Characterization of *Bipolaris* species, their effects on switchgrass biomass yield and chemical components

A Dissertation Presented for
the Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

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December 2012

DEDICATION

This dissertation is dedicated to the glory of almighty God and my family.

AKNOWLEDGEMENTS

To God from whom all blessings flow I give all the praise and glory for the successful completion of my Doctoral program at the University of Tennessee. I thank God for His protection over my entire family, His provisions for us and His strength and wisdom for me during the period of my study.

It would not have been possible to reach this stage of my study without the tremendous support and guidance I received from my major professor – Dr. Bonnie Ownley; I say thank you for your patience and understanding. I also want to acknowledge members of my research committee; these include Dr. Arnold Saxton, Dr. Kimberly Gwinn, Dr. Robert Trigiano, Dr. Phillip Wadl and Dr. Nicole Labbé. Each of them made great contributions that helped in numerous ways. My appreciation also goes to Mary Dee, Wanjing Liu, Kristie Mantooth, Andrea Vu, Denita Hadziabdic, Sarah Boggess, Debbi Dean, Lindsey Kline and all of the members of Dr. Labbé's lab for their contributions towards the success of this project.

My gratitude goes to the University of Tennessee Institute of Agriculture Access and Diversity Scholarship managers for their financial support. I thank the Southeastern Sun Grant Center, the U.S. Department of Transportation, and AgResearch at the University of Tennessee, Institute of Agriculture for providing the necessary funds for my research.

I thank Pastor Paul Hahn, his wife, all associate pastors and members of the Redeemer Church of Knoxville, for the prayers and supports in various ways for my family and me throughout the course of my study at The University of Tennessee. To all my friends, Lilian, Jeneen, Sabina, Helen, Ibukun, Jamiu, Ifeyinwa, and Toyin - thank you for your help and support during my stay in Knoxville.

ABSTRACT

Switchgrass (*Panicum virgatum* L.) is a promising biofuel crop; however, limited attention has been directed toward switchgrass pathogens and their impact on biomass yield. *Bipolaris* is one of the fungal pathogens that pose a potential threat to switchgrass production in the U.S. This research is focused on the impact of *Bipolaris* on switchgrass. The research objectives were 1) Assessment of isolates of four *Bipolaris* species for morphology and virulence diversity on switchgrass and impact on biomass yield, 2) Assessment of *Bipolaris* infection on switchgrass chemical components, 3) Assessment of switchgrass germplasm for disease resistance, and 4) Genetic characterization of *Bipolaris* species. Twenty-five representative isolates of *Bipolaris oryzae*, *B. sorokiniana*, *B. spicifera*, and *B. victoriae* varied in morphology and virulence on switchgrass. *Bipolaris oryzae* was the most virulent species and *B. spicifera* was the least. Low, intermediate and high virulence groups were identified across all isolates. Low-virulent isolates had limited negative impact on switchgrass whereas high-virulent isolates greatly impacted switchgrass, even at low inoculum density. High-virulent isolates should be targeted for disease resistance development in switchgrass. However, these virulence groups had similar effect on switchgrass chemical components. *Bipolaris* infection resulted in a significant increase in ash and lignin contents of switchgrass biomass, and a decrease in carbohydrate content, thereby reducing biomass fitness for conversion to drop-in fuels. Out of the nine switchgrass germplasms screened for disease resistance, six, namely Germplasm A, Germplasm B, Germplasm C, 'Alamo' (Bamert), 'Trailblazer', and 'Summer', were moderately resistant to *B. spicifera* (LA18) or *B*. *oryzae* (SK12), whereas none of the germplasms had resistance to *B. sorokiniana* (APCNR34) and *B. victoriae* (JA12). The moderately resistant

germplasms can further be explored as resistance donors in breeding programs for development of cultivars with biofuel-valuable traits. Simple sequence repeats markers were developed from a small insert genomic library for *B. sorokiniana*. Sixteen polymorphic loci used to characterized fifteen *B. sorokiniana* isolates successfully cross-amplified at least one isolate of *B. victoriae*, *B. spicifera* and *B. oryzae*. These markers are valuable for genetic variability studies of *Bipolaris* species and a useful tool to formulate breeding strategies for development of resistant switchgrass cultivars.

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CHAPTER 1: INTRODUCTION AND GENERAL INFORMATION

1.1 Introduction

Interest in biofuels as a renewable form of energy has increased due to concern about the negative impact of gasoline on environmental quality. Burning fossil fuel is one of the largest sources of greenhouse gas emissions and a contributor to global warming (Kumar et al., 2009). Consequences of global warming include frequent and severe natural disasters, higher incidences of diseases, rising sea levels, and shortages of food and water (Lefton and Weiss, 2010). Biofuel is oxygenated, clean, and sustainable transportation fuel, and it is expected to reduce greenhouse gas emission by at least 86% compared to fossil fuel (Balat, 2009; Kumar et al., 2009). Moreover, consumption of biofuel will reduce dependence on foreign oil, and support local agricultural and forest-product industries (Lefton and Weiss, 2010).

Ethanol is one of the most common biofuels, and can be obtained by direct conversion of biomass. Today, ethanol is mostly used as a blending agent with a petroleum product (gasoline or diesel) to reduce petroleum consumption and air pollution (Chu and Lee, 2007). Ethanol is blended with gasoline to form E10 blend (10% ethanol and 90% gasoline); some vehicles can run on E85 or E95 (Balat, 2009). Efforts to reduce gasoline consumption in the U.S., and increase reliance on clean renewable fuels from bioenergy crops has led to a mandatory annual production of 16 out of 36 billion gallons of bioethanol from lignocellulosic feedstock by the year 2022 (Robertson et al., 2008; Voith, 2009). Lignocellulosic feedstock is nonfood-plant material grown for biofuel production (Sluiter et al., 2010; Yuan et al., 2008). These feedstocks are perennials, which are more desirable than annuals as bioenergy crops because biomass can

be harvested repeatedly, without having to be replanted (Yuan et al., 2008). Lignocellulosic feedstocks do not compete with food supplies and require limited cultivation costs, making them more acceptable than sugar and starch feedstocks. The desirable traits of cellulosic feedstocks are projected to lead to an increase in cellulosic biofuel production as a proportion of renewable fuels (Fig. 1.1).

Switchgrass (*Panicum virgatum* L.) is considered as one of the most promising biofuel crops due to its high biomass yield, high net energy balance, adaptability to poor soils and marginal cropland, and its status as a native perennial grass (Bouton, 2008; Yuan et al., 2008). Ecological and agricultural benefits of switchgrass include decreasing soil erosion, improving water quality, and complementing food- and feed-based agriculture (Yuan et al., 2008). Moreover, it has been reported that switchgrass produced 540% more renewable energy than energy consumed, and greenhouse gas emissions were 94% lower than that of gasoline (Schmer et al., 2008).

1.2 Switchgrass

Switchgrass is a perennial, herbaceous, warm-season, long-lived grass that reproduces from seed and rhizomes. This grass is a native to North and Central America (Lemus et al., 2002; Sharma et al., 2003), and once established, produces biomass for 15 to 20 years. It is well adapted to a wide range of soils and climatic conditions with excellent drought and cold tolerance (Bouton, 2008). It requires limited herbicides and fertilizer to produce high yields, compared to other warm-season grasses (Bouton, 2008; Sharma et al., 2003). Switchgrass is classified into upland and lowland ecotypes. Lowland ecotypes are tall, can reach 8 to 10 feet,

thick-stemmed, flourish in wet low areas with high fertility, and when mature, will tolerate long period of flooding (Lemus et al., 2002). Upland ecotypes are short, rhizomatous, thin-stemmed, and found on upland areas with low fertility and dry conditions (Lemus et al., 2002). Switchgrass performs well when harvested less frequently; one to two harvests *per annum* is recommended (Bouton, 2008). Switchgrass is used by wildlife as cover, and can provide suitable forage for hay and grazing. It is used in landscape plantings, and to control stream bank erosion (Bouton, 2008). Recently, the use of switchgrass as a renewable biofuel resource has increased. The U.S. Department of Energy has chosen switchgrass as one of its dedicated energy crops.

Consistent supply of high quality feedstock biomass plays a significant role in attaining a sustainable biofuel future. To meet the national bioethanol mandate, researchers have focused on improving switchgrass as feedstock for biofuel (Bouton, 2007; 2008; Fu et al., 2011). Programs for improvement of switchgrass have focused on increased yield, good seedling establishment, increased feedstock quality, improved biomass production, and bioconversion processes for the biofuel industry (Bouton, 2008; Dien et al., 2006; Faga et al., 2010; Hu et al., 2010; Perrin et al., 2008; Vogel et al., 2006). However, little attention has been directed towards switchgrass diseases. An attempt to grow switchgrass with biofuel-valuable traits has led to monoculture production. Monoculture often leads to increased susceptibility to plant pathogens due to lack of genetic diversity and heterogeneity (Gonzalez-Hernandez et al., 2009). Moreover, the ambitious biofuel goal requires a substantial increase in agricultural land used for commercial-scale switchgrass production (Marshall and Sugg, 2010), and a corresponding increase in disease risk can be expected (Stewart and Cromey, 2011).

1.3 Switchgrass Pathogens

Plant pathogens cause significant yield losses of economic crops, with no exceptions for dedicated energy crops. It has been presumed that because switchgrass is a native plant, coupled with the use of genetically diverse cultivars, the impact of disease will be limited (Mitchell et al., 2008), hence little consideration has been given to research on switchgrass diseases. However, host genetic diversity is not sufficient in preventing diseases particularly when the pathogen is soilborne and seedborne (Cox et al., 2005). If a proper management program is not put in place, diseases may threaten successful production of switchgrass.

Reports of fungal isolations from switchgrass have increased to include 152 species that contain 378 isolates (Farr and Rossman, 2012). Most of these species are pathogenic; however, the significance and management of most of these fungi is not known. Nevertheless, fungal diseases affecting switchgrass yield, seed production, forage quality and longevity have been reported (Zeiders, 1984; Gravert et al., 2000; Gustafson et al., 2003; Krupinsky et al., 2004; Thomsen et al., 2008; Crouch et al., 2009; Vu et al., 2010; Vu et al., 2011a). In Tennessee, several pathogenic fungi have been isolated and identified from naturally infected seedlings and mature switchgrass plants. Similar fungal pathogens were isolated from switchgrass seeds produced across the U.S. These fungal pathogens included species of *Alternaria*, *Bipolaris*, *Curvularia*, *Fusarium*, *Pithomyces* and *Sclerotinia*. Most of these switchgrass pathogens are soilborne and seedborne. They produce long-lived, overwintering structures that enable their survival in soil for long periods and under unfavorable conditions, making management of the diseases they cause more challenging. These fungi are also pathogenic on several important

grain and grass crops such as barley, oat, corn, rice, wheat, ryegrass and Bermuda-grass (Farr and Rossman, 2012) (Table 1.1).

1.4 *Bipolaris* species

Species of *Bipolaris* are among the pathogens that pose a potential threat to switchgrass production in the U.S. *Bipolaris* species are difficult to control because they are both seedborne and soilborne. In order to identify fungal pathogens associated with switchgrass, a three-year survey of diseases of agronomic switchgrass in Tennessee was conducted during the growing season from 2007 to 2009. *Bipolaris* was one of the predominant pathogenic fungi isolated from naturally infected switchgrass seedlings and mature plants (Vu et al., 2010; Vu, 2011). Furthermore, in a survey to identify the incidence and prevalence of seedborne fungal pathogens in switchgrass seeds produced in the U.S., *B. oryzae* was the second most prevalent pathogenic species isolated (Vu, 2011). Other *Bipolaris* species isolated included *B. sorokiniana*, *B. spicifera* and *B. victoriae*. Since switchgrass is mainly grown from seeds, the absence of certification programs for switchgrass seeds may likely allow wide distribution of seedborne pathogens among switchgrass growers. *Bipolaris* can survive in soil for a long period, even in the absence of a host plant, and could serve as inoculum for the next growing season. Stand establishment is a problem in switchgrass production (Parrish and Fike, 2005) and *Bipolaris* species are likely to play a significant role.

Different species of this pathogen isolated from grower fields have the potential to cause yield losses (Vu et al., 2010). *Bipolaris sorokiniana*, which causes spot blotch and root rot, was reported as the most prevalent and important disease of switchgrass in Pennsylvania (Zeiders,

1984). In the same report, the authors suggested that if switchgrass is grown to any great extent in the humid areas of the eastern and northeastern U.S., spot blotch will probably be the most important disease. This disease has also been reported on switchgrass in Iowa, Nebraska and Virginia (Farr and Rossman, 2012). Recently, *B. sorokiniana* was reported to cause spot blotch, root rot, and stunted growth on switchgrass in Tennessee (Vu et al., 2011b). *Bipolaris sorokiniana* was isolated from switchgrass leaves, stems and roots (Vu, 2011). Worldwide, *B. sorokiniana* is an important pathogen on a wide range of other grass species and cereals (Kumar et al., 2002; Pratt, 2003) (Table 1.1). Isolates of this fungus also causes foliar spot blotch, root rot, seedling blight and head blight of cereal. Yield losses of wheat due to *B. sorokiniana* range from 16 to 33% (Valjavec-Gratian and Steffenson, 1997; Kumar et al., 2002; Ghazvini and Tekauz, 2007). High humidity and warm temperatures, conditions commonly found in environments where these host plants are grown; favor disease caused by *B. sorokiniana* (Ghazvini and Tekauz, 2007).

Bipolaris oryzae, which causes brown spot and seedling blight on rice, has been isolated from leaf spot of switchgrass in different locations of the U.S. (Ghimire et al., 2011; Krupinsky et al., 2004; Tomaso-Peterson and Balbalian, 2010; Waxman and Bergstrom, 2011), including Tennessee (Vu, 2011). Other species of *Bipolaris* associated with diseases of switchgrass are *B*. *zeae* and *B*. *heveae*, reported from North Dakota and Oklahoma, respectively (Krupinsky et al., 2004; Ghimire et al., 2011). Recently, the first report of leaf spot caused by *B*. *spicifera* on switchgrass was published (Vu et al 2011c). Moreover, *B*. *victoriae* has just been isolated from switchgrass seeds and identified (Vu, 2011).

Recent reports on *Bipolaris* diseases of switchgrass from different regions of the U.S. serve as a warning about potential disease outbreaks. Currently, about one billion gallons of cellulosic biofuel is produced in the U.S. annually (Fig. 1.1), although switchgrass represents only a fraction of the feedstock, there has already been an increase in reports of fungal pathogens. As the U.S. approaches the biofuel mandate, 16 billion gallons of lignocellulosic biofuel per year, switchgrass production will significantly increase and a corresponding increase in disease risk is anticipated (Stewart and Cromey, 2011). The exact impact of *Bipolaris* diseases on switchgrass yield and production of ethanol or other biofuels is unknown. Proactive research is required to determine potential disease threats and develop effective management strategies. This background of information led to our overall goal: to understand the *Bipolaris*-switchgrass pathosystem and the effect of *Bipolaris* infection on biofuel production.

1.5 Research objectives and justifications

This research had four main objectives:

Objective one: Assessment of isolates of four *Bipolaris* species for morphology and virulence diversity on switchgrass and impact on biomass yield

Justification

Evaluation of virulence diversity in multiple isolates of different *Bipolaris* species is essential for the development of effective resistance to *Bipolaris* in switchgrass. Although pathogenic diversity of *Bipolaris* species on other plant hosts has been published, only limited information on the virulence of *B. sorokiniana* on switchgrass has been reported (Zeiders, 1984). There is no report documenting the variation in virulence of other *Bipolaris* species on switchgrass.

Information on the pathogenicity and virulence diversity present among isolates of different *Bipolaris* species that infect switchgrass is a necessary first step to develop cultivars with effective resistance against the pathogens and also to determine appropriate sustainable agricultural practices for disease management.

Objective two: Assessment of *Bipolaris* infection on switchgrass chemical components

Justification

Assessment of *Bipolaris* infection on biomass chemical components that are converted to ethanol or other biofuels is important in evaluating the economic losses due to *Bipolaris* infection and impact on biofuel industries.

Objective three: Assessment of switchgrass germplasm for disease resistance against *Bipolaris*

Justification

Genetic resistance is considered the most practical means of reducing economic losses caused by *B. sorokiniana* in grasses (Zeiders, 1984). To date, there is no report on screening different switchgrass cultivars for disease resistance against *Bipolaris* species. Examination of switchgrass cultivars from different regions of the U.S., for potential sources of resistance, is therefore crucial for effective control of diseases caused by *Bipolaris* species.

Objective four: Genetic characterization of *Bipolaris* species

Justification

Simple sequence repeats (SSRs) are tandem repeat units of one to six base pairs that are found abundantly and are highly polymorphic in many eukaryotic genomes (Karaoglu et al., 2005; Wang et al., 2007). The diversity of SSR motifs gives each species a unique "signature" of repeat

distributions. An understanding of genetic diversity of *Bipolaris* species will be useful for developing and breeding durable resistant switchgrass cultivars. These markers also have the potential for differentiation of species and isolates. However, based on publication and sequence database searches, potential SSRs have not been reported for *Bipolaris* or related genera.

