screened to provide information to plant breeding programs involved development of disease resistance and superior biofuel traits.

Introduction

Fusarium leaf spot (FLS) on switchgrass has the ability to reduce stand establishment and hinder plant health. Switchgrass plants infected with *Fusarium* species was first isolated from 'Alamo' grown on the University of Tennessee (Knoxville, TN) in 2007. Pathogenicity assays confirmed FLS was also caused by *F. equiseti* (Vu 2011), *F. acuminatum*, and *F. graminearum* recovered from

infected agronomic switchgrass plots in Vonore, Tennessee in 2009 (Vu et al. 2010). *Fusarium armeniacum* and *F. tricinctum*, obtained from commercial switchgrass seed in 2010, caused FLS (Vu et al. 2011; Collins unpublished). *Fusarium* species collected from commercial seeds produced across the U.S., reduced biomass yields by 1% to 65.2% (Collins unpublished). A major challenge associated with switchgrass ethanol production includes stand establishment (Parrish and Fike 2005). In our previous study, isolates of *Fusarium* species reduced seed germination or plant stand by 1% to 42.6%, with *Fusarium graminearum* having the greatest impact (Collins unpublished).

Fusarium species are seedborne and soilborne, making their management difficult. In order to address this problem, genetic resistance will be needed to reduce *Fusarium* induced biomass and economic losses (Tan and Wu 2012). Several lowland and highland switchgrass cultivars are already commercially sold. Identification of resistant switchgrass cultivars will play a vital role in the promotion of breeding programs. Breeding programs would result in the development of new switchgrass cultivars with increased *Fusarium* disease resistance and increases in desired biofuel traits. This study focused on assessing four switchgrass cultivars for resistance against *F. graminearum*.

Materials and Methods

Plant material

All switchgrass cultivars ('Alamo', 'Whippet', 'Blade 1101', and 'Blade 1102') used in the experiments were produced from surface sterilized seed (10% Clorox® for 20 min on a magnetic stirrer and rinsed with sterile deionized (DI) water). To increase seed germination, all seed were subjected to cold stratification (soaked for 1 h in sterile DI water, placed in Petri dishes on sterile filter paper moistened with sterile DI water, and kept at 4.4 °C for 12-h). For the soil inoculation assay, plants were produced and maintained in growth chambers (28°C and 12-h photoperiod).

Sample collection and assay

Switchgrass cultivars were screened against a virulent *F. graminearum* isolate, TBW107. This fungus was isolated from naturally infected commercial 'Blackwell' switchgrass seed. Fungal inoculum was prepared by harvesting conidia from cultures grown on potato dextrose agar (PDA). Cultures were flooded with 5 ml of sterile DI water containing 10 µl Tween-20 (Fisher Scientific, Dubuque, Iowa) and gently scraped with a sterile rubber policeman. A hemocytometer was used to measure conidial concentration. Conidia were concentrated to 1×10^5 conidia/ml of sterile DI water.

Approximately 50 ml of a 1×10^5 conidial suspension was mixed with a 2,500 g mixture of ProMix potting soil (Premier Horticulture Inc., Quakertown, Pennsylvania) and Turface ProLeague (Profile Products, Buffalo Grove, Illinois) for each cultivar assay. For controls, 25 ml of sterile DI water was mixed with 1,250 g of the 50/50 soil mix. Inoculated soil was distributed into $9 \times 9\text{-cm}^2$ pots and covered with non-inoculated soil to allow seeds to germinate before coming into contact with the pathogen. Twenty surface sterilized seeds were placed in each pot and covered with a thin layer of non-inoculated soil. Each pot was covered with plastic and the plastic secured with a rubber band to increase relative humidity and enhance seed germination. After 5 days, the plastic was removed. Pots were placed in growth chambers and kept at 28°C with a 12-h photoperiod for 10 weeks. The study was set-up in a completely randomized factorial design with ten replicates for inoculated pots and five replicates for controls.

Assessment of switchgrass cultivars for disease susceptibility to *F. graminearum* was based on the percent seed germination, percent diseased plant area, post-emergence seedling death, plant height, and biomass yield (fresh and dry). Emerged seedling totals per pot were recorded 7, 14, and 32 days after planting. Diseased and dead seedling data were taken at 32 and 70 days after planting. Percent of emerged seedlings was calculated from the number of seeds sown. Percent of post-emergence seedling death was calculated from the total number of emerged seedlings per pot.

Switchgrass cultivars infected with *F. graminearum* were graded on a disease rating scale from 0 to 5 (Ahmed et al. 2009; Iftikhar et al. 2012), with 0 = no symptoms; 1 = 1% to 5% disease symptoms on plants within a pot; 2 = 6% to 25% disease symptoms; 3 = 26% to 50% disease symptoms; 4 = 51% to 80% disease symptoms; and 5 = 81% to 100% disease symptoms or complete plant death. Data for plant height and fresh weight were taken 10 weeks after inoculation. The average plant height per pot was obtained by measuring plants from the crown to the top of the longest leaf. Fresh weight was determined by weighing the mass of all plants within each pot cut at the crown. Oven dry weight was measured after the fresh biomass of each pot was wrapped in aluminum foil and dried for 7 days in an oven at 45°C.

Based on disease severity and germination switchgrass varieties were scaled and graded (Table 5.1) (Fajolu 2012). Scale 1-2: Moderately resistant (MR) = 0 - 1 disease rating and >80% germination; Scale 3: Susceptible (S) = 1.1 - 2 disease rating and >60% germination; Scale 4: Highly Susceptible (HS) = 2.1 - 3 disease rating and >40% germination; Scale 5: Highly Susceptible (HS) = 3.1 - 5 disease rating and <39% germination.

Statistical analysis

The experiment was arranged as a completely randomized design (CRD) with a 2 (inoculated and control) x 4 (cultivars) factorial. Data were analyzed with a mixed model procedure ANOVA (SAS 9.4 Inc, Cary, NC). Mean separation was calculated with least significant difference to determine cultivar differences in disease susceptibility. Statistical tests used a *P* value of 0.05.

Results

Four switchgrass cultivars were tested against *F. graminearum* isolate TBW107. Germination, post-emergence seedling death, diseased plant area, plant height, fresh weight, and oven dry weight were measured to determine switchgrass resistance or susceptibility against *F. graminearum* isolate TBW107. Post-emergence seedling death and plant height were not significant at a *P* value of 0.05.

Seed germination

Of the four switchgrass cultivars infected with *F. graminearum*, the highest seedling emergence was observed in 'Blade 1101' with a mean of 66.5%, 'Blade 1102' had the next highest seedling emergence, with a mean of 62.5%, 'Alamo' had a seedling emergence of 59.5%, and the least emergence was observed in 'Whippet', with a mean value of 54.5% ($P < 0.05$) (Fig. 5.1A). Compared with the

uninoculated control, percent seedling emergence was significantly lower for all cultivars infected with *F. graminearum*, except cultivar 'Whippet'.