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Table 1.1 Fungal pathogens of switchgrass[a] in Tennessee, and other grain and grass host plants[b]

Fungal pathogen	Barley (*Hordeum vulgare*)	Corn (*Zea mays*)	Oats (*Avena sativa*)	Rice (*Oryza sativa*)	Rye (*Secale cereale*)	Sorghum (*Sorghum bicolor*)	Wheat (*Triticum aestivum*)	Bermuda grass (*Panicum dactylon)*	Ryegrass (*Lolium perenne*)	Stiltgrass (*Microstegium vimineum*)
Alternaria alternata		X	X	X	X	X	X	X		
Bipolaris oryzae				X			X			
Bipolaris sorokiniana	X	X	X	X	X		X	X	X	X
Bipolaris spicifera	X	X	X	X	X	X	X	X		
Bipolaris victoriae	X	X	X	X		X				
Curvularia lunata	X	X		X		X	X	X	X	X
Fusarium acuminatum	X	X	X		X	X	X	X		
Fusarium equiseti	X	X	X	X	X	X	X	X		
Fusarium graminearum	X	X	X	X	X	X	X		X	
Fusarium oxysporum	X	X	X	X	X	X	X	X	X	
Pithomyces chartarum		X		X			X	X	X	

[a] Vu et al., 2011

[b] Farr and Rossman, 2012

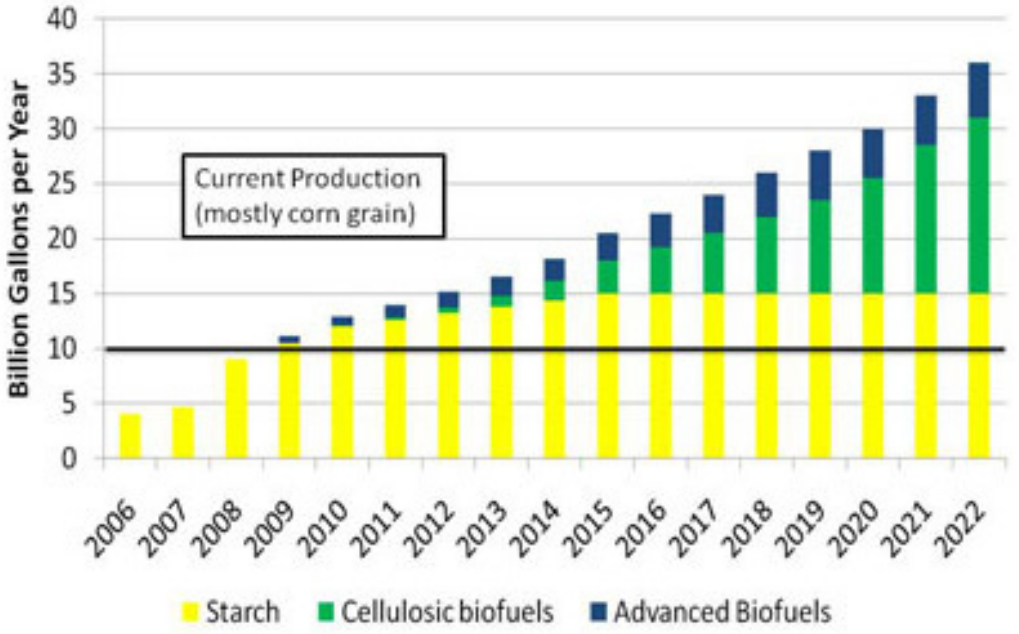

Fig. 1.1 Historical and projected biofuel production. Source:

http://bioenergy.msu.edu/bioenergy/policy

CHAPTER 2: ASSESSMENT OF ISOLATES OF FOUR *BIPOLARIS* SPECIES FOR MORPHOLOGY AND VIRULENCE DIVERSITY ON SWITCHGRASS AND IMPACT ON BIOMASS YIELD

2.1 Abstract

Bipolaris is an important pathogen on switchgrass (*Panicum virgatum* L.), causing root rot, seedling blight, foliar disease, and reductions in plant stand and biomass. Pathogenic *Bipolaris* are both seedborne and soilborne making disease control challenging. Information on the variability in pathogenicity and virulence in *Bipolaris* species pathogenic on switchgrass will aid in development of disease management practices. The morphology and virulence diversity of 25 representative isolates of *Bipolaris oryzae*, *B. sorokiniana*, *B. victoriae* and *B. spicifera* obtained from infected switchgrass across the U.S. was evaluated on 'Alamo'. *Bipolaris* isolates within and between species vary greatly in morphology and degree of virulence. Data from detached leaf, whole plant inoculation and seed inoculation assays consistently implicate *B. oryzae* as the most virulence species and *B. spicifera* as the least whereas *B. sorokiniana* and *B. victoriae* were moderately virulent. *Bipolaris oryzae* infection resulted in the highest percent disease area on detached leaf and whole plant assays, and had the most negative impact on plant stand, height and biomass in seed inoculation assays. Moreover, there were extreme virulence patterns among and within *Bipolaris* species. Three virulence groups were identified across all isolates: low, intermediate and high virulence. Low-virulent isolates had limited impact on switchgrass biomass causing only 5% reduction in biomass at low inoculum density, but reduced plant stand by 38%. Disease severity caused by these isolates did not increase

significantly at higher inoculum density. On the other hand, high-virulent isolates greatly impacted switchgrass, even at low inoculum density and caused up to 52% reduction in plant stand, and 70% reduction in biomass when compared with control plants. More so, their impact on plant growth and disease severity significantly increased at higher inoculum density and caused up to 74% and 88% reduction in plant stand and biomass, respectively. The presence of such isolates in the field can be problematic since only a few conidia are required for disease development. High-virulent isolates should be targeted for disease resistance development in switchgrass. Moreover, information on variability in virulence in *Bipolaris* will aid in development of disease management strategies, and thereby reduce the impact of this fungal pathogen on switchgrass.

2.2 Introduction

Switchgrass (*Panicum virgatum* L.) is considered the most promising biofuel crop due to its high biomass yield, high net energy balance, adaptability to poor soils and marginal cropland, and its status as a native perennial grass (Bouton, 2008; Yuan et al., 2008). However, reports of isolation of fungi from switchgrass have increased to include 152 species (Farr and Rossman, 2012). Most of these species are known pathogens; however, the significance and management of most of these fungi on switchgrass is not known.

Based on a survey to identify fungal pathogens of switchgrass in the U.S., *Bipolaris* species were identified as among the predominant fungi isolated from naturally infected seedlings and mature plants from Tennessee, and from seeds produced in several different states (Vu et al., 2010; Vu et al., 2011a). *Bipolaris* species are difficult to control because they are both

seedborne and soilborne. Since switchgrass is mainly grown from seeds, the absence of certification programs for switchgrass seeds would likely allow wide distribution of seedborne pathogens among switchgrass growers. Soilborne fungi can survive in soil for a long period, even in the absence of a host plant, and could serve as inoculum for the next growing season. Different *Bipolaris* species isolated from switchgrass have the potential to cause yield losses (Vu et al., 2010). *Bipolaris sorokiniana*, which causes spot blotch, stunted growth and root rot, was reported as one of the most prevalent and important diseases of switchgrass (Vu et al., 2011b; Zeiders, 1984). Worldwide, *B. sorokiniana* is an important pathogen on a wide range of other grass species and cereal crops including wheat, barley, rye, and Bermuda grass, causing economic yield losses (Kumar et al., 2002; Pratt, 2003). *Bipolaris oryzae*, which is responsible for brown spot and seedling blight on rice, has been isolated from leaf spot of switchgrass in different parts of the U.S. (Ghimire et al., 2011; Krupinsky et al., 2004; Tomaso-Peterson and Balbalian, 2010; Waxman and Bergstrom, 2011). Other *Bipolaris* species implicated in causing switchgrass leaf spot are *B*. *spicifera* (Vu et al., 2011c) and *B. victoriae* (Vu, 2011).

Information on pathogenicity and virulence of fungi isolated from switchgrass will aid in development of disease management strategies. Although pathogenic and virulence diversity of *Bipolaris* species on other plant hosts has been published, only limited information on the virulence of *B. sorokiniana* on switchgrass has been reported (Zeiders, 1984). Three to eight pathotypes of *B. sorokiniana* have been identified in different geographic locations and on various barley genotypes (Arabi and Jawhar, 2004; Fetch and Steffenson, 1994; Ghazvini and Tekauz, 2007; Valjavec-Gratian and Steffenson, 1997). Likewise, this species exhibits different virulence patterns on different wheat genotypes (Asad et al., 2007; Mahto et al., 2012; Poloni

et al., 2009). Isolates of *B. sorokiniana* collected from diverse geographic regions displayed different degrees of virulence on wheat (Mahto et al., 2012). Moreover, *B. sorokiniana* isolates vary in colonial morphology (Mahto et al., 2012; Poloni et al., 2009). Isolates within the same morphological group differ in virulence (Mahto et al., 2012). Isolates of *B. oryzae* differ in their aggressiveness on rice (Kamal and Mia, 2009; Kumar et al., 2011). Four morphology categories of *B. oryzae* isolates identified from rice hosts were correlated with different level of aggressiveness (Kumar et al., 2011). Information on the morphology and virulence diversity of *B. spicifera* and *B. victoriae* are limited (Koo et al., 2003; Safari-Motlagh and Kaviana, 2008).

Knowledge on detailed variation in virulence of *Bipolaris* species on switchgrass is lacking. In addition, the impact of *Bipolaris* on switchgrass biomass yield and overall quality is unknown. Evaluation of biomass yield reduction is important to assess the potential impact of *Bipolaris* on switchgrass production. This information will provide insights into possible agricultural and disease management strategies. Thus, the present study is an assessment of the diversity in morphology, pathogenicity, and virulence of *Bipolaris sorokiniana*, *B. oryzae*, *B. victoriae* and *B. spicifera* isolates recovered from commercial switchgrass seed and naturally infected field plants, and the impact of these fungi on feedstock biomass is determined.

2.3 Materials and methods

2.3.1 Fungal isolates

Bipolaris species included in this study were isolated from infected switchgrass plant materials, identified and characterized using simple sequence repeats (Fajolu et al., 2012) (Table 2.1).

2.3.2 Morphological characteristics

Morphological variability of *Bipolaris* was evaluated based on colonial and conidial properties. Colony morphology was characterized by mycelia color, growth pattern, and sector formation. Prior to colony characterization, each isolate was grown on potato dextrose agar (PDA) at 25°C for seven days. Conidial variability was based on size, shape, pigment, and pattern of conidial formation on infected switchgrass leaf blades in a detached leaf assay.

2.3.3 Plant materials

Switchgrass 'Alamo' plants were grown from seeds that were scarified and surface-sterilized with 60% sulfuric acid (Gwinn et al., 1991), or surface-sterilized in 50% Clorox® for 1 h. Twenty seeds were planted in 9 × 9-cm^2 pots containing 50% ProMix potting soil (Premier Horticulture Inc., Quakertown, Pennsylvania) and 50% Turface ProLeague (Profile Products, Buffalo Grove, Illinois). Plants were grown in environment-controlled growth chambers with a 12-h photoperiod at 25°C.

2.3.4 Detached leaf assay

Isolates of *B. oryzae* (n=10), *B. spicifera* (n=2), *B. victoriae* (n=1), and *B. sorokiniana* (n=12) were prescreened for pathogenicity and virulence on switchgrass (Table 2.1). The test was arranged in a randomized block design (RBD) and blocks were separated by replicate.

Leaf blades from 8-to-10-week-old plants grown from seed scarified with 60% sulfuric acid were harvested. Equal-sized blades (~5-cm length) were surface-sterilized in 95% ethanol for 30 s, followed by 1 min in 10% Clorox, 30 s in 95% ethanol, and a dip in sterile deionized (DI) water.

Three sections of surface-sterilized leaf blade were placed on 9-cm diameter sterile filter paper disks in a sterile petri dish; both leaf sections and filter paper were moistened with sterile DI water.

Each leaf section was inoculated at the center of the adaxial surface with a 7-mm plug of mycelium cut from the edge of actively growing fungi on PDA using a sterile core-borer. The test was carried out with four replicates of petri dishes for each isolate and the control. Control leaf sections were inoculated with PDA without fungal growth. Petri dishes of the same replicate were incubated in a moist chamber at room temperature to enhance high humidity and disease reaction. Leaf sections were checked daily for disease reaction. Assessment for pathogenicity and virulence were based on percent diseased area on leaf sections at 5-day post inoculation (dpi). Each petri dish containing leaf blades was photographed with a Samsung GX1S camera mounted on a copy stand. Brightness and contrast of the images were modified to enhance color differences between green and diseased areas of the leaf. Image adjustment was done at the same brightness and contrast settings for all pictures taken. Percent disease area on each leaf section was measured using Assess 2.0 image analysis software for plant disease quantification (Lamari, 2008). Leaves were viewed also under a dissecting microscope to assess for sporulation. Non-aggressive isolates were excluded from subsequent experiments.

2.3.5 *Bipolaris* virulence on switchgrass seeds and whole plants

Two additional isolates of *B. oryzae* were excluded from the virulence assay because of inability to produce enough spores for whole plant inoculation. Eighteen virulent isolates were evaluated on switchgrass seeds surface-sterilized in 50% Clorox for 1 h, and whole plants grown

from seeds surface-sterilized with 60% sulfuric acid. The evaluation was conducted with two levels of inoculum concentration, 10^1 and 10^5spores/ml sterile DI water. The experimental design was isolate nested within species, and inoculum concentrations were combined in a factorial arrangement of treatments using a completely randomized design (CRD) with three replications.

2.3.5.1 Inoculum preparation

Inoculum for seed and whole plant experiments was obtained by growing each virulent isolate on Rabbit food agar (RFA). Isolates of *B. oryzae* grow and produce spores well on RFA under UV light (Hau and Rush, 1980). To ensure uniformity, all isolates were treated in the same manner.

RFA was prepared by steeping 50 g of forti-diet mouse and rat food (Kaytee Inc., Chilton, Wisconsin) in 500 ml DI water for 20 min, followed by filtration through cheesecloth. Agar (15 g) was added to the filtrate, which was brought up to 1 L with DI water and autoclaved. Each virulent isolate was grown on RFA for 72 h at room temperature. To enhance sporulation, plates were exposed to UV light for 12 h followed by 12h of complete darkness and the cycle was repeated for 5 to 7 days (Hau and Rush, 1980).

On the inoculation day, conidia were dislodged by flooding the surface of the plates with sterile DI water containing Tween 20 (Fisher Scientific, Dubuque, Iowa) and gently scraping the fungal colony with a sterile spatula (Fisher Scientific). Spores were harvested under a biosafety cabinet (ESCO Technologies Inc., Hatboro, Pennsylvania). A drop of Tween 20 added to the conidial suspension acted as a surfactant to enhance dispersal of fungal spores on the plants or seeds. For each isolate, a conidial suspension of 10^5spores/ml sterile water was prepared and the

spore concentration was measured with a hemocytometer. An inoculum concentration of 10^1 spores/ml sterile DI water was obtained by serial dilution of a sample from the 10^5spores/ml conidial suspension.

2.3.5.2 Seed inoculation and virulence assessment

About 100 surface-sterilized seeds were incubated in 5 ml of conidial suspension in a sterile petri dish and gently shaken for 24 h on a shaker at 65 rpm. Twenty inoculated seeds were sown in each pot containing 50% ProMix potting soil and 50% Turface ProLeague with three replicate pots per isolate. Control seeds were incubated in sterile water and Tween 20 without conidia, and sown in the same manner as the treated seeds. Pots were kept in the growth chamber at 25°C with a 12-h photoperiod for 10 weeks. Virulence evaluation was based on the following: 1) percent seed germination, 2) disease rating of leaf blotch or spot, and root rot on emerged seedlings (Clive, 1971), 3) plant height measured from the crown to top of leaf and 4) biomass yield (fresh weight) per pot.

2.3.5.3 Whole plant inoculation and virulence assessment

Leaves of 6-week-old seedlings were trimmed aseptically and inoculated by spraying with a conidial suspension using an aerosol sprayer until leaf run-off. For each isolate, three replicate pots, each containing about 15 seedlings, were inoculated. Control plants were sprayed with sterile DI water and Tween 20 suspension. Inoculated and control plants were enclosed in plastic bags to maintain high relative humidity for five days, bags were then removed and plants were maintained in a walk-in growth chamber at 25°C with a 12-h photoperiod and 50 to 60% relative humidity. Plants were monitored daily for disease development. Virulence of each

isolate was evaluated at 21 dpi by visually estimating percentage diseased plant area (Clive, 1971) from five randomly selected plants within each pot. The height of the selected plants was measured from the crown to top of leaf and roots were examined for necrotic lesions or root rot. Biomass yield was determined by weighing the fresh weight of all plants, excised at the crown, within each pot.

2.3.5.4 Recovery of originally inoculated isolates from diseased plants

On the day of virulence assessment, symptomatic leaf and root sections from infected plants were excised and surface-sterilized for 1 min in 95% ethanol followed by 3 min in 20% Clorox, 1 min in 95% ethanol and a dip in sterile DI water. Samples of air-dried, surface-sterilized infected tissue were placed on 2% water agar amended with 10 mg/liter rifampicin and 5 µl/liter 2, 4 EC Danitol miticide (Valent, Walnut Creek, California), and incubated at 25°C for 2-3 days. Fungal hyphae from symptomatic plant tissue were sub-cultured onto PDA. Colonial and conidial characteristics were examined and compared with that of the originally inoculated isolates to confirm that disease symptoms were associated with the inoculated isolates.

2.3.6 Statistical analysis

Data were analyzed by ANOVA using mixed model (SAS 9.3 Inc., Cary NC). Data from detached leaf assays were analyzed as a RBD with sampling and isolate nested within species. Block was separated by replicate and each leaf section was a sample. Least squares means were computed and separation with least significant difference was performed to determine the differences in pathogen aggressiveness. ANOVA for the virulence assay included the main effects of species, isolate, and spore concentration, and the interactions of species × spore

concentration and isolate × spore concentration. The virulence assay was analyzed as a CRD with factorial design and sampling. Mean separation was computed to determine differences in isolate virulence and differences in the interaction between pathogen and spore concentration combinations. The significance level for statistical tests was P=0.05.

2.4 Results

2.4.1 Morphological characteristics

The morphological variability of *Bipolaris* species included in this study is shown in Figure 2.1. Two isolates of *B*. *spicifera* displayed distinct colony morphology; gray-to-black growth and white cottony growth (Fig. 2.1A-B). On diseased switchgrass leaf blades, conidia were formed in clusters on conidiophores (Fig. 2.1C). Conidia were oval-shaped, with brown pigmentation and had one to three septations (Fig. 2.1D). Of the four *Bipolaris* species studied, *B*. *spicifera* produced the smallest conidia. *Bipolaris sorokiniana* isolates were classified into three groups based on colony morphology; 1) black growth with brown sectors, 2) gray growth with white sectors and, 3) gray suppressed growth (Fig. 2.1E-G). Conidia were formed in small groups on infected switchgrass leaf blades. Conidia were oval, brown, and had three to nine septations (Fig. 2.1H-I).

The only isolate of *B*. *victoriae* available for this study had light brown mycelial growth (Fig. 2.1J). Conidia were in pairs on infected switchgrass leaf blades, with green-brown pigmentation. Conidia were oblong with three to 11 septations (Fig. 2.1K-L). Based on colony morphology, *B. oryzae* isolates included in the present study were categorized into three groups; 1) gray cottony growth with few sectors, 2) gray fluffy growth with many white sectors,

and 3) white growth (Fig. 2.1M-O). On infected switchgrass, conidia were borne singly. They were oblong, slightly curved and brown pigmented with three to ten septations (Fig. 2.1P-Q).

2.4.2 Pathogenicity variability – detached leaf assay

All 25 isolates of *Bipolaris* were pathogenic on switchgrass, as evidenced by necrotic lesions on detached leaf blades within 5 dpi (Fig. 2.2), but there were significant differences in pathogen aggressiveness, based on percent diseased leaf area, both at the species and isolate level ($P<0.0001$). Among the species, *B. oryzae* and *B. victoriae* were the most aggressive and *B. spicifera* was the least whereas *B. sorokiniana* was moderately aggressive ($P<0.05$). However, across all isolates, *B. oryzae* isolate SK12 was the most aggressive with a mean of 83% diseased leaf area whereas the only representative isolate of *B. victoriae* had a mean of 64%. Isolates with a mean less than 20% diseased area were considered non-aggressive and were excluded from whole plant and seed virulence assays (data not shown). Non-aggressive isolates included *B. oryzae* AP163, *B. spicifera* MH12073, and *B. sorokiniana* PSB3, SLA28, and ST2-2 with means ranging from 10.9 to 18.9% diseased leaf area. In general, the majority of the isolates were categorized as moderately aggressive on the detached leaf assay.

Isolates of all species sporulated on detached leaf blades within five days post-inoculation. However, only trace sporulation was observed with isolates of *B. oryzae* at edge of the leaf blade or around the inoculum plug. Extensive sporulation was observed with isolates of other species. Moreover, even with pronounced necrotic lesions, mycelial growth was sparse on leaves inoculated with *B. oryzae* whereas with other *Bipolaris* species there was extensive

mycelial growth (Fig 2.2). The sparse mycelial growth of *B. oryzae* indicates that a fungal product (such as a toxin) rather than fungal growth is required to damage leaf tissue.

Lesions produced by the four *Bipolaris* species were accompanied by chlorosis of the leaf tissue. Lesions produced by virulent or moderately virulent isolates extended beyond the inoculum plug. Lesions fully covered the leaf sections at 5 dpi for virulent isolates and at 9 dpi for moderately virulent isolates. Lesions produced by non-virulent isolates were less extensive after 14 dpi.

2.4.3 Virulence variability

All 18 isolates of *Bipolaris* tested differed in their virulence on switchgrass when applied to seeds or whole plants. When applied to seeds, the differential virulence response on switchgrass was determined from percent seed germination, disease symptoms on emerged seedlings, plant height, and biomass yield. *Bipolaris* virulence after whole plant inoculation was measured as percent diseased plant area, plant height, and biomass yield. Pathogens re-isolated from symptomatic plants were similar to the originally inoculated isolates both in conidial and colonial morphology (Fig. 2.3).

2.4.3.1 Percent seed germination

Inoculation of seeds with *Bipolaris* impacted germination both at low (10^1 spores/ml) and high (10^5 spores/ml) inoculum density. Based on analysis of variance, there were significant differences in germination among species, isolates, spore concentration, and the pathogen × spore concentration interaction (Table 2.2). At the species level, reduction in seed germination

ranged from 38 to 52% and 42 to 74% at low and high inoculum density, respectively. *Bipolaris oryzae* was the most virulent with the greatest negative impact on seed germination, and *B. spicifera* was the least virulent with the least effect on seed germination rate ($P<0.05$) (Fig. 2.4A). Isolates within species varied greatly in virulence. Within *B. oryzae*, isolate SK226 was the most virulent causing the least seed germination with a mean of 8% and WT30 was the least virulent resulting in the highest seed germination rate with a mean of 56% at combined low and high inoculum density effects. Among *B. sorokiniana* isolates, WT76 was the most virulence and AP150 the least with seed germination means of 20 and 52%, respectively. Only single isolates of *B. victoriae* and *B. spicifera* were available for evaluation in the virulence assay, therefore determining a difference within species was not possible. However, across all isolates, *B. oryzae* SK226 was the most virulent and *B. oryzae* WT30 the least virulent.

The effect of spore concentration was significant and there were significant species × spore concentration and isolate × spore concentration interactions (Table 2.2). The higher the pathogen concentration, the lower the germination rates of infected seeds and the greater the virulence of the pathogen. However, only seeds infected with *B. oryzae* or *B. sorokiniana* had statistical differences in germination rate at different spore concentrations ($P<0.05$). *Bipolaris oryzae* and *B. sorokiniana* reduced germination by an additional 22 and 15%, respectively, when pathogen concentration was increased from 10^1 to 10^5 spores/ml. Seed germination at both low and high inoculum density was statistically the same for *B. spicifera* and *B. victoriae* infection. Among all isolates, germination of *Bipolaris* infected seeds varied from 12 to 60% at 10^1 and, 3 to 53% at 10^5 spores/ml ($P<0.05$). Moreover, isolates responded differently in their impact on germination rates as spore concentration increased. The greatest response was

observed in seeds inoculated with *B. oryzae* isolate AP105 with 58% seed germination at 10^1 spore concentration, which was significantly reduced to 7% at 10^5 spores/ml. On the other hand, for *B. sorokiniana* isolate SLCO8, there was no difference in seed germination with increased spore concentration, with 38% for both.

2.4.3.2 Disease symptoms on emerged seedlings

Only a few isolates induced visible symptoms on seedlings from *Bipolaris* infected seeds. Symptoms observed included leaf spots on plants grown from seeds inoculated with 10^5 spores/ml of *B. victoriae*; stem necrosis and seedling blight caused by *B. oryzae* at 10^1 and 10^5spore concentration, respectively; and root necrosis induced by 10^5 spores of *B. sorokiniana* (Fig 2.5A-D). At low inoculum density, the majority of the emerged seedlings had no foliar or root symptoms.

2.4.3.3 Percent diseased plant area

Bipolaris species impacted plant vigor following foliar inoculation. All isolates tested on whole plants induced either spot blotch or leaf spot symptoms on switchgrass within 5 dpi at 10^5 spore concentration. Leaf lesions were accompanied by chlorosis of leaf tissues (Fig. 2.6A). Some diseased leaves progressed to dead leaf tissues at 21 days after inoculation. Other symptoms included stem and root necrosis (Fig. 2.6B). The percent diseased plant area ranged from 1 to 75% for all isolates tested. Foliar inoculation with 10^1 spore concentration induced only a few or no lesions with slight chlorosis, and diseased plant area ranged from 1 to 25%.

Differences in percent diseased area among the species, isolates, spore concentrations, and pathogen × spore concentration interactions were highly significant (Table 2.2). Based on mean separation, across spore concentration and isolates, *B. oryzae* contributed most to these differences and was the most virulent with diseased plant area greater than those caused by the other three species ($P<0.05$) (Fig. 2.7A). At high inoculum density, *B. oryzae* caused an average of 45% diseased plant area whereas with *B. sorokiniana* and *B. victoriae* averages of 31 and 28% diseased plant area were observed, respectively. *Bipolaris spicifera* was the least virulent species with only 11% of plant area with disease symptoms. There were significant differences in percent diseased area for isolates within each species (Table 2.2). Within *B. oryzae*, isolates SK12 and WT30 had the most and least diseased area with means of 32 and 6.6%, respectively, across both pathogen inoculum rates. Isolates APCNR 34 and JA5, within *B. sorokiniana*, had the highest and lowest disease reaction with means of 26 and 9.1%, respectively. Across all isolates, SK12 was the most virulent whereas LA18 (*B. spicifera*) and WT30 induced the least disease reaction ($P<0.05$).

The effects of spore concentration and pathogen × spore concentration interaction were significant (Table 2.2). The higher the pathogen concentration, the greater the diseased plant area, and the greater the pathogen virulence ($P<0.05$). All *Bipolaris* species tested responded differently in percent diseased area with increased spore concentration. The greatest difference in diseased area was found in *B. oryzae*; increasing from 1.8 to 45 (25-fold increase) and the least was observed in *B. spicifera* with a 9-fold increase in diseased area as the spore concentration increased from 10^1 to 10^5. *Bipolaris sorokiniana* and *B. victoriae* had an increase of 15-fold and 18-fold in diseased area, respectively. Likewise, all isolates induced greater

disease at higher inoculum density. The greatest response difference was observed with isolate *B. oryzae* isolate WT62 which caused a 45-fold increase in diseased area at higher inoculum concentration. Isolate LA18 had the least, with an increase of 9-fold in disease as inoculum concentration was increased.

2.4.3.4 Plant height

Inoculation of switchgrass seeds or whole plants with *Bipolaris* impacted plant height. For seed inoculation, there were significant differences in plant height for species, isolates, spore concentration and the isolate × spore concentration interaction (Table 2.2). Inoculation of seeds with *B. spicifera* did not impact plant height whereas the other three *Bipolaris* species caused a significant reduction in plant height of emerged seedlings (Fig. 2.4B). *Bipolaris oryzae* reduced plant height by 37.6% whereas the reduction in plant height by *B. sorokiniana* and *B. victoriae* was only 22.7 and 26.2%, respectively ($P<0.05$). There was no significant species × spore concentration interaction (Table 2.2). Isolates varied in their ability to impact plant height. Infection with *B. oryzae* isolate SK226 resulted in the most severe stunting of growth, indicating that this was the most virulent isolate. The high inoculum rate of SK226 reduced plant height to 3.3 cm, compared to the control with 80.7 cm. There was a significant isolate × spore concentration interaction and isolates responded differently to increased inoculum density. For example, *B. sorokiniana* isolate WT76 caused the greatest reduction in plant height (73.7 to 24.3 cm) as the spore concentration increased from 10^1 to 10^5 spores/ml. Of the 18 isolates tested, only two isolates of *B. sorokiniana* and four isolates of *B. oryzae* caused a significant

response in plant height to increased spore concentration whereas for other isolates, there were no significant differences in plant height for both low and high inoculum density.

For whole plant inoculation, only the main effects of species and isolate were significant (Table 2.2). Among the species, height of plants inoculated with any of the four *Bipolaris* species were significantly stunted compared to controls, with the greatest high reduction caused by *B. oryzae* ($P<0.05$) (Fig. 2.7B). Infection with *B. spicifera* reduced plant height by 32%, and reduction in plant height by the other three species ranged from 38 to 42%. Among all isolates, *B. oryzae* SK12, *B sorokiniana* WT76 and *B. spicifera* LA18 had the tallest plants with means ranging from 42.5 to 42.7 cm, but reduced plant height compared with the control by about 34%. Isolate SLCO8 (*B. sorokiniana*) produced the shortest plants with a mean of 34.7 cm, and reduced plant height by 45% when compared to control plants ($P<0.05$). The majority of the isolates resulted in similar plant height values. The effect of spore concentration on plant height was not significant and there was no significant pathogen × spore concentration interaction (Table 2.2). Effect of *Bipolaris* on plant height and biomass yield is illustrated (Fig. 2.8).

2.4.3.5 Biomass yield

In addition to the negative impact on plant stand, height and disease, *Bipolaris* infection significantly reduced switchgrass biomass yield (fresh weight) per pot. For seed inoculation, differences in biomass yield among species, isolates, spore concentrations and the pathogen × spore concentration interaction were significant (Table 2.2). *Bipolaris spicifera* was the least virulent with the highest biomass yield, and *B. oryzae* the most virulent with the lowest biomass yield whereas, *B. sorokiniana* and *B. victoriae* were moderately virulent and had a similar

impact on biomass yield (P<0.05) (Fig. 2.4C). At low pathogen concentration, *B. spicifera* reduced biomass by only 5% whereas the other three species reduced biomass by 64 to 70%. Higher pathogen concentration increased biomass reduction to 12.5% with *B. spicifera*, 67% for *B. sorokiniana* and *B. victoriae,* and 88% with *B. oryzae*. Only *B. oryzae* contributed to the significant species × spore concentration interaction and had decreased biomass as inoculum concentration increased. Other species did not differ in biomass yield at low and high inoculum density (P<0.05). Across all isolates, infection with *B. spicifera* isolate LA18 and *B. oryzae* isolate SK226 had the greatest and least biomass yield with means of 30 g and 1.6 g, respectively. Moreover, only isolates AP105, SK12, WT62 (*B. oryzae*) and WT76 (*B. sorokiniana*) produced a significant decrease in biomass yield in response to increased disease pressure whereas infection with other isolates resulted in no changes in biomass yield at concentrations of 10^1 and 10^5 spores/ml.

For whole plant inoculations, there were significant differences in biomass yield among species and isolates (Table 2.2). *Bipolaris spicifera* had the greatest biomass yield, but reduced biomass by 36% and *B. victoriae* the least, and reduced biomass by 62%. *Bipolaris sorokiniana* and *B. oryzae* had the same impact on biomass and resulted in 50% biomass reduction (P<0.05) (Fig. 2.7C). Across all isolates, the impact of *Bipolaris* on biomass varied considerably and percent reduction ranged from 32 to 71%. Isolates AP124, SK12, APCNR34, WT76 and LA18 had statistically the same biomass and the highest yield with means ranging from 17.0 to 18.1 g, but caused 32 to 36% biomass reduction. Across both spore concentrations, isolates AP105 and WT95 (*B. oryzae*), APCNR150 and SLCO8 (*B. sorokiniana*) and JA12 (*B. victoriae*) had the lowest biomass yield with means of 7.8 to 10.1 g, which resulted in 62 to 71% biomass reduction. The

main effect of spore concentration was not significant, and there was no significant interaction between pathogen and spore concentration (Table 2.2).

2.5 Discussion

Bipolaris isolates within and between species varied in colonial and conidial morphology. The morphological characteristics of *Bipolaris* species studied differed from those previously reported from different host plants and geographical regions (Arzanlou and Khodaei, 2012; Kumar et al., 2011; Mahto et al., 2012; Poloni et al., 2009). Conidial morphology of *B. spicifera* isolated from sunflower in Iran differs from those observed in this study (Arzanlou and Khodaei, 2012). However, conidial arrangement was similar to the arrangement described on infected Bermuda seeds (Koo et al., 2003). Three colonial groups of *B. sorokiniana* were identified in Nepal (Mahto et al., 2012) and five groups in Brazil (Poloni et al., 2009). These groups were different from the colonial groups observed in the present study. The morphological categories of *B. oryzae* identified in this study differed from those identified among isolates collected from rice in India (Kumar et al., 2011). Morphology differences are probably influenced by different host genotypes and climatic conditions (Kumar et al., 2011).

Pathogenicity is the capacity of a pathogen to infect and cause damage in a host whereas, virulence is the degree of damage caused to a host by pathogen infection (Sacristán and García-Arenal, 2008). Pathogens that induce high virulence grow rapidly within a host, possess high transmission rate to another host, and are able to overcome host resistance factors (Sacristán and García-Arenal, 2008; Fisher et al., 2012). This study revealed distinct variations in pathogenicity and virulence among *Bipolaris* species and among isolates within species. Results

of detached leaf, seed inoculation and whole plant inoculation assays conducted were in agreement in determining the virulence identity of *Bipolaris* isolates studied.