Disease severity

Symptoms observed on switchgrass seedlings included leaf spot, leaf and stem streaking, chlorosis, and stunted growth. Infected 'Blade1101' switchgrass, which had little foliar disease, had significantly less diseased plant material than that of infected 'Whippet' and 'Alamo' switchgrass (Fig. 5.1B). 'Whippet' was the most susceptible cultivar to *F. graminearum* infection ($P < 0.05$). Compared to the uninoculated control, *F. graminearum* infected cultivars 'Blade1102', 'Alamo', and 'Whippet' had significant increases in foliar disease.

Plant yield

Significant differences in fresh weight and oven dry weight were found between cultivars. Infected 'Blade 1101' had the highest fresh and dry biomass yields and 'Whippet' the least with means of 2.53 g/pot and 0.79 g/pot and 1.09 g/pot and 0.34 g/pot, respectively (Fig. 5.1C and Fig. 5.1D). 'Blade 1102' infected with *F. graminearum* had significantly reduced fresh weight at 0.79 g/pot and dry weight at 0.27 g/pot when compared to the control (Fig. 5.1C and Fig. 5.1D). 'Whippet' had significant dry weight reductions at 0.17 g/pot when compared to the control (Fig. 5.1D).

Discussion

Four switchgrass cultivars were screened for disease resistance against a virulent isolate (TBW107) of *Fusarium graminearum* (Table 5.1). No cultivars were resistant to *F. graminearum* infection. The highest level of resistance was observed in 'Blade 1101'. 'Blade 1102' and 'Alamo' had intermediate disease responses, while 'Whippet' was the most susceptible to *F. graminearum* infection. Isolate TBW107 had no effect on post-emergence seedling death but did negatively affect seed germination, fresh weight, and oven dry weight. 'Blade 1101' was the most resistant cultivar, with TBW107 only impacting seed germination. Based on these results, 'Blade 1101' may have some genetic resistance to *F. graminearum* TBW107. This cultivar should be further studied to identify the source(s) responsible for resistance to *F. graminearum* infection. 'Blade1102' and 'Alamo' were rated as susceptible and 'Whippet' was highly susceptible. The four cultivars exhibited varying degrees of reduction in seed germination, fresh weight, dry weight, and disease severity. Other *F. graminearum* isolates and *Fusarium* species should be screened against these cultivars as well as other switchgrass cultivars to identify additional sources of resistance against *Fusarium* species.

The differential disease response of switchgrass cultivars to *F. graminearum* indicates resistance to *Fusarium* infection may be due to different genes or different genotypes contained within each synthetic cultivar. Other hosts of *F. graminearum*, such as wheat, also exhibit variance in responses to infection with

different isolates. In one study, nine wheat cultivars were screened against six isolates of *F. graminearum* from the U.S. and China. Variance was found in both isolate and variety, with only four of the nine cultivars maintaining resistance (Gui-Hua and Shaner 1996). In another study, 131 recombinant inbred lines of wheat were screened against *F. graminearum*. Several genes were found to aid resistance response (Schroeder and Christensen 1963). Screening switchgrass cultivars against fungal pathogens for identification of resistance is beneficial for breeding programs to develop resistant varieties for biofuel production. 'Blade1101' should be explored as a potential resistance donor in plant breeding programs. Integrated disease management strategies with genetic resistance should be emphasized as switchgrass continues to be grown on a commercial scale.

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Appendix

Table 5.1 Interaction of switchgrass cultivars infected with *Fusarium graminearum* isolate TBW107^a

Isolate	Cultivar	Germination (%)	Disease Severity	Scale ^b	Grade ^c
TBW107	'Blade 1101'	66.5 ± 3.9 A	0.7 ± 0.22 B	2	MR
	'Bade 1102'	62.5 ± 3.9 AB	1.1 ± 0.22 B	3	S
	'Alamo'	59.5 ± 3.4 AB	1.7 ± 0.22 B	3	S
	'Whippet'	54.5 ± 3.4 B	1.9 ± 0.22 A	4	HS

^aData reported are mean values of ten replicates ± standard error. For each variable, values with the same letters are not different according to an F-protected LSD at P=0.05.

^{b,c} Scale 1-2: Moderately resistant (MR) = 0 - 1 disease rating and >80% germination; Scale 3: Susceptible (S) = 1.1 - 2 disease rating and >60% germination; Scale 4: Highly Susceptible (HS) = 2.1 - 3 disease rating and >40% germination; Scale 5: Highly Susceptible (HS) = 3.1 - 5 disease rating and <39% germination

Figure 5.1 Mean disease response of four switchgrass cultivars to Fusarium graminearum infection (A) effect of inoculation X cultivar on seed germination, (B) diseased area, (C) fresh weight, (D) oven dry weight^a

^aData reported are mean values of ten replicates $\pm$ standard error. Values with the same letters are not different according to an F-protected LSD at $P = 0.05$.

^bCultivars were infected with *Fusarium graminearum* isolate TBW107.