Among the four *Bipolaris* species evaluated, *B. oryzae* was consistently the most virulent species in the detached leaf assay, had the highest percent diseased area in the whole plant assay, and had the highest negative impact on plant stand, height and biomass yield from the seed inoculation assay. *Bipolaris oryzae* has a similar impact on other agronomically important crops such as rice, where it reduces seed germination and plant vigor; causes root rot, seedling blight, leaf spot and abnormal seedling formation, which results in economic yield losses worldwide (Kamal and Mia, 2009; Kumar et al., 2011; Nghiep and Gaur, 2004; Safari-Motlagh and Kaviani, 2008). Moreover on other crops, *B. oryzae* was more virulent than other *Bipolaris* species. On rice, *B. oryzae* was more aggressive than *B. victoriae*, *B. indica* and *B. bicolor* (Safari-Motlagh and Kaviani, 2008).

In the seed inoculation assay, *B. oryzae* caused up to 74% reduction in plant stand, which was consistent with the reduction in seed germination of rice from 20.5 to 84.5% (Kamal and Mia, 2009). One of the major problems in switchgrass cultivation is stand establishment (Parrish and Fike, 2005). It is obvious that seedborne pathogens like *Bipolaris* play a significant role in this problem even at low inoculum density, and with low-virulent species. Moreover, the height and vigor of established switchgrass plants were greatly impacted by *Bipolaris*, reducing biomass yield. All *Bipolaris* species had almost the same impact on plant height when applied to whole plants, where they caused significant height reduction. However, with seed inoculation, *B.*

oryzae had the greatest impact and caused up to 70% height reduction. In addition to stunted growth, the seedling blight observed on emerged seedlings also impacted biomass yield.

All four *Bipolaris* species reduced switchgrass biomass yield when applied to seed or whole plants but, *B. oryzae* had the greatest impact with up to 88% biomass reduction at higher inoculum density when compared to control plants. In a previous report on the severity of *B. oryzae* on switchgrass, the potential to reduce biomass yield was noted (Krupinsky et al., 2004). *Tilletia maclaganii*, which causes smut on switchgrass, reduced biomass/tillering by 38 to 82%, but dry biomass loss was about 40% (Thomsen et al., 2008). Other fungi implicated in switchgrass biomass reduction includes isolates of *Fusarium*, *Alternaria*, *Xylaria,* and *Phaeosphaeria* species causing up to 61% total dry mass reduction (Kleczewski et al., 2012). High biomass yield with limited fertilizer application is one of the switchgrass qualities that make it a suitable bioenergy crop (Bouton, 2008). Recently, fungal pathogens have been shown to pose a potential threat to switchgrass biomass production. Energy from switchgrass is biomass-dependent and to economically grow switchgrass for bioenergy, biomass yield must be maximized to increase its net energy yield. Therefore, cost-effective disease management must be ensured to alleviate losses due to switchgrass pathogens. *Bipolaris oryzae* is a concern for switchgrass production because it was found on switchgrass seeds of different cultivars obtained from different part of the U.S. (Vu, 2011). *Bipolaris oryzae* appears to be more prevalent and widely distributed than other *Bipolaris* species and some other fungal pathogens of switchgrass (Vu, 2011). This pathogen also causes disease in rice, and is prevalent in growing regions and able to infect most rice cultivars grown worldwide (Nghiep and Gaur, 2004).

Bipolaris sorokiniana ranks second to *B. oryzae* in degree of virulence on 'Alamo'. In a previous report, *B. sorokiniana* was the most prevalent and important foliar disease of switchgrass in Pennsylvania (Zeiders, 1984). Although *B. sorokiniana* varies in virulence on a variety of cereals and grasses, it has constantly been evolving. Between the year 1997 and 2004, three pathotypes or virulence groups of *B. sorokiniana* were found on barley from different countries (Arabi and Jawhar, 2004; Valjavec-Gratian and Steffenson, 1997; Zhong and Steffenson, 2001). In 2004, six pathotypes were reported (Meldrum et al., 2004). In 2007, eight virulence groups were identified with enhanced virulence (Ghazvini and Tekauz, 2007). The newly-emerged pathotypes possess unique virulence on seedlings and mature plants of resistant barley cultivars (Ghazvini, 2012; Ghazvini and Tekauz, 2007). There is the possibility that more virulent *B. sorokiniana* isolates will emerge on switchgrass.

The limited isolates of *B. spicifera* and *B. victoriae* available for this study did not allow for a conclusion regarding the virulence identity of these two species. Nonetheless, the two isolates of *B. spicifera* evaluated in this study were weakly pathogenic or had low virulence on 'Alamo'. The single isolate of *B. victoriae* evaluated was moderately virulent on switchgrass. Of the four *Bipolaris* species, *B. spicifera* and *B. victoriae* had only been reported on switchgrass from Tennessee, whereas the other two species had been reported from different states in the U.S. (Farr and Rossman, 2012). *Bipolaris spicifera* and *B. victoriae* have been isolated from important grain crops and grasses such as wheat, corn, barley, oat, rye, rice, sorghum and bermudagrass (Farr and Rossman, 2012) (Table 1.1).

The susceptibility of switchgrass to *Bipolaris* was influenced by the pathogen concentration. At low concentration, the majority of *Bipolaris* isolates tested did not produce visible lesions on leaves or roots after inoculation, this is a reflection of either symptomless infection or that switchgrass is resistance to *Bipolaris* at low inoculum density. Based on isolate response to increase in inoculum concentration, *Bipolaris* can be classified into three groups. The first group comprises the majority of isolates. Their virulence on switchgrass was inoculum dependent and required a concentration greater than 10^1 spores/ml for disease development. At low spore concentration, these isolates were not virulent and did not produce any visible symptoms. They became virulent at higher spore concentration. The second group comprises mainly *B. spicifera* and just a few isolates of other species. These isolates have low virulence both at low and high inoculum density, and had limited impact on switchgrass when compared with other isolates. Isolates in the third group were very aggressive; they were virulent on switchgrass at low and high inoculum concentrations. Although disease severity increased as inoculum density increased, these isolates induced disease symptoms and produced significant impacts on switchgrass at low inoculum density. The presence of such isolates in the field could be problematic even at a very low inoculum density since only a few conidia are required for disease development. Such isolates should be targeted for disease resistance development in switchgrass.

Virulence assessment with seed inoculation resulted in more consistent and distinct variability in pathogen impacts on switchgrass compared to that of the whole plant assays. For example, with seed inoculation, *B. oryzae* isolate SK226 was consistently the most virulent isolate. This isolate resulted in the lowest percent plant stand, most stunted growth, and caused the highest

fresh weight biomass reduction. On the other hand, with whole plant inoculation *B. oryzae* SK12 induced the greatest percent diseased plant area, but resulted in the tallest plants and caused the lowest biomass reduction. The impact of *Bipolaris* on switchgrass biomass was greater when spores were applied on seeds rather than on the whole plant. More so, seedling blight, which is a major cause of biomass reduction, was observed only from the seed inoculation experiment. Planting of infected seeds is probably more problematic to switchgrass production than infection after stand establishment. The survival of *Bipolaris* species on seeds has been reported to be the major source of primary inocula (Manamgoda et al., 2011). Recent increases in reports of fungal diseases on switchgrass (Farr and Rossman, 2012) may be attributed in part to increased distribution of fungal-infected seeds to growers. Since switchgrass propagation is mainly by seed, it is therefore important that seed certification programs and cost-effective seed treatments be established for switchgrass.

Based on the results of the virulence assays conducted in this study, all *Bipolaris* isolates, within or across species, can be classified into three virulence groups. Isolates were identified with extreme virulence; those that were highly virulent and those with low virulence, whereas the majority of isolates had an intermediate level of virulence. Information on the pathogenicity and virulence variability in *Bipolaris* and their role in switchgrass production will aid in development of disease management strategies, and thereby reduce the impact of this fungal pathogen on switchgrass. Information obtained from this study is valuable for the incorporation of disease resistance in switchgrass breeding programs.

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Table 2.1 Fungal isolates studied for morphology, pathogenicity and virulence diversity

Species	Isolate ID	Source[a]	Origin
Bipolaris sorokiniana	SLA26	Alamo	Colorado
	SLA28[b]	Alamo	Colorado
	SLA32	Alamo	Colorado
	SLCO1	Alamo	Colorado
	SLCO8	Alamo	Colorado
	ST2-2[b]	Alamo	Colorado
	JA 5	Alamo	Oklahoma
	PSB3[b]	Alamo	Tennessee
	APCNR34	Cave-in-Rock	Illinois
	APCNR150	Cave-in-Rock	Illinois
	WT65	Cave-in-Rock	Iowa
	WT76	Cave-in-Rock	Iowa
Bipolaris spicifera	LA18	Alamo	Oklahoma
	MH12073[b]	Alamo	Tennessee
Bipolaris victoriae	JA12	Alamo	Oklahoma

Table 2.1 Continued

Species	Isolate ID	Source[a]	Origin
Bipolaris oryzae	AP105	Cave-in-Rock	Illinois
	AP124	Cave-in-Rock	Illinois
	AP163[b]	Cave-in-Rock	Illinois
	WT27[c]	Cave-in-Rock	Iowa
	WT30	Cave-in-Rock	Iowa
	WT62	Cave-in-Rock	Iowa
	WT95	Cave-in-Rock	Iowa
	SK12	Kanlow	Missouri
	SK13[c]	Kanlow	Missouri
	SK226	Kanlow	Missouri

[a] Switchgrass cultivars from which isolates were obtained.

[b] Non-aggressive isolates on detached leaf assay.

[c] Isolates did not produce sufficient number of spores for whole plant inoculation.

[b, c] Isolates not included in the virulence assay.

Table 2.2 Analysis of variance of the virulence of *Bipolaris* isolates on switchgrass seeds and whole plants

	Type 3 Test of Fixed Effects					
	Percent	Percent diseased	Plant height[a]		Biomass yield[a]	
	Germination[b]	plant area[c]	Seed[b]	Whole plant[c]	Seed[b]	Whole plant[c]
Effect	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values
Species	<0.0001/ 99.00	<0.0001/ 23.10	<0.0001/ 15.19	<0.0001/ 116.39	<0.0001/ 98.13	<0.0001/ 52.09
Isolate(species)	<0.0001/ 17.74	<0.0001/ 4.85	<0.0001/ 5.21	0.0008/ 2.99	<0.0001/ 10.59	<0.0001/ 10.60
Spore concentration	<0.0001/ 50.92	<0.0001/ 186.09	0.0277/ 5.04	0.3168/ 1.01	0.0141/ 6.31	0.4918/ 0.48
Species*spore concentration	0.005/ 4.04	<0.0001/ 21.69	0.0773/ 2.20	0.6672/ 0.60	0.0266/ 2.92	0.4145/ 1.00
Isolate(species)* spore concentration	<0.0001/ 4.58	<0.0001/ 4.38	0.0028/ 2.71	0.8500/ 0.62	0.0342/ 1.95	0.2534/ 1.25

[a] P and F values of analysis of variance from seed and whole plant inoculation.

[b]DF value of analysis of variance from seed inoculation assay was 76.

[c]DF value of analysis of variance from whole plant inoculation assay was 80.

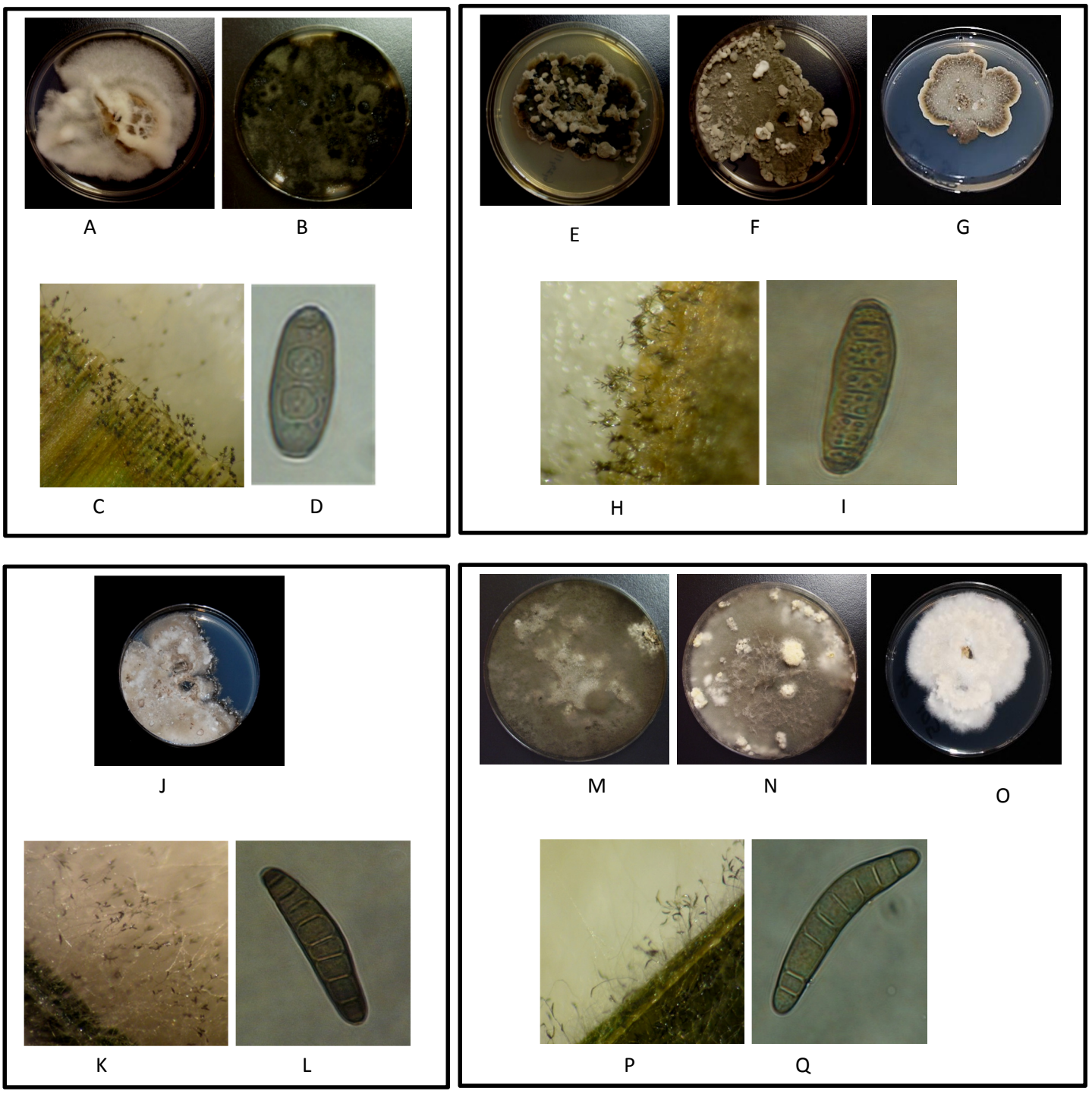


Fig. 2.1 Colonial and conidial morphology of *Bipolaris* species. (A-D) *B. spicifera*; (E-I) *B. sorokiniana*; (J-L) *B. victoriae*; and (M-Q) *B. oryzae*. Magnification of individual conidia was × 400 and conidia on infected leaf blades was × 40.

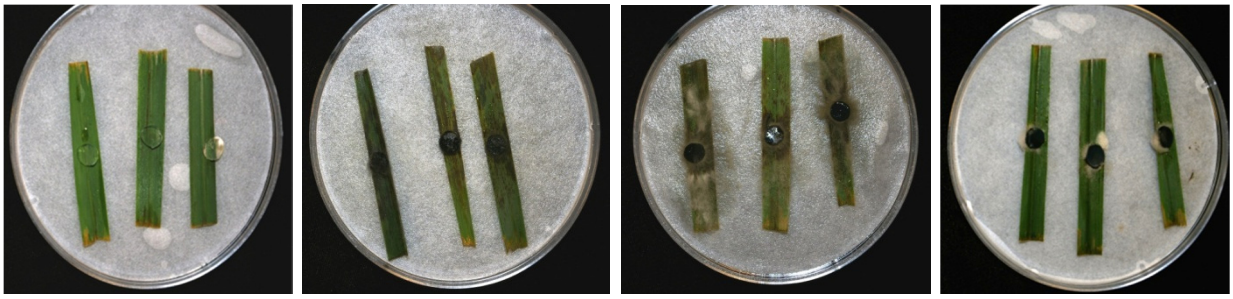
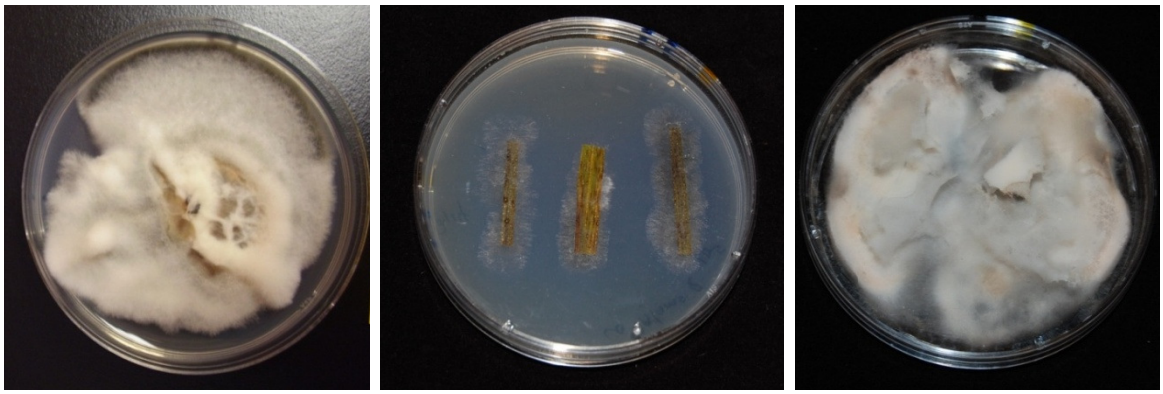


Fig. 2.2 Detached leaf assay at five days after inoculation. (A) Control leaf sections inoculated with agar plugs. (B) Leaf sections inoculated with *Bipolaris oryzae* had pronounced lesions without obvious mycelial growth. (C) Leaf sections inoculated with *B. sorokiniana* had extensive mycelial growth and lesions. (D) Example of a non-aggressive isolate.



A B C

Fig. 2.3 Re-isolation of *Bipolaris* from inoculated symptomatic switchgrass. (A) Original *B. spicifera* isolate LA18 used to inoculate switchgrass. (B) Fungal growth from inoculated symptomatic switchgrass leaf. (C) *B. spicifera* isolate LA18 recovered from inoculated symptomatic plant.

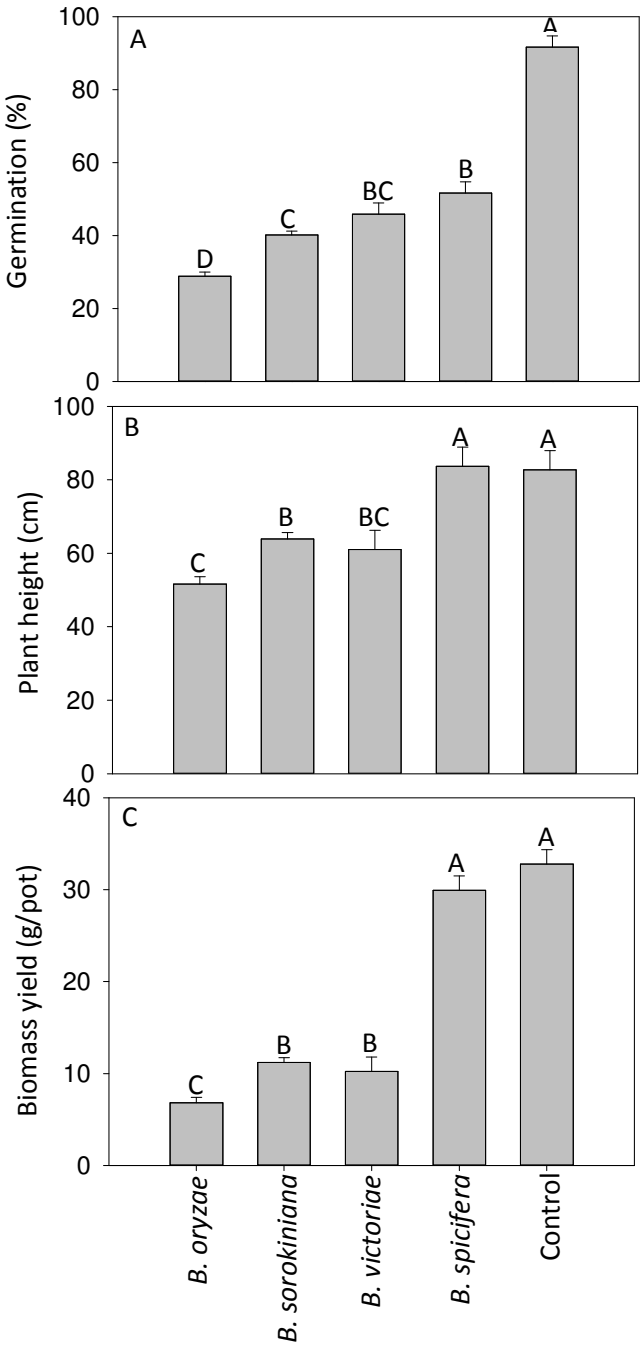


Fig. 2.4 Impact of *Bipolaris* species on switchgrass following seed inoculation. Main effect of *Bipolaris* with combined inoculum densities on seed germination (A), plant height (B), and biomass yield (C). For each variable, bars with a common letter are not significantly different at P=0.05 (n=3 replicates).



Fig. 2.5 Disease symptoms on emerged seedlings from inoculated seeds. (A) Leaf spot on plant grown from seeds inoculated with 10^5 spores of *Bipolaris victoriae*. (B) Seedling blight and (C) Stem necrosis caused by 10^5 and 10^1 spores/ml of *B. oryzae*, respectively. (D) Root necrosis induced by 10^5 spores/ml of *B. sorokiniana*.



Fig. 2.6 Disease symptoms on whole plants at 21 days after inoculation. Leaf lesions (A) and root necrosis (B).

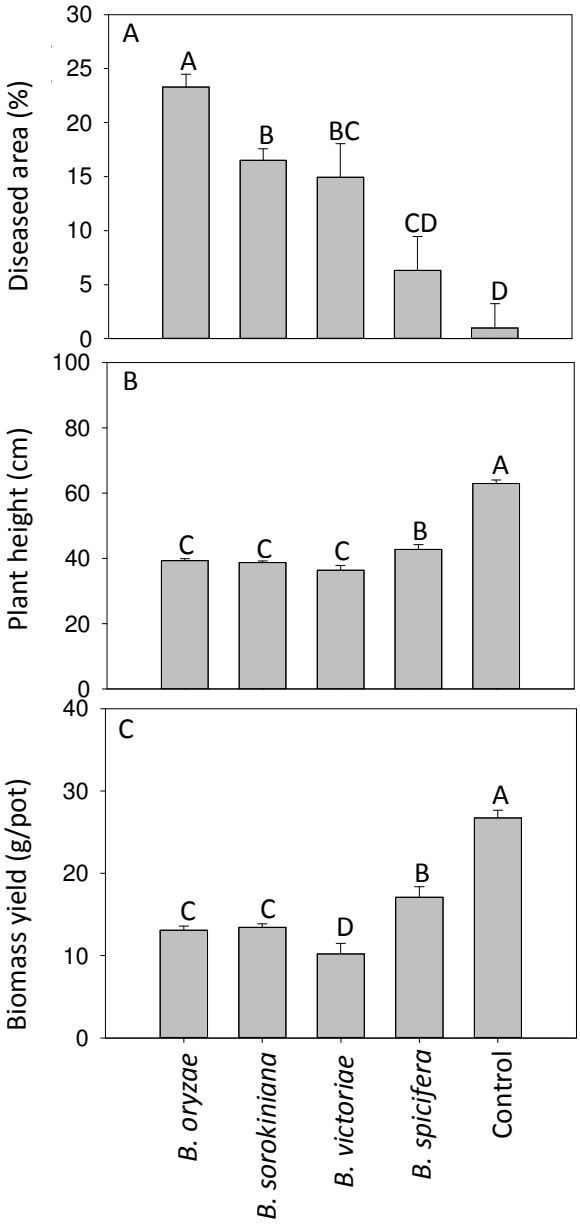


Fig. 2.7 Impact of *Bipolaris* species on switchgrass following whole plant inoculation. Effect of *Bipolaris*, with the means for inoculum densities combined, on percentage diseased area (A), plant height (B), and biomass yield (fresh weight) (C). For each variable, bars with a common letter are not significantly different at P=0.05 (n=3 replicates).



Fig. 2.8 Plant height and biomass reduction due to infection with *Bipolaris*. Healthy control (Left) and *B. sorokiniana* infected plant (Right).

CHAPTER 3: ASSESSMENT OF *BIPOLARIS* INFECTION ON SWITCHGRASS CHEMICAL COMPONENTS

3.1 Abstract

Bipolaris is an important pathogen of switchgrass (*Panicum virgatum* L.) in the United States. The impact of four *Bipolaris* isolates on feedstock fermentable sugars, lignin and ash contents was evaluated on switchgrass 'Alamo'. A two-stage acid hydrolysis method was used to hydrolyze switchgrass samples for chemical analysis. The major fermentable sugars, glucose and xylose, were significantly reduced in *Bipolaris* infected biomass. Galactose and arabinose contents of *Bipolaris* infected switchgrass were about 1% higher than that of the uninfected switchgrass control. In general, *Bipolaris* caused up to 7% reduction in total biomass carbohydrate content. Since feedstock carbohydrate content is directly proportional to ethanol yield, *Bipolaris* will reduce ethanol production by 7%. Lignin content of infected biomass was not significantly different from that of the uninfected control biomass. However, there was an increase in lignin content with high inoculum density of the pathogen. Increased disease severity will increase recalcitrance of switchgrass, making bioconversion of biomass to biofuels more challenging. There was a significant increase in the ash content of *Bipolaris* infected biomass. A lower ash content of feedstock is desirable for bioprocessing of biomass-to-ethanol or other biofuel products. Research towards development of proper management strategies and sustainable agricultural practices should be emphasized in order to alleviate losses from switchgrass pathogens. Failure will threaten the success of the emerging biofuel industry.

3.2 Introduction

Understanding the impact of fungal pathogens on bioenergy crops, such as switchgrass, necessitates the study of effects of these pathogens on the quality of the biomass, the chemical components of biomass, which are converted to biofuel.

Interest in biofuels as a renewable form of energy has increased due to the concern about the negative impact of gasoline on environmental quality. Efforts to reduce gasoline consumption in the U.S., and increase reliance on clean renewable fuels from bioenergy crops has led to a mandatory annual production of 16 out of 36 billion gallons of bioethanol from lignocellulosic feedstock by the year 2022 (Robertson et al., 2008). Lignocellulosic biomass is nonfood-plant material grown for biofuel production and has gained attention over starch- or sugar-based feedstock due to competition between energy and food supplies (Yuan et al., 2008). Lignocellulosic biomass is composed mainly of cellulose, hemicellulose and lignin (Sluiter et al., 2010). Cellulose, a polymer of glucose, is the most abundant structural constituent in plant cells and represents the majority of fermentable glucose in a feedstock (Kumar et al., 2009; Thammasouk et al., 1997). Hemicellulose is a polymer of different sugars including xylose, arabinose, mannose, galactose, glucose and uronic acid. Hemicellulose is the source of other fermentable sugars available in a feedstock (Kumar et al., 2009; Mosier et al., 2005). Lignin, a phenolic compound polymer, provides structural support to the plant and represents the recalcitrant element of a feedstock (Kumar et al., 2009; Thammasouk et al., 1997). Minor components of lignocellulosic biomass are protein, ash, and extractives. Extractives are a soluble nonstructural component of a biomass that includes chlorophyll, waxes, nonstructural sugars, and nitrogenous compounds. Extractives have the potential to interfere with the

compositional analysis of a biomass sample and hence, must be removed prior to the analysis (Kumar et al., 2009; Sluiter et al., 2005a). Compositional analysis of lignocellulosic biomass is needed to evaluate and optimize the bioconversion process and biofuel yields. It is also useful in comparative studies of the composition of different biomass feedstocks (Sluiter et al., 2010; Thammasouk et al., 1997).

Switchgrass (*Panicum virgatum* L.) is a promising source of lignocellulosic feedstock for biofuel production in the U.S. due to its high biomass yield, high net energy balance, and its status as a native perennial grass (Bouton, 2008). Recently, reports of fungal isolates recovered from switchgrass have increased in number to 378 (Farr and Rossman, 2012). Most of these isolates are pathogenic; however, the significance and management of most of these fungi is unknown. *Bipolaris* isolates are among the major pathogens infecting switchgrass. A study on the incidence and prevalence of seedborne and soilborne fungal pathogens of switchgrass in the U.S. identified *Bipolaris* as one of the predominant and widely distributed species (Vu et al., 2010; 2011). Assessment of *Bipolaris* infection on switchgrass biomass composition is important to evaluate the potential impact of this pathogen on biofuel production. Accurate estimates of the cellulose and hemicellulose contents of a biomass are important because they provide the value of carbohydrate available for microbial fermentation into ethanol (Sluiter et al., 2010; Thammasouk et al., 1997) or other biofuel products. The objective of this study was to assess the effects of two isolates of *B. sorokiniana*, and one each of *B. oryzae* and *B. spicifera*, on switchgrass biomass composition.

3.3 Materials and Methods

3.3.1 Fungal isolates

*Bipolari*s isolates used in this study were obtained from infected switchgrass, and identified and genetically characterized using simple sequence repeat markers (Fajolu et al., 2012). Four *Bipolaris* isolates that included two isolates of *B. sorokiniana* (AP34 and WT76) and an isolate each of *B. oryzae* (SK12) and *B. spicifera* (LA18), were used to infect switchgrass 'Alamo'. Spore inoculum for each isolate was prepared according to section 2.3.5.1 of this dissertation. This study was carried out at two levels of inoculum concentration; 10^1 spores/ml sterile water (low disease pressure) and 10^5 spores/ml sterile water (high disease pressure).

3.3.2 Inoculation of switchgrass with *Bipolaris* isolates

Switchgrass plants were grown from seeds scarified and surface-sterilized with 60% sulfuric acid (Gwinn et al., 1991). Five-week-old seedlings were inoculated with spore suspensions containing a drop of Tween 20, using an aerosol sprayer until leaf run-off. For each isolate, at each inoculum concentration, three replicate pots, each containing about 20 seedlings, were inoculated. Control plants were sprayed with sterile water and Tween 20 suspension, see section 2.3.5.3.

Inoculated plants were enclosed in plastic bags for five days to enhance high relative humidity and disease reaction, bags were then removed. Plants were maintained in a walk-in growth chamber at 25°C with a 12-h photoperiod and 50 to 60% relative humidity for an additional 10 weeks. The experimental design was a complete randomized design (CRD) with three replications; inoculum concentrations were combined in a factorial arrangement of treatments.

3.3.3 Switchgrass preparation for compositional analysis

All analyses were conducted according to the National Renewable Energy Laboratory (NREL) procedures (U.S. Dept. of Energy, 2012).

Switchgrass plants were harvested 10 weeks after inoculation by clipping the stems from the crown. Plants from each replicate pot were harvested separately and treated as a separate sample throughout the study. Switchgrass samples were dried in an oven (SII Dry Kilns, Lexington, North Carolina) at 45°C for seven days. Over-dried samples were ground in a Wiley Knife mill (Thomas Scientific, Swedesboro, New Jersey) to pass through a 40-mesh screen (Hames et al., 2008). The resulting sample was about 0.425-mm particle size. Milled samples were stored in airtight zip lock bags at room temperature prior to analysis.

3.3.4 Determination of total solid and moisture content

A total solid is the amount of solids remaining after the sample is oven-dried to a constant weight at 105°C whereas moisture content is the amount of water volatilized at 105°C in a sample (Sluiter et al., 2008a). Crucibles were completely dried in an Isotemp oven (Fisher Scientific, Dubuque, Iowa) at 105°C for at least 4 h. Dried crucibles were cooled in desiccators; the weight of each crucible was determined and recorded. A 0.5-g milled sample was weighed into a pre-dried crucible and placed in the Isotemp oven at 105°C for 24 h. Percent total solids and moisture content were calculated from the weight loss of samples after drying at 105°C – equations 1 and 2.

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

3.3.5 Determination of ash content

Ash is the amount of inorganic material present in a biomass sample. This could be structural ash, which is inorganic material bound in the structure of the biomass, or extractable ash, which is inorganic material removed during sample extraction (Sluiter et al., 2005b). To determine the total ash content, crucibles were placed in an Isotemp programmable forced-draft furnace (Fisher) at 575°C for at least 4 h. The crucibles were then cooled in desiccators and the weight of each was recorded. A sample weight of 0.5 g was measured into the tared crucible and placed in the furnace at 575°C for 24 h. Percent ash content after combustion was calculated using equation 4. To determine the structural ash content, a similar procedure was carried out with an extractives-free sample.

3.3.6 Determination of extractives content

Soluble extractives were removed from switchgrass samples using a two-step NREL extraction procedure with deionized (DI) water and 95% ethanol as solvents (Sluiter et al., 2005a). Briefly, 2.5 g of milled sample was mixed with enough glass beads to fill a 10-ml extraction cell (Thermo Scientific, Sunnyvale, California). The weight of sample plus glass beads was recorded. During the same period, the percent total solids of samples were determined. Sequential water and ethanol extraction were carried out automatically using a Dionex Accelerated Solvent Extractor

(Thermo Scientific). During the extraction cycles, the solvent soluble extractives were collected in collection vials.

After extraction, residual switchgrass sample and glass beads were recovered from each extraction cell into a known-weight pan and dried at 40°C for three days. The weight of each dried sample plus pan and beads was recorded. A 10-mesh brass sieve was used to remove glass beads from the extracted samples. Percent total solids of extractives-free samples were determined. Extracted samples were stored in airtight zip lock bag at room temperature prior to hydrolysis. The percent extractives content of each sample was calculated based on the change in weight of oven-dried samples before and after extraction – equations 5 and 6.