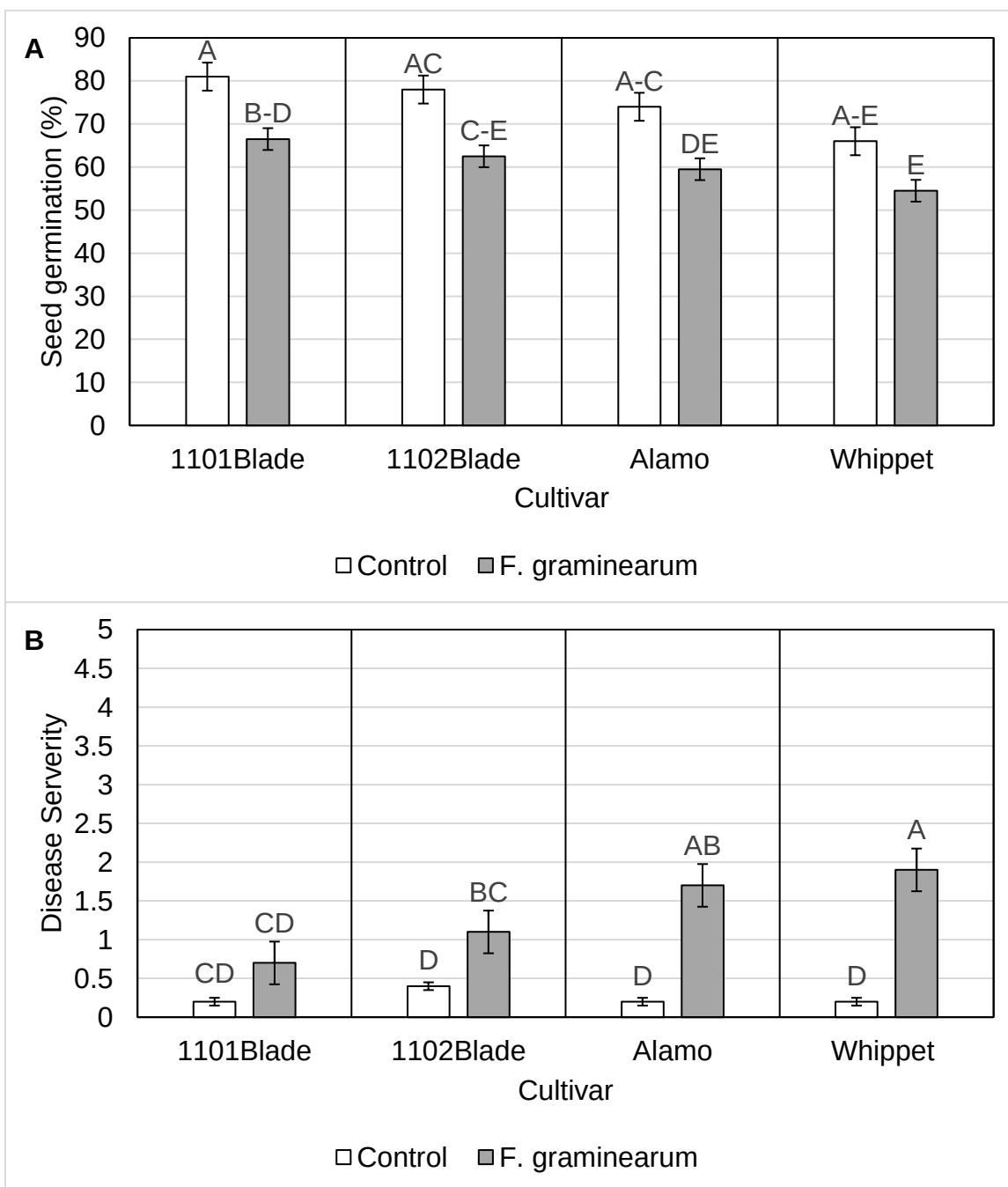


Figure 5.1 Continued

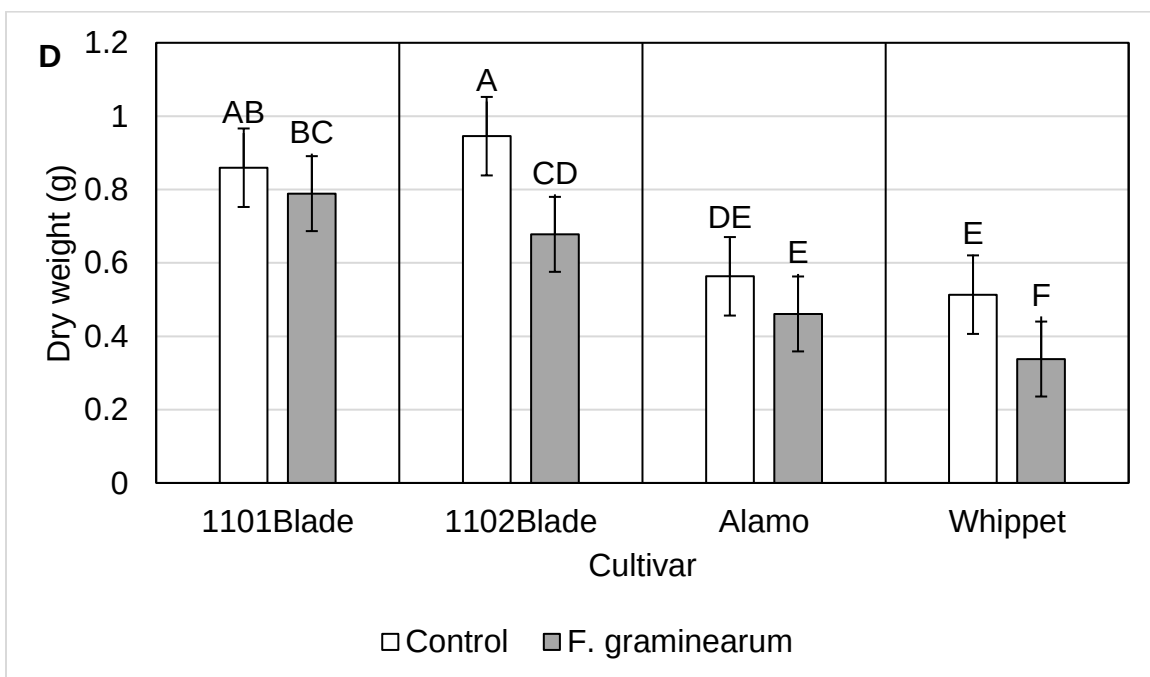
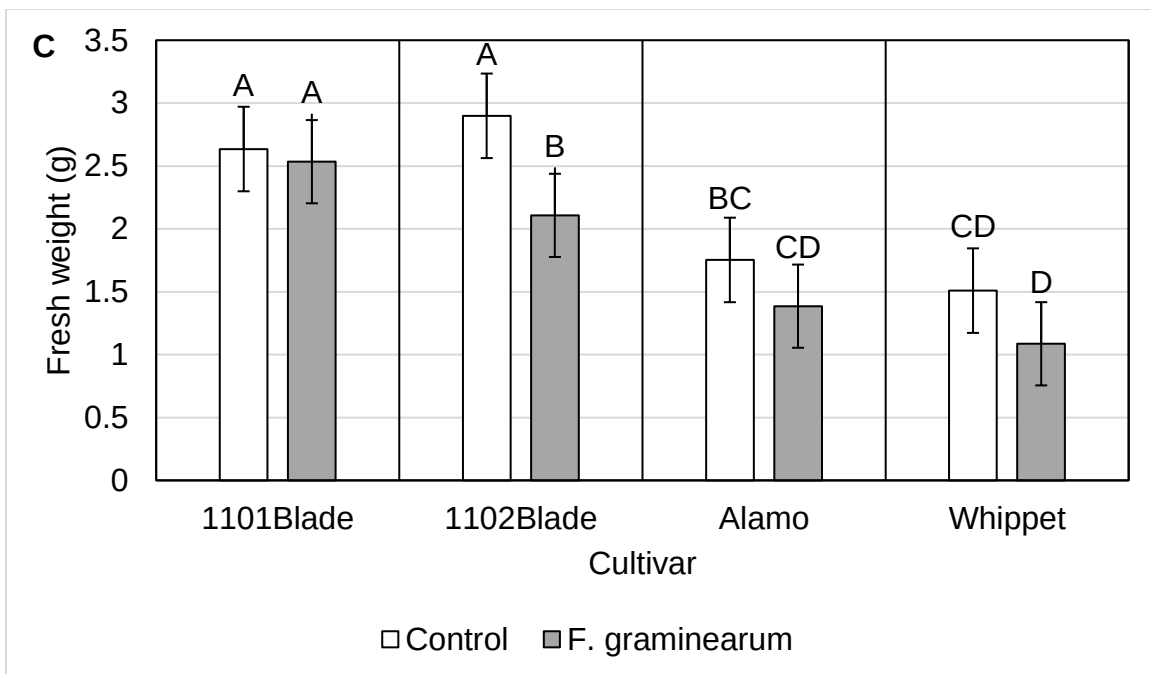