3.3.7 Determination of structural carbohydrate and lignin content

Hydrolysis

A two-stage hydrolysis was used to hydrolyze the polymeric carbohydrate into monosaccharides that were then measured to determine the carbohydrate content of the extractives-free samples. The lignin component was released also and determined (Sluiter et al., 2008b). At the first stage, 300 mg of extractives-free sample in a pressure tube (Ace Glass, Vineland, New Jersey) was hydrolyzed with 3 ml of 72% sulfuric acid (Sigma, St. Louis, Missouri) at 30°C for 1 h. This was done by placing the pressure tube into a water bath. A Teflon rod (Ace Glass) was used to stir the mixture every 10 min to ensure uniform hydrolysis. After 1 h of hydrolysis, the acid was diluted to 4% by adding 84 ml of DI water to the sample mixture. A Teflon cap and O-ring seal (Ace glass) were screwed on the pressure tubes, which were then placed into an autoclave for the second stage hydrolysis at 121°C for 1 h. A set of sugar

recovery standards (SRS) were prepared and included in the second stage of the hydrolysis to correct for losses due to degradation of sugars during dilute acid hydrolysis. An 87-mg sample of each standard sugar, including glucose, xylose, galactose, arabinose and mannose, was measured into a pressure tube, and 3 ml of 72% acid and 84 ml of DI water were added. The tube was capped as described previously and autoclaved along with the sample tubes.

Lignin content

After completion of hydrolysis, the hydrolyzate was allowed to cool to room temperature and vacuum-filtered through a filtering crucible. Crucibles were previously dried at 575°C and weighed. The filtrate was used to determine the acid soluble lignin and carbohydrate contents of the extractives-free sample. The solid fraction contained in the crucible was used to determine the acid-insoluble residue (AIR) and acid-insoluble lignin (AIL). To determine AIR, crucible plus the solid residue was oven-dried at 105°C for 24 h, after which the weight was recorded. Acid-insoluble lignin was calculated after sample combustion at 575°C – 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, New York). Prior to UV absorbance measurement, samples were diluted by adding 950 µl of DI water to 50 µl of sample. This was done to obtain an absorbance range of 0.7 to 1.0. Absorbtivity of switchgrass biomass (110 liters/g-cm) at 205 nm was applied to ASL calculations. Total lignin content of extractives-free sample was the sum of ASL and AIL – equations 9 and 10.

Carbohydrate content

About 50 ml each of the hydrolysis filtrate, including the test samples and SRS, was neutralized with calcium carbonate to pH 5-6. The neutralized sample was filtered through a 0.2-µm Millex syringe filter (Fisher Scientific) into a 1.5-ml high-performance liquid chromatography (HPLC) vial and capped. Samples were stored at -20°C prior to HPLC analysis. A series of calibration standards at concentrations 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 analysis to verify the 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, California) was used and held at 80 – 85°C during analysis. Mobile phase was HPLC grade water, and components were eluted isocratically at a flow rate of 0.25 ml/min for 60 min. Injection volume varied from 10 to 50 µl depending upon sample concentration. The detector was maintained at column temperature. Samples were analyzed for cellobiose, glucose, xylose, galactose, arabinose, and mannose. The chromatogram peaks for each sugar were recorded (Fig. A-1). A standard curve was generated for each sugar by plotting calibration standard concentrations against chromatogram peaks (Fig. A-2). The resulting equation was used to obtain the concentration of each sugar as determined by HPLC. The percent of each sugar on an extractives-free sample was calculated using equations 11 – 16.

3.3.8 Summative mass closure of switchgrass biomass

Seven biomass components were measured on the extractives-free samples; these include glucose, xylose, galactose, arabinose, mannose, lignin, and ash. The summative mass of switchgrass biomass was calculated by adding the measured components.

3.3.9 Statistical analysis

Data were analyzed by ANOVA as a CRD factorial using mixed model (SAS 9.3 Inc, Cary, NC). Mean separation was computed with least significant difference method to determine differences in biomass structural components as affected by *Bipolaris* isolates and differences in the interaction between isolate and inoculum concentration combinations. The significance level for statistical tests was P=0.05.

3.4 Results

In the present study, chemical composition of switchgrass following treatment with *Bipolaris* pathogen was examined. The four *Bipolaris* isolates used had previously been characterized as virulent (*B. oryzae* SK12), moderately virulent (*B. sorokiniana* AP34, WT76) and less virulent (*B. spicifera* LA18) (section 2.4). The impact of these pathogen categories, at low (10^1 spores/ml) and high (10^5) disease pressure, on switchgrass composition was investigated. Switchgrass structural components reported here were based on extractives-free biomass samples whereas; the total ash was based on original switchgrass sample. Compositions of *Bipolaris* infected biomass samples were similar (Figs. 3.1 - 3.3). For each sample, glucose was the predominant sugar, constituting an average of 39.5% dry mass of the extracted sample. On

average, other components in the switchgrass samples were 28.5% xylose, 2.3% galactose, 4.3% arabinose, 19.4% lignin and 3.1% ash content. The mannose content was below the limit of detection. The absence of cellobiose during HPLC analysis indicates a complete hydrolysis of biomass samples. The measured components account for about 97% of the dry matter in the extractives-free biomass. The remaining 3% probably accounts for microcomponents of the biomass not analyzed in this study.

Analysis of variance identified significant differences in structural ash content among treatments (Table 3.1); however, only the control treatment contributed to this difference (Fig. 3.1A). Switchgrass plants treated with *Bipolaris* isolates did not differ in their structural ash content and means ranged from 3.1 to 3.3%. The healthy control plants had the least structural ash content with a mean of 2.5%. Pathogen spore concentration was not significant and there was no pathogen × inoculum concentration interaction (Table 3.1). On the other hand, differences in the total ash content among isolates, isolate spore concentration, and isolate × inoculum concentration interaction were significant (Table 3.1). At the isolate level, switchgrass infected with *Bipolaris* isolates did not differ in their total ash content and means ranged from 9.1 to 9.4%. The uninfected control plant had the lowest total ash content with a mean of 7.1% (Fig. 3.1 B). The higher the pathogen concentration, the lower the total ash content of switchgrass infected with *Bipolaris* isolates with means 9.08 ± 0.07 at 10^1 spores/ml and 8.62 ± 0.07 at 10^5 spores/ml. Plants inoculated with different isolates responded differently in total ash at different pathogen concentration. The greatest difference in total ash was found in plants inoculated with *B. oryzae* SK12 with an increase from 8.9 to 9.8%; followed by plants

inoculated with *B. sorokiniana* AP34 increasing from 8.9 to 9.6% at spore concentration 10^5 and 10^1, respectively. Other isolates did not differ in total ash at different inoculum concentrations.

Based on ANOVA, there were significant differences in ASL among isolate, isolate spore concentration, and isolate × inoculum concentration interaction (Table 3.1). At the isolate level, switchgrass biomass infected with *B. oryzae* SK12 had the highest ASL content with a mean value of 5.4%, whereas, switchgrass inoculated with other isolates did not differ with means ranging from 5.0 to 5.3%. Uninfected control plants had the lowest ASL content with a mean of 4.6% (Fig 3.2A). The higher the pathogen spore concentration, the lower the ASL content of switchgrass infected with *Bipolaris* isolates with means of 5.16 ± 0.06 at 10^1 spores/ml and 4.96 ± 0.06 at 10^5 spores/ml. Only isolate *B. oryzae* SK12 contributed to the isolate × inoculum concentration interaction observed, plants inoculated with this isolate had a lower ASL content at 10^5 spore concentration (mean= 5.0%) and higher ASL content at 10^1 spore concentration (mean= 5.7%). Other isolates did not differ in ASL content at different inoculum concentrations. Differences in AIL content among infected and control plants were not significant but pathogen spore concentration, and the isolate × spore concentration interaction were significant (Table 3.1). In contrast to ASL, the higher the isolate spore concentration, the higher the AIL content of biomass infected with *Bipolaris* isolates with means 13.95 ± 0.1 at 10^1 spores/ml and 14.65 ± 0.1 at 10^5 spores/ml. Moreover, only *B. sorokiniana* isolates AP34 and WT76 contributed to the isolate × inoculum concentration interaction observed. There was an increase in AIL content of plants inoculated with *B. sorokiniana* as spore concentration increased from 10^1 to 10^5 with means of 13.8% and 14.5% for isolate AP34, and 13.3% and 14.8% for isolate WT76, respectively. Plants inoculated with other isolates did not differ in ASL content at different

inoculum concentrations. AIL content across treatments ranged from 14.0% to 14.7% (Fig. 3.2B). Total lignin content did not differ significantly among infected and control plants, and there was no isolate × spore concentration interaction. However, the effect of spore concentration was significant (Table 3.1). The higher the spore concentration, the higher the total lignin content of switchgrass samples with means of 19.11 ± 0.11 at 10^1 spores/ml and 19.61 ± 0.11 at 10^5 spores/ml. Lignin content across treatments ranged from 19.1 to 19.7% (Fig. 3.2C).

The two major fermentable sugars, glucose and xylose, were significantly lower for *Bipolaris* infected biomass compared to the control (Tables 3.1 and Fig. 3.3A-B). *Bipolaris*-treated plants had less glucose content, with means between 38.3 and 40.2%. *Bipolaris* infection reduced glucose content by an average of 5% when compared with uninfected control plants. The spore concentration was not significant and there was no pathogen × spore concentration interaction (Table 3.1). The xylose content of *Bipolaris* infected plants was about 1-3% less than that of uninfected control plants (Fig. 3.3B). The effect of spore concentration was significant; however, only *B. spicifera* isolate LA18 contributed to the observed difference; with higher xylose content at higher spore concentration. Nevertheless, the xylose content was still lower than that of the control. For other isolates, xylose content of infected switchgrass did not differ at low and high pathogen concentrations. There was no interaction between pathogen and spore concentration (Table 3.1).

Unlike xylose and glucose, galactose and arabinose contents of infected switchgrass were significantly higher than that of the uninfected switchgrass (Table 3.1). However, galactose and

arabinose content were about 1% greater than that of the control (Fig. 3.3C and D). For galactose, there was a significant difference among the isolates, and there was an interaction between pathogen and spore concentration, but the main effect of spore concentration was not significant. Only the effect of isolate was significant for arabinose content; spore concentration was not significant, and there was no significant interaction between isolate and spore concentration.

Total carbohydrate content (sum of all sugars) differences were significant among isolates (Table 3.1 and Fig 3.3E). Total carbohydrate of uninfected control plants (mean = 79.9%) was higher than those inoculated with *Bipolaris* isolates (means = 73.2 to 76.1%). *Bipolaris* infection reduced total carbohydrate content by 3.8 to 6.7% when compared with uninfected control plants. The spore concentration was not significant and there was no pathogen × spore concentration interaction.

3.5 Discussion

Estimation of switchgrass biomass chemical components enables evaluation of the impact of different treatments of switchgrass on ethanol or other biofuel yield. In addition, estimation of biomass composition is useful for economic analysis of the biomass-ethanol process (Sluiter et al., 2010). In the present study, the impact of *Bipolaris* infection on switchgrass structural components was evaluated. Extractive-free switchgrass samples were analyzed for carbohydrate, lignin and ash content. A comparison of the chemical composition in the present study with switchgrass composition previously reported showed that whole ‘Alamo’ plants

contained similar amounts of glucose, arabinose and ash, but contained more lignin and less xylose and galactose (Hu et al., 2010).

Of the total extractives-free biomass, the carbohydrate content was 73 to 76% for *Bipolaris* infected switchgrass and 80% for the uninfected control plants. Since all the switchgrass samples treated with *Bipolaris* isolates had reduced total carbohydrate content when compared to the control plants, it was concluded that *Bipolaris* isolates were responsible for the 4-7% reduction observed. On other grass crops such as ryegrass, the fungal pathogens *Drechslera* and *Rhynchosporium* reduced carbohydrate content by at least 15% (Lam, 1985). During infection, fungi redirect plant carbohydrate metabolism to favor the pathogen's nutrient availability (Berger et al., 2007), which may account for the reduction in biomass carbohydrate content. In addition, reduction in carbohydrate content of diseased plants can be attributed to disruption in photosynthesis caused by the development of chlorosis and necrosis of leaf tissues, thereby reducing the surface area for photosynthesis (Heydari and Balestra, 2011; Berger et al., 2007). A decrease in photosynthesis will automatically decrease carbohydrate produced during photosynthesis.

According to our data, it is presumed that *Bipolaris* infection will reduce ethanol yield by at least 4%. The basis of this assumption is that feedstock carbohydrate content is directly proportional to ethanol yield (Sluiter et al., 2010). This observation was based on single isolate infection; there is a likelihood of greater carbohydrate reduction with multiple pathogen infection. Disease severity does not significantly influence the impact of *Bipolaris* on switchgrass carbohydrate content. A similar effect was observed at both low (10^1 spores) and

high (10^5 spores) disease pressure. This indicates that at low inoculum density, most likely in the absence of visible symptoms, switchgrass pathogens can significantly reduce biomass carbohydrate content and thereby reduce ethanol yield.

On the other hand, lignin content was influenced by the inoculum concentration of *Bipolaris* pathogens. A significant increase in lignin content observed in switchgrass samples infected with 10^5 spores probably reflects an increased level of plant defense against the pathogen compared with switchgrass treated with 10^1 spores. In response to pathogen attack, plants increase lignin deposition to enhance cell defenses against fungal penetration and prevent the spread of fungal toxins and enzymes (Bhuiyan et al., 2009; Smith et al., 2007). Lignification is one of the first lines of defense against invasive pathogens (Bhuiyan et al., 2009), and it occurs both in compatible and incompatible fungal-plant interactions. Enhanced resistance of *Eucalyptus nitens* (forest tree) against *Mycophaerella* species was attributed to increased deposition of lignin at an early stage of infection and thus served to restrict pathogen spread. In a compatible reaction with a closely related tree species, *E. globules*, deposition of lignin occurred in a later stage of disease development and *E. globules* was reported to be more susceptible to *Mycophaerella* than *E. nitens* (Smith et al., 2007). At high disease pressure, fungal attack will increase recalcitrance of switchgrass and will make bioconversion of biomass to ethanol more challenging.

Feedstock with low ash content is desirable for bioprocessing of biomass-to-ethanol because ash interferes with acid hydrolysis (Sluiter et al., 2010). Increased ash content of *Bipolaris* infected switchgrass biomass may have been due partly to the presence of fungal materials. In

studies of the chemical composition of fungal mycelia, from related (*Penicillium* - Ascomycota) and non-related (*Ganoderma* - Basidiomycota) fungi, the mycelium contained 3.6% ash of the total fungal dry weight (Tashpulatov et al., 2000; Tseng et al., 1984). Therefore, the fungal mycelia ash content may have contributed to the final biomass ash content.

The impact of *Bipolaris* isolates on switchgrass structural composition was irrespective of their virulence identity; a similar effect was observed with more and less virulent isolates. Although *Bipolaris* had a limited impact on switchgrass chemical composition, in previous research (section 2.4 of this dissertation) a greater impact was observed with biomass yield. When *Bipolaris* was applied to switchgrass seed or applied to whole plants, significant reductions in stand establishment and plant height were observed, thereby reducing biomass yield (fresh weight) by up to 88%. In order to obtain an accurate view of the overall impact of switchgrass pathogens on the biomass-to-ethanol conversion process, it is important to consider the effects on the physical (biomass yield) and chemical properties of switchgrass. From these studies, it is clear that *Bipolaris* not only negatively affects biomass quantity but also impacts its quality by reducing biomass fitness for bioethanol conversion. As the U.S. approaches the biofuel mandate, 16 billion gallons of cellulosic biofuel per year, switchgrass monoculture will increase significantly, and a concomitant increase in disease threats to switchgrass is expected (Stewart and Cromey, 2011). Research towards development of proper management strategies and sustainable agricultural practices should be emphasized in order to alleviate losses from switchgrass pathogens. Failure will threaten the success of the emerging biofuel industry.

3.6 References

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Table 3.1 Analysis of variance of structural components of switchgrass infected with *Bipolaris* pathogens[a]

Type 3 Test of Fixed Effects[b]										
Effect	Total ash[c]	Structural ash	ASL[d]	AIL[e]	Total lignin	Glucose	Xylose	Galactose	Arabinose	Carbohydrate[f]
	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values	P/F- values
Isolate	<0.0001/	<0.0001/	<0.0001/	0.0596/	0.0719/	<0.0001/	0.0221/	<0.0001/	0.0038/	<0.0001/ 11.34
	86.95	10.93	10.32	2.71	2.54	29.90	3.63	21.75	5.49	
Spore	<0.0001/	0.1562/ 2.17	0.0330/	<0.0001/	0.0038/	0.1847/	0.0298/	0.7571/ 0.1	0.0575/	0.0972/ 3.03
concentration	23.15		5.25	23.14	10.71	1.89	5.47		4.06	
Isolate*spore	0.0120/ 4.25	0.2078/ 1.62	0.0492/	0.0486/	0.0681/	0.9290/	0.2498/	0.0089/	0.1036/	0.7304/ 0.51
concentration			2.88	2.89	2.59	0.21	1.47	4.56	2.22	

[a] Structural components of extractives-free samples of switchgrass

[b]DF value of analysis of variance was 20

[c]Total ash was based on original switchgrass sample

[d] ASL: Acid soluble lignin

[e] AIL: Acid insoluble lignin

[f] Total carbohydrate: summation of glucose, xylose, galactose and arabinose contents.