Figure 5.1 Continued

CHAPTER SIX

ASSESSMENT OF *FUSARIUM ARMENIACUM*, *FUSARIUM GRAMINEARUM*, AND *BIPOLARIS ORYZAE* INFECTION ON SWITCHGRASS CHEMICAL COMPONENTS

Abstract

Fusarium and *Bipolaris* are important pathogens of switchgrass (*Panicum virgatum* L.) in the U.S. The impact of *Fusarium armeniacum*, *F. graminearum*, and *B. oryzae* isolates on feedstock extractives, fermentable sugars, lignin and ash contents were evaluated on switchgrass 'Alamo'. Extractives were removed from biomass due to the phenolic compounds. Many of the phenolic compounds exhibit antimicrobial properties that hinder the fermentation process. Extractives content was found to be more abundant in *B. oryzae*-infected switchgrass when compared to the control. A two-part acid hydrolysis method was used to hydrolyze switchgrass samples for chemical analysis. The major fermentable sugars, glucose and xylose, were significantly reduced in *B. oryzae*-infected biomass and xylose significantly reduced in *F. armeniacum* infected biomass. Galactose and arabinose were not different than the uninoculated switchgrass control. Since feedstock carbohydrate content is directly proportional to ethanol yield, switchgrass infected with *B. oryzae* and *F. armeniacum* are expected to have reduced ethanol production compared to healthy plants. Lignin content of infected biomass was not significantly different from that of the uninfected control biomass. There was a significant increase in the total ash content of *B. oryzae*-infected biomass and significantly reduced total ash content in *F. graminearum* infected biomass. A lower ash content of feedstock is desirable for bioprocessing of biomass-to-ethanol or other biofuel products. Management strategies and

sustainable agricultural practices are needed to mitigate losses from switchgrass pathogens.

Introduction

To more fully understand the impact of fungal pathogens on switchgrass and other bioenergy crops, the quality of the biomass and biomass chemical components should be investigated. As energy consumption continues to shift from gasoline to renewable resources, biofuels will become an important part of the renewable energy market. Efforts are currently underway to reduce gasoline consumption in the U.S. Currently, the U.S produces 70 million gallons of bioethanol from lignocellulosic feedstock annually. By 2022 the U.S. will produce 36 billion gallons of bioethanol (Robertson et al. 2008). Lignocellulosic feedstock is in higher demand than starch- or sugar- based feedstock because it does not compete with food supplies and produces comparable energy to that of its counterparts (Yuan et al. 2008).

Lignocellulosic biomass is obtained from nonfood-plant material, and is composed mainly of cellulose, hemicellulose, and lignin (Sluiter et al. 2010). The most abundant structure in plant cells is cellulose. Cellulose is a polymer of glucose that makes up a significant portion of the fermentable glucose used in biofuel production (Kumar et al. 2009; Thammasouk et al. 1997). Hemicellulose is a polymer of xylose, arabinose, mannose, galactose, glucose, and uronic acid that are sourced for feedstock fermentation (Kumar et al. 2009; Mosier et al.

2005). Lignin provides structural support for the plant. Lignin is a phenolic compound polymer that is an intractable element of feedstock (Kumar et al., 2009; Thammasouk et al., 1997). Other components of lignocellulosic biomass are ash, extractives, and protein. Extractives are composed of soluble nonstructural components that include chlorophyll, sugars, nitrogenous compounds, and waxes. Extractives are removed from biomass samples in order to avoid interference during compositional analysis (Kumar et al., 2009; Sluiter et al., 2005a) and because they interfere with biofuel conversion. To optimize the bioconversion process and biofuel yields, evaluations of lignocellulosic biomass composition is needed.

Switchgrass (*Panicum virgatum* L.) is a promising source of lignocellulosic feedstock for biofuel production in the U.S. due to its ability to thrive on marginal soils, produce high biomass yields, high net energy balance, carbon cycling, and its status as a native perennial grass (Bouton 2008). Since its selection as a model biofuel candidate, several pathogens have been identified on switchgrass (Farr and Rossman 2016). As research into switchgrass disease increases, management practices will be developed to promote healthier crops. *Bipolaris* and *Fusarium* are among the major pathogens currently known to infect switchgrass (Vu et al., 2010; 2011). Assessment of *Bipolaris* and *Fusarium* infection on switchgrass biomass composition is necessary to evaluate the impact of these pathogens on biofuel production. The carbohydrates found in cellulose and hemicellulose are imperative for microbial fermentation and their

estimates important for future breeding and management practices (Sluiter et al. 2010; Thammasouk et al. 1997). The objective of this study was to assess the effects of single isolates of *B. oryzae*, *F. armeniacum*, and *F. graminearum*, on switchgrass biomass composition.

Materials and Methods

Fungal isolates

Virulent isolates of *F. graminearum*, *F. armeniacum*, and *B. oryzae* were obtained from infected commercial switchgrass seed, and identified through PCR amplification of the Translation Elongation Factor 1- α protein sequence.

Inoculum preparation

Each isolate was grown on potato dextrose agar (PDA) amended with 10 mg/liter rifampicin and 8 μ l/liter 2,4 EC Danitol miticide (Valent, Walnut Creek, CA) at 25°C for 3 weeks. Conidia were obtained from cultures by flooding plates with 5 ml of sterile deionized (DI) water containing 10 μ l Tween-20 (Fisher Scientific, Dubuque, IA). Plates were scraped with a sterile rubber policeman to release conidia, and conidia were concentrated to 1×10^5 conidia/ ml of sterile DI water for use as inoculum.

Soil inoculation and planting

For each isolate, approximately 50 ml of 1×10^5 conidial suspension (50 ml sterile DI water for controls) was mixed with 2,500 g of 50% of ProMix potting soil

(Premier Horticulture Inc., Quakertown, PA) and 50% Turface ProLeague (Profile Products, Buffalo Grove, IL). Inoculated soil was distributed into 9 × 9-cm² pots and covered with non-inoculated soil to promote seed germination before seed contact with the pathogen. Thirty surface sterilized seeds were placed in each pot and covered with a thin layer of non-inoculated soil, covered with plastic, and secured with a rubber band to increase relative humidity and enhance seed germination. The plastic was removed after 5 days. Plants were maintained in growth chambers with a 12-h photoperiod at 28°C for 16 weeks.

Switchgrass preparation for compositional analysis

Sixteen weeks after inoculation, plants were harvested by clipping the stems from the crown. Switchgrass samples were dried in an oven (SII Dry Kilns, Lexington, NC) at 45°C for 7 days and ground in a Wiley Knife Mill (Thomas Scientific, Swedesboro, NJ) Milled samples were placed in airtight Ziploc® bags at room temperature until analysis. Analyses were conducted according to the National Renewable Energy Laboratory (NREL) procedures (U.S. Dept. of Energy, 2012).

Total solids and moisture content

To determine total solids and moisture content, crucibles were completely dried in an oven at 105°C for 4 h. Dry crucibles were weighed and kept in desiccators, to prevent weight discrepancies. For each isolate, a 0.5-g milled sample was placed into a dried crucible and placed into an oven at 105°C for 24 h. Percent

total solids and moisture content was calculated from the weight loss of samples after drying at 105°C for 24 hr (See equations 1 and 2).

Compositional analyses of biomass were reported on a dry weight basis with the oven dry weight (ODW) of each sample calculated using equation 3. ODW was the weight of biomass corrected for the amount of moisture present in the sample at the time of weighing (Sluiter et al., 2008a).

Ash content

To determine total ash content, crucibles were placed in an Isotemp programmable forced draft furnace (Fisher) at 575°C for 4 h. Dry crucibles were weighed and placed in desiccators. For each isolate, a 0.5-g milled sample was weighed into a dried crucible and placed into an Isotemp programmable forced draft furnace (Fisher) at 575°C for 24 h. Percent total ash after combustion was calculated using equation 4.

Structural ash content was determined by placing crucibles in an Isotemp programmable forced draft furnace (Fisher) at 575°C for 4 h. Crucibles were weighed and placed in desiccators. A 0.5-g extractives – free sample was weighed into a dried crucible and placed into an Isotemp programmable forced draft furnace (Fisher) at 575°C for 24 h. Percent ash content after combustion was calculated using equation 4.

Extractives content

To calculate percent total solids, a 2.5-g milled sample was mixed with enough glass beads to fill a 10-ml extraction cell, and weighed. Deionized water and 95% ethanol extraction was carried out automatically using a Dionex Accelerated Solvent Extractor. During extraction cycles, solvent soluble extractives were collected in collection vials. After extraction, residual switchgrass and glass beads were recovered and placed in a pre-weighed pan. The pan was dried at 40°C for 3 days, after which, a 10- mesh brass sieve was used to remove the glass beads from each sample. Percent total solid of the extractives-free sample was calculated. The percent extractives content was calculated based on the change in weight of the oven-dried samples before and after extraction using equations 5 and 6.

Hydrolysis

A 300-mg extractive-free sample was hydrolyzed with 3 ml of 72% sulfuric acid at 30°C for 1 h in a pressure tube placed into a water bath. A Teflon rod was used to stir the mixture every 10 min to ensure uniform hydrolysis. After 1 h, the acid was diluted to 4% by adding 84 ml of DI water into the mixture. A Teflon cap and an O ring were placed on the pressure tubes and the tubes autoclaved at 121°C for 1 h. A set of sugar recovery standards (SRS) were prepared to correct for losses due to degradation of sugars during acid hydrolysis. Samples (87-mg) of each standard sugar (glucose, xylose, galactose, arabinose, and mannose)

were measured into a pressure tube with 3 ml of 72% acid and 84 ml of DI water. The pressure tube was capped and autoclaved with the sample tubes.

Lignin and carbohydrate content

Acid-insoluble lignin (AIL) content was determined after hydrolysis. The hydrolysate was cooled to room temperature and vacuum-filtered through a filtering crucible. Filtering crucibles were dried at 575°C for 4 h and weighed. The remaining solid fraction and crucible were oven-dried at 105°C for 24 h and the weight measured. AIL was calculated using equations 7 and 8. Acid soluble lignin (ASL) was determined by measuring absorbance of the hydrolysis filtrate at 205 nm on a UV – visible spectrophotometer (Milton Roy, Rochester, NY). Solid residue was diluted by adding 905 µl of DI water to 50 µl of the sample to obtain an absorbance range of 0.7 and 1.0. Absorptivity of switchgrass biomass (100 liters / g-cm) at 205 nm was applied to ASL calculations. Total lignin content of an extractives-free sample was the sum of ASL and AIL, using equations 9 and 10.

Carbohydrate content was determined by neutralizing 50 ml each of the hydrolysis filtrate (samples and SRS) with calcium carbonate to a pH of 5 to 6. The samples were filtered through a 2-µm Millex syringe filter (Fisher Scientific) into a 1.5 ml – high performance liquid chromatography (HPLC) vial and capped. Calibration standards with a concentration between 0.1 and 4.0 mg/ ml were prepared for each sugar to be quantified. Calibration verification standards (CVS)

were included in the HPLC to verify quality and stability of the calibration standard curves. Calibration standards, CVS, SRS, and samples were analyzed on a Perkin Elmer Flexar HPLC system fitted with a refractive index detector. An Aminex HPX – 87P carbohydrate column (BioRad, Hercules, CA) was used and held at 80 to 85°C during analysis. The mobile phase was HPLC grade water, and components were eluted isocratically at a flow rate of 0.25 ml/ min for 60 min. The detector was maintained at column temperature. Samples were analyzed for cellobiose, glucose, xylose, galactose, arabinose, and mannose. Chromatogram peaks for each sugar was recorded. A standard curve was generated for each sugar by plotting calibration standard concentrations against chromatogram peaks. The percent of each sugar in an extractives-free sample was calculated using equations 11 through 16.

Summative mass closure of switchgrass biomass

Glucose, xylose, galactose, arabinose, mannose, lignin, and ash were measured on the extractives-free samples. The summative mass of switchgrass biomass was calculated by combining measured components.

Statistical analysis

Data were analyzed with ANOVA as a completely randomized design using a mixed procedure model (SAS 9.4 Inc, Cary, NC). Mean separation was computed with least significant difference methods to determine differences in

biomass structural components affected by *Fusarium* and *Bipolaris* isolates and differences. The significance level for statistical tests was $P = 0.05$.

Results

Chemical Composition

The chemical composition of switchgrass biomass treated with virulent *B. oryzae* (isolate SK12), *F. armeniacum* (RSA117), and *F. graminearum* (TBW107) was examined (Table 6.1). Switchgrass structural components reported are based on an as received sample basis. For each sample, glucan was the predominant sugar at 25.8% to 27.5% dry mass, followed by xylan (16.3% to 17.8%), arabinan (3.2% to 3.4%), and galactan (1.7%). Total ash and structural ash ranged from 10.6% to 11.6% and 9.8% to 11.5%, respectively. Lignin accounted for 12.9% to 14% and extractives 22.4% to 25.7%. Mannose content was below the limit of detection, and absence of cellobiose during HPLC analysis indicates complete hydrolysis of samples. The measured components accounted for about 95.3% to 97.1% of the dry matter in the extractives-free biomass. The missing 2.9% to 4.3% dry mass accounts for microcomponents of the biomass not analyzed in this study. During this study, no significant differences were found for lignin, arabinan, galactan, mannose, and cellobiose dry mass among pathogen-infected biomass samples, or between infected biomass and the uninoculated control. The switchgrass composition of infected material was comparable to that of

previously reported switchgrass biomass and contained similar amounts of galactose, arabinose and lignin (Hu et al. 2010; Fajolu 2012).