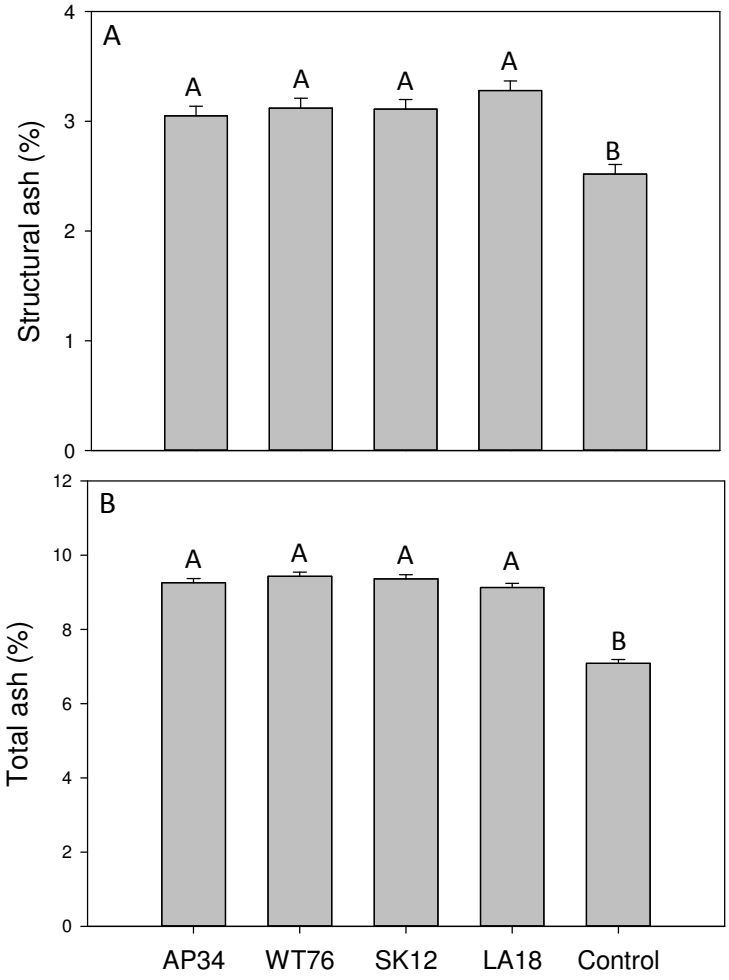

Fig. 3.1 Comparison of extractive-free ash content (A) and total ash of original switchgrass sample (B) between *Bipolaris* infected switchgrass and uninfected control. Each component is a percent dry weight in the extractive-free or original switchgrass sample. For each variable, bars with a common letter are not significantly different at P=0.05 (n= 3 replicates).

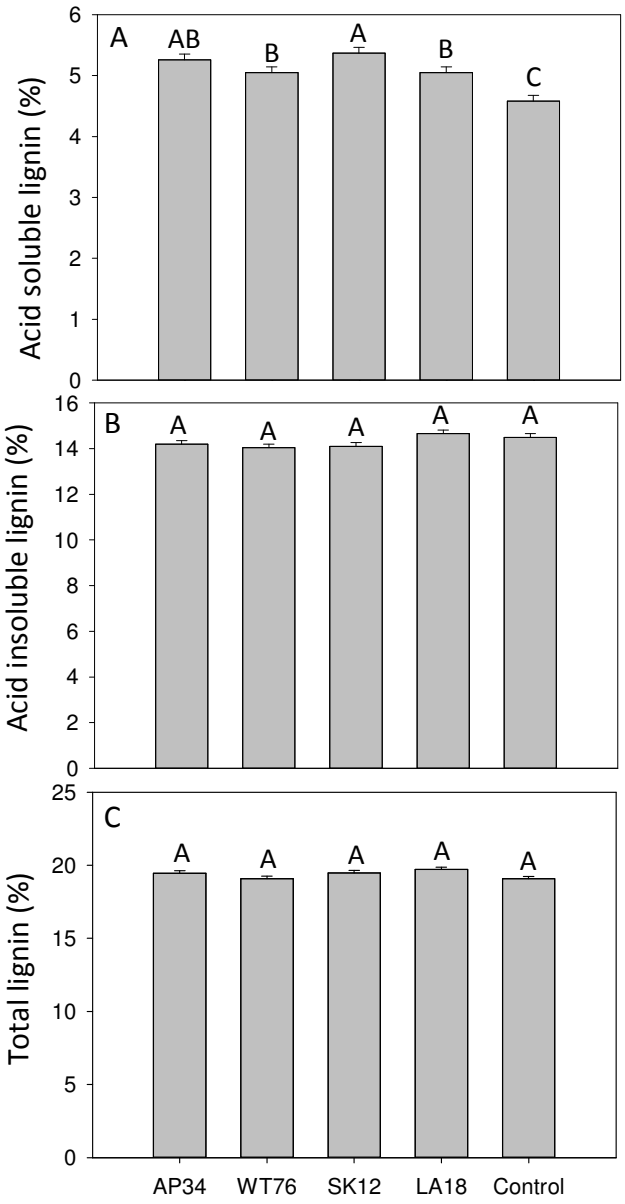

Fig. 3.2 Comparison of acid soluble lignin (A), acid insoluble lignin (B), and total lignin (C) between *Bipolaris* infected switchgrass and uninfected control. Total lignin was the sum of acid soluble lignin and acid insoluble lignin. Each component is a percent dry weight in the extractive-free switchgrass. For each lignin content, bars with a common letter are not significantly different at P=0.05 (n= 3 replicates).

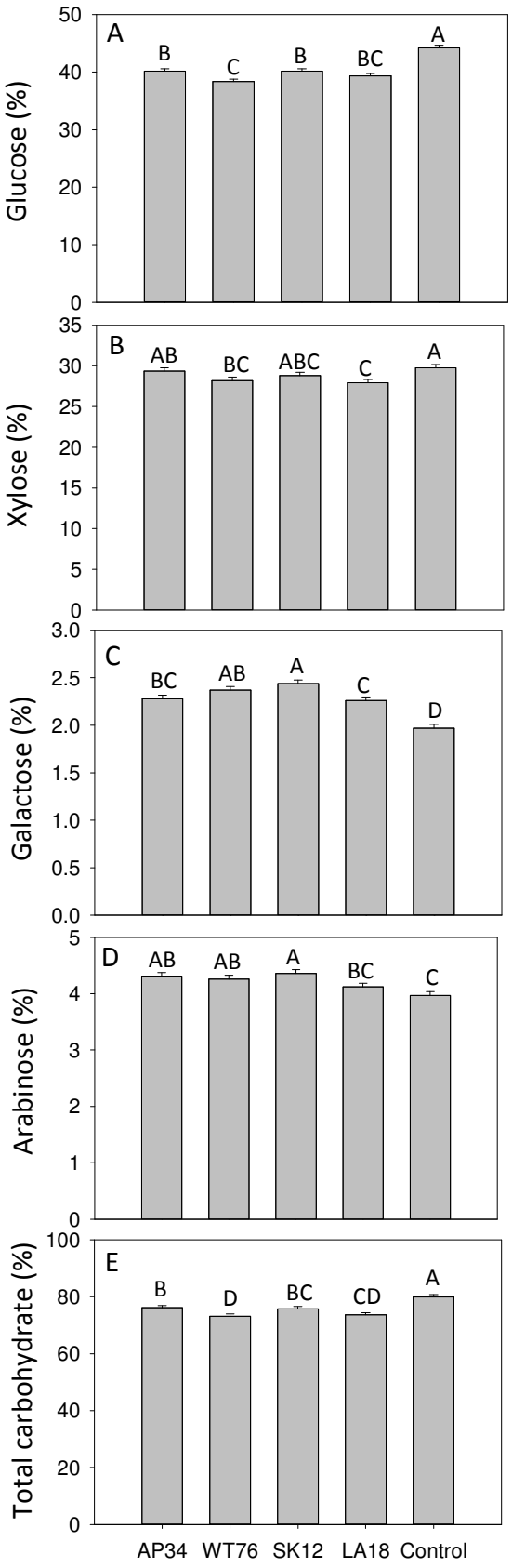
A
Glucose (%)
50
40
30
20
10
0
B
C
B
BC
A
B
Xylose (%)
35
30
25
20
15
10
5
0
AB
BC
ABC
C
A
C
Galactose (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
BC
AB
A
C
D
D
Arabinose (%)
5
4
3
2
1
0
AB
AB
A
BC
C
E
Total carbohydrate (%)
100
80
60
40
20
0
B
D
BC
CD
A
AP34
WT76
SK12
LA18
Control

Fig. 3.3 Comparison of individual sugar components and total carbohydrate between *Bipolaris* infected switchgrass and uninfected control. Each component is a percent dry weight in the extractive-free switchgrass. For each variable, bars with a common letter are not significantly different at P=0.05 (n= 3 replicates).

3.7 Appendix

$$\%totalsolids_{sample} = \frac{(weight\ of dry\ sample\ and\ crucible - weight\ of\ dry\ crucible)}{weight\ of\ sample} \times 100 \quad \text{Eqn. 1}$$

$$Moisture\ content_{sample} = 100 - \%total\ solids \quad \text{Eqn. 2}$$

$$Oven\ dry\ weight\ (ODW)_{sample} = \frac{weight\ of\ sample \times\ \%\ total\ solids}{100} \quad \text{Eqn. 3}$$

$$\%Ash\ content_{sample} = \frac{(weight\ of\ crucible\ plus\ ash - weight\ of\ crucible)}{ODW_{sample}} \times 100 \quad \text{Eqn. 4}$$

$$Extracted\ sample = (weight\ of\ pan + beads + sample) - (weight\ of\ pan + beads) \quad \text{Eqn. 5}$$

$$\%extractive = \frac{[(sample \times \%\ totalsolidsbeforeextraction) - (extractedsample \times \%totalsolids)]}{(sample \times \%total\ solids\ before\ extraction)} \times 100 \quad \text{Eqn. 6}$$

$$ODW = \frac{weight\ of extractive - free sample\ used\ for\ hydrolysis\ \times\ \%total\ solid\ of extractives - free\ sample}{100}$$

$$\%AIR = \frac{(weight\ of\ crucible\ plus\ solid\ residue\ at\ 105^0C - weight\ of\ crucible)}{ODW} \quad \text{Eqn. 7}$$

$$\%All = \frac{(weight\ of\ crucible\ plus\ residue - Weight\ of\ crucible) - (Weight\ of\ crucible\ plus\ ash - weight\ of\ crucible)}{ODW} \times 100$$

Eqn. 8

$$\%ASL = \frac{UV_{abs} \times Volume\ filtrate \times Dillution}{\varepsilon \times ODW} \times 100 \quad \text{Eqn. 9}$$

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

$$Dillution = \frac{Volume\ of sample + volume\ of\ diluting\ DI\ water}{Volume\ of\ Sample}$$

$$Total\ lignin\ content\ of\ an\ extractive - free\ sample = \%ASL + \%AIL \quad \text{Eqn.10}$$

$$\%CVS\ recovered\ from\ HPLC\ analysis\ = \frac{Concentration\ detected\ by\ HPLC,\ mg/ml}{Known\ concentration\ of\ standard,\ mg/ml} \times 100$$

Eqn. 11

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

$$\%Rsugar = \frac{Concentration\ of\ sugar\ detected\ by\ HPLC,\ mg/ml}{Known\ concentration\ of\ sugar\ before\ hydrolysis,\ mg/ml} \times 100 \quad \text{Eqn. 12}$$

%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 (Cx_1) is calculated thus:

$$C_{x1} = \frac{Concentration\ of\ a\ sugar\ as\ determined\ by\ HPLC,\ mg/ml}{(\%Rsugar/100)} \quad \text{Eqn. 13}$$

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

$$C_{x2} = \frac{C_{x1}}{(\%CVS\ recovery)} \quad \text{Eqn. 14}$$

To correct for hydration, each sugar concentration was multipled 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).

$$\mathrm{C_{anhydro}} = C_{x2} \times anhydro\ correction\ factor \quad \text{Eqn. 15}$$

$$\%sugar\ extractive - free = \frac{\mathrm{C_{anhydro}} \times \mathrm{Volume\ of\ filtrate}}{ODW} \times 100 \quad \text{Eqn. 16}$$

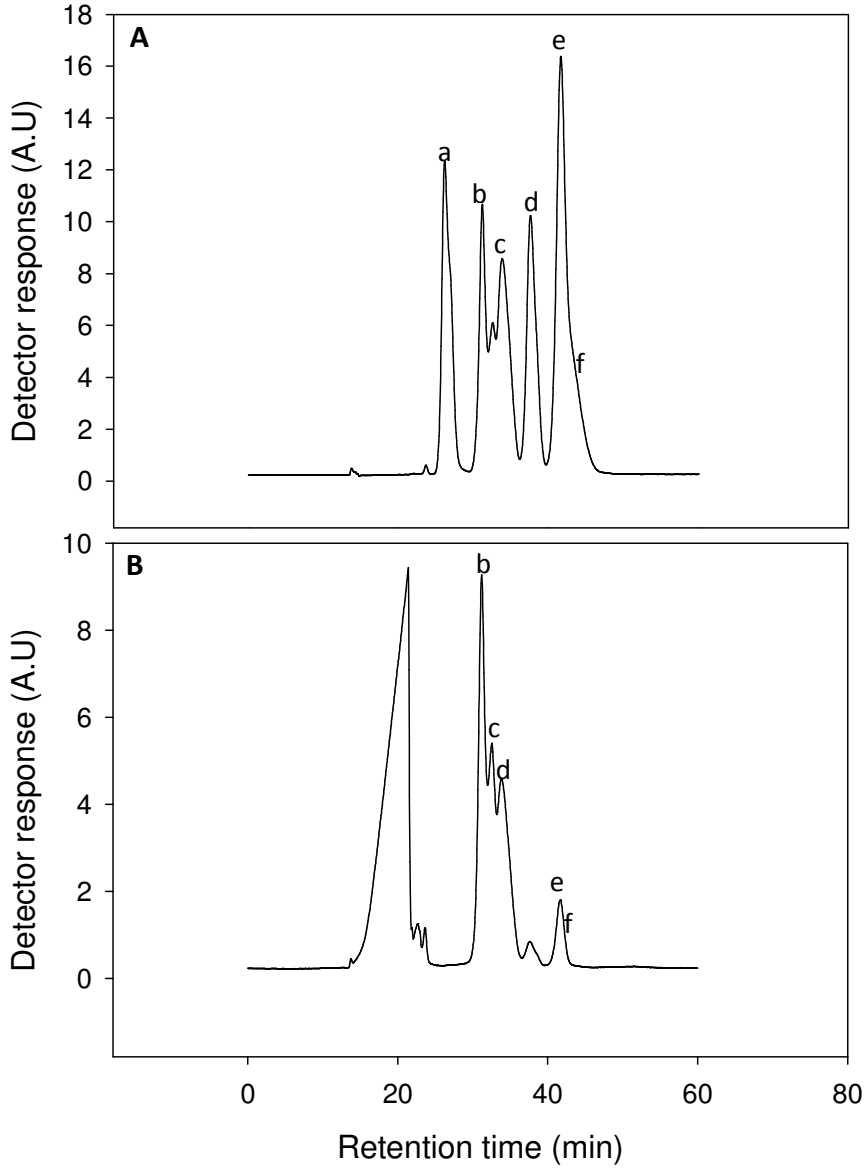

Fig. A-1 High-performance liquid chromatography (HPLC) chromatogram of sugars in calibration standard (A) and infected switchgrass sample following hydrolysis (B). Peaks correspond to cellobiose (a), glucose (b), xylose (c), galactose (d), arabinose (e), mannose (f).

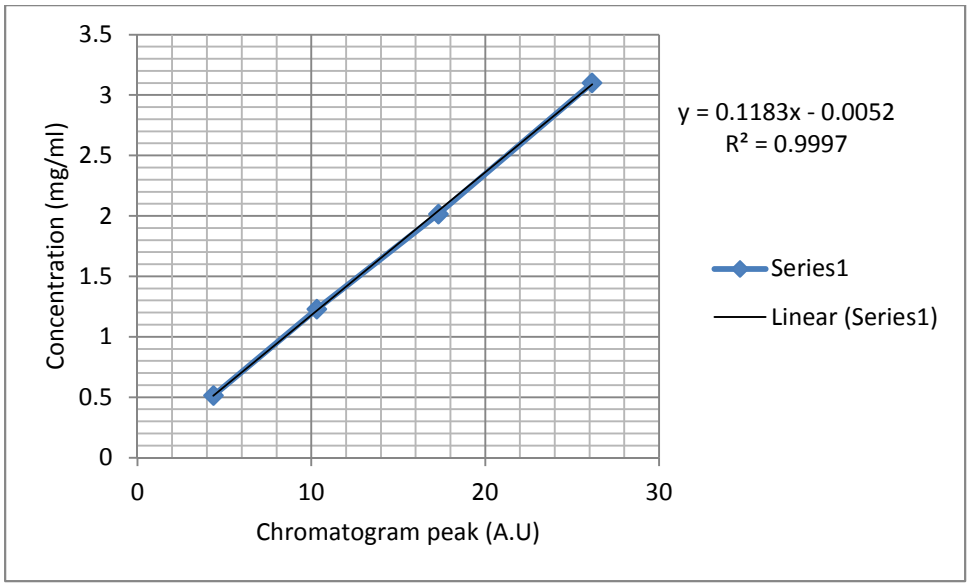

Fig. A-2 An example standard sugar curve. Glucose standard curve obtained by plotting calibration standard concentrations against chromatogram peaks.