Extractives content

Percent extractives content were similar between *Fusarium*-infected biomass and the control. Biomass infected with *Bipolaris oryzae* had elevated levels of extractive content (22.65%) when compared to biomass inoculated with *F. armeniacum* (23.1%) *F. graminearum* (22.60%) and uninoculated (22.35%) samples (Fig. 6.1).

Ash content

The chemical analyses of percent total ash yielded a significant increase in *B. oryzae* infected biomass (0.44%) and a decrease in *F. graminearum* (0.57%)-infected biomass when compared to the control (Fig. 6.2). There was no significant difference in total ash between *F. armeniacum*-infected biomass and the uninoculated control.

Carbohydrate content

No significant differences were found between *B. oryzae* and *Fusarium*-infected biomass and the uninoculated control for arabinan, galactan, mannose, and cellobiose. Significant reductions (1.69%) of glucan were found in *B. oryzae*-infected biomass as compared to those in control (Fig. 6.3 A). Xylan reductions

were found in both *F. armeniacum* and *B. oryzae* at a rate of 0.89% and 1.53%, respectively (Fig 6.3 B).

Discussion

Evaluating the chemical composition of healthy and infected switchgrass may prompt development of management and treatment practices, improving ethanol yield. Biomass composition analysis is also useful in economic analysis during the biomass-ethanol conversion process (Sluiter et al. 2010). The chemical composition of pre-extractives removed *Bipolaris oryzae* and *Fusarium* infected biomass were analyzed in this study. Switchgrass samples were analyzed for extractives, carbohydrate, lignin and ash content. Chemical components of infected biomass samples were compared to healthy switchgrass biomass. Compared to previous studies switchgrass chemical components contained similar amounts of galactose, arabinose and lignin (Hu et al. 2010; Fajolu 2012). Switchgrass samples treated with *B. oryzae* had reduced glucan and xylan content when compared to control plants. This reduction in carbohydrate content is attributed to the metabolism of plant sugars into nutrients for the pathogen (Berger et al. 2007). Reductions in carbohydrate content may also occur when photosynthesis is disrupted or reduced due to chlorosis and necrosis and reduced area of plant tissue (Heydari and Balestra 2011; Berger et al. 2007). As feedstock carbohydrate content is directly proportional to ethanol yield, *B. oryzae* and *F. armeniacum* infections will result in reduced ethanol (Sluiter et al. 2010). Greater carbohydrate reduction may occur in cases of multiple pathogen

infection. As ash interferes with acid hydrolysis, the reduced ash content found in *F. armeniacum* and *F. graminearum*-infected biomass is a desirable trait during biomass-to-ethanol processing (Sluiter et al. 2010). The cause of the decreased ash content is not known and requires further research. *Bipolaris oryzae*-infected biomass had increased ash content that is likely due to the presence of fungal materials within the sample, contributing to the biomass ash content. Chemical composition of fungal mycelia has been found to contain 3.6% ash (Tashpulatov et al. 2000; Tseng et al. 1984). The increase in extractives content found in *B. oryzae* infected biomass is attributable to plant defense. Several antimicrobial compounds have been identified in switchgrass extractives (Vu 2011), with greater amounts of extractives produced in response to plant infection. Management strategies and sustainable agricultural practices should be implemented to lessen biomass and ethanol loss caused by pathogens.

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Appendix

Eqn. 1

$\% \text{ total solids}_{\text{sample}} = (\text{weight of dry sample and crucible} - \text{weight of dry crucible}) \times 100 / \text{weight of sample}$

Eqn. 2

$\text{Moisture content}_{\text{sample}} = 100 - \% \text{ total solids}$

Eqn. 3

$\text{Oven dry weight (ODW)}_{\text{sample}} = \text{weight of sample} \times \% \text{ total solids} / 100$

Eqn. 4

$\% \text{ Ash content}_{\text{sample}} = ((\text{weight of crucible plus ash} - \text{weight of crucible}) / \text{ODW}_{\text{sample}}) \times 100$

Eqn. 5

$\text{Extracted sample} = (\text{weight of pan} + \text{beads} + \text{sample}) - (\text{weight of pan} + \text{beads})$

Eqn. 6

$\% \text{ Extractive} = [(\text{sample} \times \% \text{total solids before extraction}) - (\text{extracted sample} \times \% \text{ total solids})] / (\text{Sample} \times \% \text{total solids before extraction}) \times 100$

$\text{ODW}_{\text{sample}} = (\text{weight of extractive} - \text{free sample used for hydrolysis}) \times \% (\text{total solids of extractives} - \text{free sample}) / 100$

Eqn. 7

$\% \text{AIR content}_{\text{sample}} = ((\text{weight of crucible} + \text{solid residue at } 105^{\circ}\text{C} - \text{weight of crucible}) / \text{ODW}_{\text{sample}}) \times 100$

Eqn. 8

$\% \text{ AIL} = ((\text{weight of crucible} + \text{residue} - \text{weight of crucible}) - (\text{weight of crucible} + \text{ash} - \text{weight of crucible}) / \text{ODW}_{\text{sample}}) \times 100$

Eqn. 9

$\% \text{ ASL content}_{\text{sample}} = ((\text{UVabs} \times \text{volume filtrate} \times \text{dilution}) / \text{E} \times \text{ODW}_{\text{sample}}) \times 100$

Note:

UV abs = UV-Visible absorbance at 205nm

Volume hydrolysis filtrate = 84 ml of DI water and 3 ml of sulfuric acid= 87 ml

ϵ = Absorbivity of switchgrass biomass at 205 nm = 110 liters/g-cm

Eqn. 10

Dilution = (volume of sample + volume of diluting DI water) / Volume of sample
Total lignin content of an extractive-free sample = % ALS + % AIL

Eqn. 11

% CVS recovered from HPLC analysis = ((concentration detected by HPLC, mg / ml) / Known concentration of standard, mg / ml) x 100

The amount of each component sugar (SRS) recovered after dilute acid hydrolysis (%R sugar) is calculated thus:

Eqn. 12

% R sugar = ((concentration of sugar detected by HPLC, mg / ml) / Known concentration of sugar before hydrolysis, mg / ml) x 100

%R sugar is used to account for degraded sugars during dilute acid hydrolysis, and for any dilution made prior to HPLC analysis. Concentration of a sugar (mg/ml) in the hydrolyzed extractives-free sample after correction for loss during 4% acid hydrolysis (Cx1) is calculated thus:

Eqn. 13

C_{x1} = Concentration of a sugar as determined by HPLC, mg / ml / (% R sugar / 100)

The sugar concentration was adjusted for %CVS recovery obtained in equation 11 as follows:

Eqn. 14

C_{x2} = C_{x1} / (%CVS recovery)

To correct for hydration, each sugar concentration was multiplied by an anhydro correction factor of 0.9 for six carbon sugars (glucose, galactose, and mannose) and a factor of 0.88 for five carbon sugars (xylose and arabinose).

Eqn. 15

C_{anhydro} = C_{x2} X anhydro correction factor

Eqn. 16

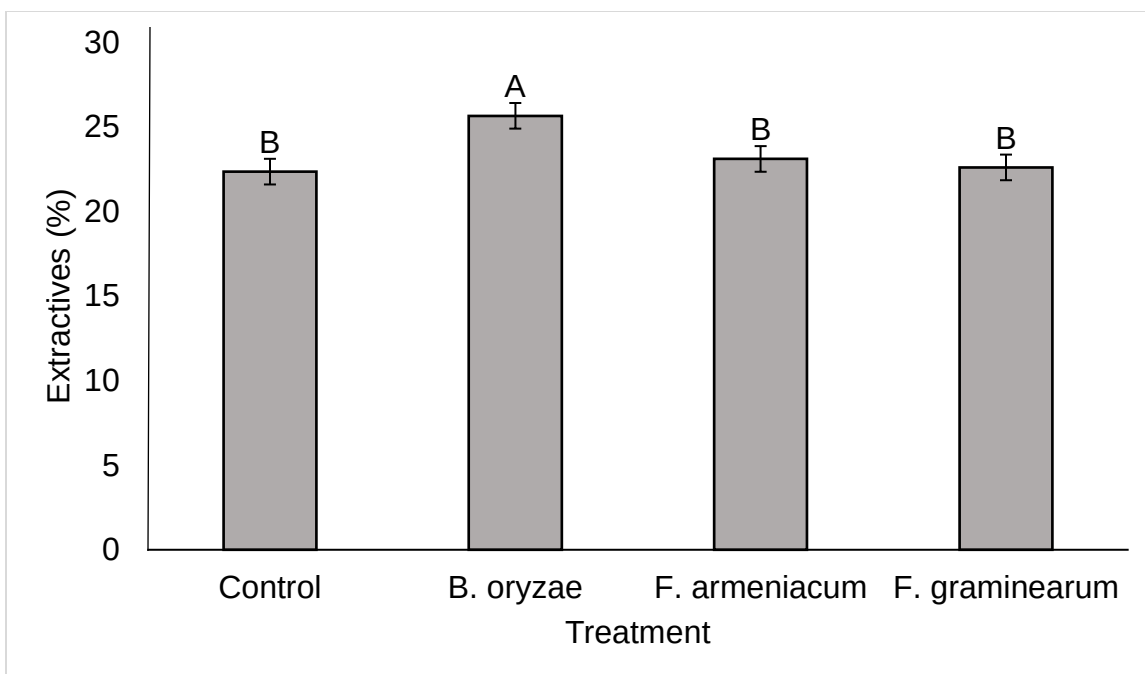
% Sugar extractive – free = (C_{anhydro} X Volume of filtrate / ODW) x

Table 6.1 Chemical composition of infected biomass (percent as received basis)^a

Sample ID	Total Ash	Structural Ash	Extractives	Glucan	Xylan	Galactan	Arabinan	Total Lignin	Mass Closure
Control	11.1±0.1	10.5±0.0	22.4±0.5	27.5±0.9	17.8±0.4	1.7±0.2	3.4±0.2	13.4±0.0	96.7
<i>F. armen</i>	10.8±0.0	9.8±0.3	23.1±0.0	27.2±0.2	16.9±0.2	1.7±0.1	3.2±0.0	14.0±0.1	96.0
<i>F. gram</i>	10.6±0.1	10.2±0.3	22.6±0.2	26.4±0.0	17.7±0.1	1.7±0.0	3.3±0.1	13.4±0.2	95.3
<i>B. oryzae</i>	11.6±0.1	11.5±0.1	25.7±0.6	25.8±0.5	16.3±0.4	1.7±0.0	3.3±0.0	12.9±0.0	97.1

^aData reported are mean values of two replicates ± standard error.

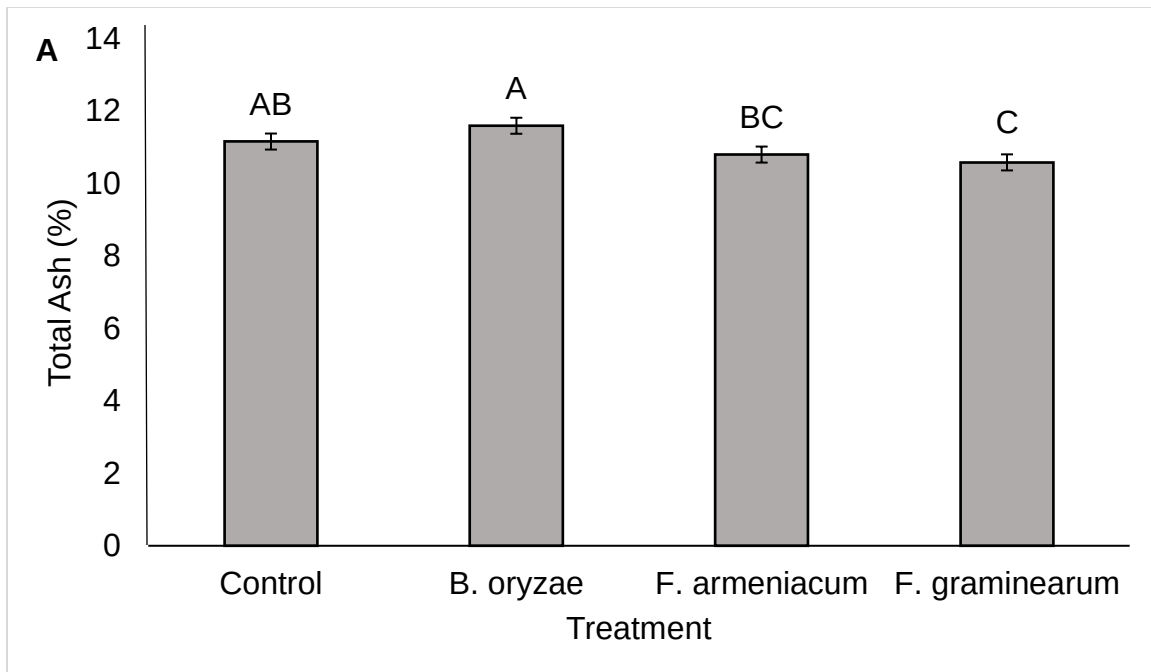
^bFungal isolates used – *Fusarium graminearum* isolate TBW107, *Fusarium armeniacum* isolate RSA117, and *Bipolaris oryzae* isolate SK12.



^aData reported are mean values of two replicates $\pm$ standard error. For each variable, bars with a common letter are not significantly different at $P = 0.05$.

^bFungal isolates used – *Fusarium graminearum* isolate TBW107, *Fusarium armeniacum* isolate RSA117, and *Bipolaris oryzae* isolate SK12.

Figure 6.1 Comparison of extractives content between infected switchgrass and uninfected control. Each component is a percent dry weight in the original switchgrass sample^a



^aData reported are mean values of two replicates $\pm$ standard error. For each variable, bars with a common letter are not significantly different at $P = 0.05$.

^bFungal isolates used – *Fusarium graminearum* isolate TBW107, *Fusarium armeniacum* isolate RSA117, and *Bipolaris oryzae* isolate SK12.

Figure 6.2 Comparison of as received ash content between infected switchgrass and uninfected control. Each component is a percent dry weight in the original switchgrass sample^a

Figure 6.3 Comparison of individual sugar components between infected switchgrass and uninfected control. Each component is a percent dry weight in the extractive-free switchgrass^a.

^aData reported are mean values of two replicates $\pm$ standard error. For each variable, bars with a common letter are not significantly different at $P = 0.05$.

^bFungal isolates used – *Fusarium graminearum* isolate TBW107, *Fusarium armeniacum* isolate RSA117, and *Bipolaris oryzae* isolate SK12

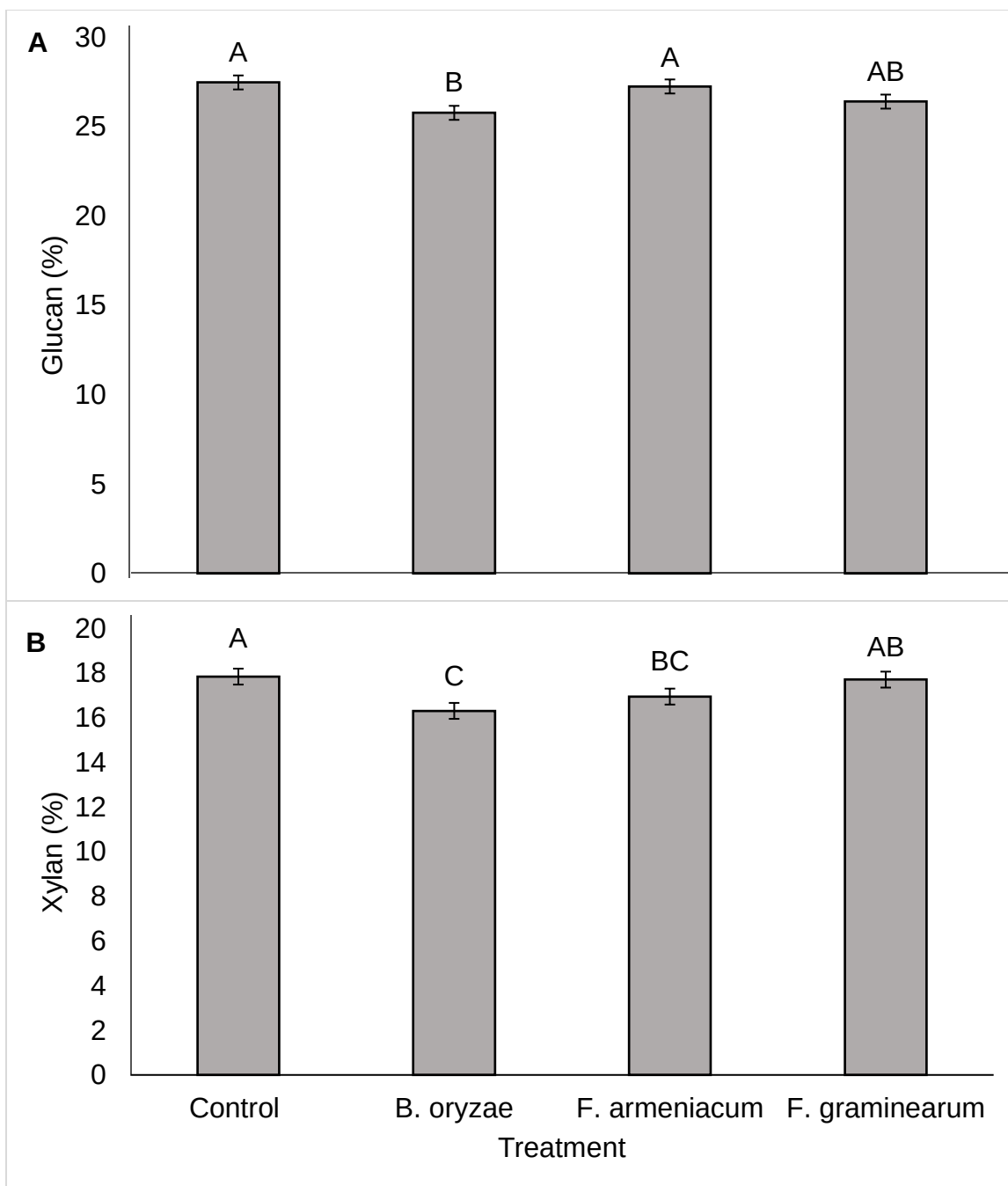


Figure 6.3 Continued

CHAPTER SEVEN

CONCLUSION

The results of this project show that several *Fusarium* species play an important role in stand establishment, providing information that could be used in future disease management practices and seed certification programs. Although further research is needed, *Alternaria alternata* was shown to act as a weak pathogen, impacting only switchgrass health. Four switchgrass cultivars were screened for *F. graminearum* resistance. One of the four cultivars was moderately resistant, two cultivars susceptible, and one cultivar highly susceptible. The moderately resistant cultivar could be used as a resistance donor in future plant breeding programs. More cultivars should be screened against virulent *Fusarium* isolates to identify resistance against *Fusarium* species. Compositional analysis of infected switchgrass yielded significant variations in extractives, ash, and sugar content. Elevated extractives content exhibited in *Bipolaris oryzae* infected switchgrass could be a cause for concern. The phenolic compounds contained within extractives are thought to have antimicrobial activity, which could hinder the fermentation process during biofuel production. Infection of switchgrass with *B. oryzae* and *F. armeniacum* resulted in reductions of carbohydrate content. It is likely that switchgrass infected with virulent isolates of *B. oryzae* and *F. armeniacum* will encumber production, as the carbohydrate content is directly proportional to biofuel yield. As bioenergy research continues, genetic resistance, seed certification programs, and disease management strategies will be needed to reduce the impact of fungal pathogens on switchgrass and increase commercial switchgrass-derived products.

VITA

Sara B. Collins was born in Denver, Colorado. She received a Bachelor of Science degree in Wildlife and Fisheries Biology from Lincoln Memorial University at Harrogate in December 2013. After taking a small amount of time off from academics, Sara further pursued an education in Plant Pathology. In December 2013, she earned a Master of Science degree in Entomology and Plant Pathology under the direction of Dr. Bonnie Ownley.