CHAPTER 4: ASSESSMENT OF SWITCHGRASS GERMPLASM FOR DISEASE RESISTANCE AGAINST *BIPOLARIS*

4.1 Abstract

Bipolaris species are important pathogens of switchgrass causing spot blotch, leaf spot, seedling blight, and rot root diseases. These pathogens can cause significant reductions in biomass yield. In order to identify potential sources of resistance against these fungal pathogens, nine switchgrass germplasms, namely, 'Alamo' (Bamert), 'Blackwell', 'Cave-in-Rock', 'Dacotah', Germplasm A, Germplasm B, Germplasm C, 'Summer', and 'Trailblazer', were screened against *B. sorokiniana* isolate APCNR34, *B. spicifera* isolate LA18, *B*. *oryzae* isolate SK12 and *B. victoriae* isolate JA12. All germplasms were susceptible or highly susceptible to *B. sorokiniana* APCNR34 and *B. victoriae* JA12. Screening of additional switchgrass germplasms may identify a source of resistance against these isolates. Six germplasms, namely 'Alamo' (Bamert), Germplasm A, Germplasm B, Germplasm C, 'Summer', and 'Trailblazer', were moderately resistant to *B. spicifera* LA18 and *B. oryzae* SK12. These germplasms should be explored further as sources of disease resistance in plant breeding programs for development of cultivars with desirable biofuel crop traits.

4.2 Introduction

Bipolaris species, causing spot blotch, leaf spot, seedling blight, and rot root diseases, are important pathogens with a broad host range, including bioenergy crops, and are distributed worldwide (Table 1.1). These fungi cause economic yield losses. In the U.S., switchgrass is a

dedicated bioenergy crop and *Bipolaris* species have been isolated from this crop across several different states, mostly in regions where switchgrass is grown on a commercial scale for biofuel (Farr and Rossman, 2012; Ghimire et al., 2011; Krupinsky et al., 2004; Tomaso-Peterson and Balbalian, 2010; Vu et al., 2011a; b; Waxman and Bergstrom, 2011; Zeiders, 1984). It has been predicted that if switchgrass is grown to any great extent in the humid areas of the eastern and northeastern U.S., spot blotch will probably be the most important disease (Zeiders, 1984). Isolates of four *Bipolaris* species collected from naturally infected switchgrass seedlings and mature plants in Tennessee, and seeds produced across the U.S., reduced biomass yield by 5 to 88% (Vu et al., 2010; Fajolu et al., 2012). Moreover, *Bipolaris* plays a significant role in stand establishment, a major challenge in switchgrass production (Parrish and Fike, 2005). In our previous study, isolates of *Bipolaris* species reduced seed germination or plant stand by 38 to 74% and *B. oryzae* had the greatest impact (Section 2.4).

Bipolaris species are both seedborne and soilborne, making their control more challenging. Genetic resistance is considered the most practical means of reducing economic losses caused by *B. sorokiniana* in grasses (Zeiders, 1984). Therefore, identification of disease resistance in switchgrass germplasm is crucial for effective control of diseases caused by *Bipolaris* species, thereby maximizing switchgrass yield. More so, identified switchgrass germplasm can serve as a resistance donor in plant breeding programs for future development of switchgrass cultivars with biofuel-valuable traits. Hence, the present study was focused on assessing nine switchgrass germplasms for a potential source of resistance against *Bipolaris*.

4.3 Materials and methods

4.3.1 Switchgrass germplasms

Nine switchgrass germplasms produced in the U.S. were evaluated for disease resistance against *Bipolaris* isolates. Switchgrass germplasms included in this study were 'Alamo' (Bamert), 'Blackwell', 'Cave-in-Rock', 'Dacotah', Germplasm A, Germplasm B, Germplasm C, 'Summer', and 'Trailblazer'. Names of germplasms A, B, and C are not disclosed because these germplasms have not been released to the public by the company that produced them. The Bamert, Germplasms A, B, and C seeds were supplied by Jonathan Walton, University of Tennessee.

Switchgrass seeds were surface-sterilized in 5.25% sodium hypochlorite (commercial Clorox as purchased) according to the method of Haynes et al. 1997, with modification. To ensure uniformity, 15 cm^3 of seeds was added to 80 ml of Clorox and a drop of Tween 20. The mixture was stirred on a magnetic stirrer for 15 min. The seeds were then rinsed with distilled water and immersed in deionized (DI) water at 4^oC for 24 h on a shaker at 150 rpm. After pre-chilling, seeds were rinsed and incubated in DI water for 24 h at room temperature on a shaker at 150 rpm.

4.3.2 Fungal isolates

Switchgrass germplasms were screened against four *Bipolaris* isolates, including *B. sorokiniana* APCNR34, *B. oryzae* SK12, *B. victoriae* JA12 and *B. spicifera* LA18. These fungi were isolated from naturally infected switchgrass seeds, and had been characterized on 'Alamo' as virulent

(*B. oryzae* SK12), moderately virulent (*B. sorokiniana* APCNR34, *B. victoriae* JA12) and less virulent (*B. spicifera* LA18) (section 2.4).

4.3.3 Inoculum preparation

Inoculum of each isolate was prepared on organic pearled barley grains (Arrowhead Mills, Melville, New York) according to the method of Ahmed et al. (2009) with minor modification. Briefly, 100 g of barley grain was soaked in DI water for 24 h in a 500-ml conical flask, and then excess water was decanted from the grain. The mouth of the flask was plugged with non-absorbent cotton wrapped in cheesecloth, covered with aluminum foil and autoclaved for 30 min at 121°C. The sterilized grains were kept at room temperature for 24 h and autoclaved a second time at 121°C for 30 min. The second sterilization was done to eliminate any heat-resistant bacterial spores that might contaminate the grains. Sterilized grains were completely cooled under a biosafety cabinet (ESCO Technologies Inc., Hatboro, Pennsylvania) and clumped grains were aseptically separated using a sterile metal spatula. For each flask, sterilized and separated grains were inoculated with ten 7-mm plugs of mycelium cut from the edge of actively growing fungi on potato dextrose agar (PDA) using a sterile core-borer. Each isolate was inoculated into a separate flask. Inoculated grains were incubated at room temperature for 14 days to allow fungal growth and colonization of barley grains. The flasks were hand shaken every day to ensure uniform colonization of the grains. After 14 days, colonized barley grains were air-dried on an absorbent under-pad (Fisher Scientific, Dubuque, Iowa) under a biosafety cabinet (ESCO Technologies Inc.). Dried colonized barley grains were used immediately or stored in airtight zip lock bags at 4°C for future use.

4.3.4 Soil inoculation and seed planting

Inoculum was mixed with ProMix potting soil (Premier Horticulture Inc., Quakertown, Pennsylvania) at a ratio of 1:10 (100 g of inoculum and 1,000 g of soil). Inoculated soil was distributed in pots and overlaid with clean non-inoculated soil to allow the seeds to germinate on pathogen-free soil before contacting the pathogen. Thirty surface-sterilized seeds were sown in each pot and covered with non-inoculated soil. Each pot was covered with plastic film, held in place with a rubber band, to retain moisture and enhance seed germination. Plastic films were removed 5-days after planting. Pots were maintained in growth chambers at 25°C with a 12-h photoperiod for six weeks. The experimental design was a completely randomized factorial design with four replicate pots.

4.3.5 Assessment for disease resistance

Assessment of switchgrass germplasm for disease resistance against *Bipolaris* was based on 1) percent emerged seedlings 2) percent diseased plant area on emerged seedlings, 3) post-emergence seedling death, 4) plant height, and 5) biomass yield. The total number of emerged seedlings per pot was recorded on day 7 and 14 after planting. Data on diseased and dead seedlings were taken on days 21 and 28 after planting. Calculation of the percent of emerged seedlings was based on the number of seeds sown. The percent of post-emergence seedling death was based on the total number of emerged seedlings per pot. The percent diseased plant area per pot was visually estimated (Clive, 1971). Based on reaction to *Bipolaris*, switchgrass germplasms were graded on a 0 to 5 scale (Ahmed et al., 2009; Iftikhar et al., 2012), where 0 = no symptoms and 100% emergence or no post-emergence seedling death; 1 = 1 to 5% disease

symptoms on all plants in a pot and >90% emergence or <10% post-emergence seedling death; 2 = 6 to 25% disease symptoms on all plants in a pot and >80% emergence or <20% post-emergence seedling death; 3 = 26 to 50% disease symptoms on all plants in a pot and >50% emergence or <40% post-emergence seedling death; 4 = 51 to 75% disease symptoms on plants in a pot and <50% emergence or >40% post-emergence seedling death; 5 = 76 to 100% dead plants. The scale was categorized into 0 = resistant, 1-2 = moderately resistant, 3 = susceptible, and 4-5 = highly susceptible germplasm. Data for plant height and biomass yield were taken at 6 weeks after inoculation. The average plant height per pot was obtained by measuring plants from the crown to the top of leaf. Biomass yield was determined by weighing the mass (fresh weight) of all plants within each pot cut at the crown.

4.3.6 Statistical analysis

Data were analyzed by ANOVA as a CRD factorial using mixed model (SAS 9.3 Inc, Cary, NC). Mean separation was computed with least significant difference method to determine differences in germplasm disease resistance and differences in the interaction between isolate and germplasm combinations. The significance level for statistical tests was P=0.05.

4.4 Results

Nine switchgrass germplasms were tested against four *Bipolaris* isolates. Switchgrass response to *Bipolaris* differed depending on the germplasm-*Bipolaris* combination. Switchgrass resistance against *Bipolaris* was measured as a percentage of 1) emergence, 2) post-emergence seedling death, 3) diseased plant area, 4) plant height, and 5) biomass yield.

Percent seedling emergence

Based on analysis of variance, there were differences in seedling emergence for the main effects of isolate and switchgrass germplasm, and for the isolate × germplasm interaction (Table 4.1). Across all switchgrass germplasms tested, the highest seedling emergence was observed in 'Dacotah', Germplasm B, and 'Trailblazer', with mean values greater than 71%. 'Alamo' (Bamert) had the next highest seedling emergence, with a mean of 61.2%, and the least emergence was observed in 'Cave-in-Rock', with a mean value of 28.5% ($P<0.05$) (Table 4.2). Among the *Bipolaris* isolates, *B. sorokiniana* APCNR34 contributed most to these differences, and resulted in the least seedling emergence overall with a mean of 50.7%. The other three isolates did not differ in seedling emergence, with mean values ranging from 57.3 to 58.3% ($P<0.05$) (Table 4.3). Compared with the uninoculated control, percent seedling emergence was significantly lower for all treatments with *Bipolaris* (Table 4.3). Moreover, seedling emergence differed depending on the combination of *Bipolaris* isolate and switchgrass germplasm (Table 4.4). While inoculation with *B. spicifera* LA18 did not significantly affect the emergence of all germplasms tested when compared with the control, *B. sorokiniana* APCNR34 reduced seedling emergence by 13 to 31% in ' Alamo' (Bamert), 'Blackwell', Germplasm B, and Germplasm C, but did not significantly affect the emergence of other germplasms. Inoculation with *B. victoriae* JA12 only reduced seedling emergence in 'Blackwell' by 17% whereas *B. oryzae* SK12 caused emergence of 'Blackwell' and 'Dacotah', to be reduced by 16 and 22%, respectively. Neither isolate had a significant effect on the emergence of other germplasms tested when compared with the control ($P<0.05$) (Table 4.4).

Post-emergence seedling death

Differences in post-emergence seedling death among the isolates, germplasms, and isolate × germplasm interactions were highly significant (Table 4.1). Among the *Bipolaris* isolates in this study, *B. sorokiniana* APCNR34 caused the most seedling death with a mean of 48% across all germplasms (Table 4.3). 'Alamo' (Bamert), Germplasm A, and Germplasm B were the least susceptible to seedling blight, with *Bipolaris* infection causing only 5.2 to 8.5% post-emergence seedling death ($P<0.05$) (Table 4.2). The highest seedling death was observed in 'Summer', with a mean of 33.5%, indicating the most vulnerable germplasm (Table 4.2). Among all switchgrass germplasms tested, infection with *B. victoriae* JA12 or *B. spicifera* LA18 caused significant seedling death only in 'Summer' with means of 48.5 and 15.9%, respectively, whereas the other eight germplasms did not differ from the control (Table 4.4). Inoculation with *B. victoriae* SK12 caused seedling death only in 'Dacotah' with a mean of 13.4%. *Bipolaris sorokiniana* APCNR34 caused significant post-emergence seedling death in all switchgrass germplasms tested with means ranging from 22.6% to 88%, with the highest death rate observed in 'Summer' ($P<0.05$) (Table 4.4 and Fig. 4.1). Seedling death caused by *B. sorokiniana* APCNR34 was observed at an early stage of emergence, even before seedling death was noticed in plants infected with other *Bipolaris* isolates.

Percent diseased plant area

All tested germplasms responded differently to different isolates. Symptoms observed on emerged seedlings included leaf or stem lesions, chlorosis, and stunted growth. There were significant differences in percent diseased area among isolates, germplasms, and the isolate ×

germplasm interaction (Table 4.1). Across all germplasms, the lowest percent diseased leaf area was recorded for Germplasm B with a mean of 23.5%, followed by 'Alamo' (Bamert) and Germplasm A, with the same means of 27.8%. The highest percent diseased area was recorded in 'Summer' with a mean of 47%, indicating the most vulnerable germplasm to *Bipolaris* infection ($P<0.05$) (Table 4.2). Among all isolates, *B. spicifera* LA18 caused the least diseased plant area with an average of 24% (Table 4.3). Inoculation of switchgrass with *B. spicifera* LA18 resulted in 30% diseased plant area in 'Cave-in-Rock' and 'Dacotah' whereas other germplasms did not differ from the control ($P<0.05$) (Table 4.4). The absence of significant diseased plant area on the other seven germplasms indicates some level of resistance to isolate *B. spicifera* LA18. On the other hand, infection with *B. sorokiniana* APCNR34 on all germplasms tested resulted in significant diseased area ranging from 40 to 90%; the least diseased area was observed in Germplasm B and the highest in 'Summer' (Table 4.4). Likewise, *B. victoriae* JA12 caused significant diseased area in all germplasms tested with means ranging from 27.5 to 62.5%. Isolate *B. oryzae* SK12 caused significant percent diseased area in five germplasms, namely 'Alamo' (Bamert), 'Cave-in-Rock', 'Dacotah', 'Summer', and 'Trailblazer' with means ranging from 25 to 43.8%. Other germplasms inoculated with *B. oryzae* SK12 did not differ from the control ($P<0.05$) (Table 4.4).

The summary of reaction of switchgrass germplasms to *Bipolaris* infection is illustrated in Table 4.4. Out of the nine germplasms screened, six ('Alamo' (Bamert), Germplasm A, Germplasm B, Germplasm C, 'Summer', and 'Trailblazer') were graded as moderately resistant and three germplasms ('Blackwell', 'Cave-in-Rock', and 'Dacotah') were graded as susceptible to isolate *B. spicifera* LA18. In response to isolate *B. oryzae* SK12, germplasms 'Alamo' (Bamert), Germplasm

A, Germplasm B, and Germplasm C were graded as moderately resistant and the other five germplasms were susceptible. All germplasms evaluated were susceptible or highly susceptible to isolates *B. sorokiniana* APCNR34 and *B. victoriae* JA12.

Plant height

There were significant differences in plant height for isolates, germplasms, and the isolate × germplasm interaction (Table 4.1). Across all switchgrass germplasms tested, 'Trailblazer' had the tallest plants with a mean value of 8.5 cm. Germplasm A, Germplasm C, and 'Summer' had the most stunted growth, with mean values 4.0 to 4.5 cm (Table 4.2). Among the *Bipolaris* isolates, *B. sorokiniana* APCNR34 resulted in the greatest reduction in plant height and *B. spicifera* LA18 caused the least with means of 3.7 and 7.0 cm, respectively; control plants were 7.8 cm (Table 4.3). The impact of *Bipolaris* on plant height differed depending on the germplasm (Table 4.5). *Bipolaris spicifera* isolate LA18 reduced plant height only in 'Cave-in-Rock' whereas height of other germplasm inoculated with this isolate did not differ with those of the uninoculated control. On the other hand, *B. sorokiniana* APCNR34 impacted the height of all germplasm causing 1.9 to 6.6 cm reduction in plant height when compared with control germplasms. *Bipolaris victoriae* JA12 and *B. oryzae* SK12 did not impact the height of Germplasm C and Germplasm A, respectively. Both isolates significantly reduce height of other germplasm. Compared with the uninoculated control, isolate JA12 reduced plant height by 1.5 to 5.0 cm and SK12 reduced plant height by 1.8 to 4.8 cm (Table 4.5).

Biomass yield

Based on ANOVA, differences in biomass (fresh weight) yield among isolates, germplasms, and isolate × germplasm interaction were highly significant (Table 4.1). Among the nine germplasms screened, 'Alamo' (Bamert) had the highest biomass yield and 'Summer' the least with means of 0.87 g/pot and 0.17 g/pot, respectively (Table 4.2). Infection of germplasms with *B. spicifera* LA18 did not impact biomass yield. *Bipolaris sorokiniana* APCNR34 caused the highest biomass reduction with a mean of 0.13 g/pot and reduced biomass by 81.9% when compared to the control. The impact of *B. victoriae* JA12 and *B. oryzae* SK12 on germplasm biomass did not differ with means of 0.37 g/pot and 0.34 g/pot, and reduced plant biomass by 48.6% and 52.8%, respectively (Table 4.3). Across all germplasm × isolate combinations, isolates JA12 and SK12 did not significantly impact biomass yield of Germplasm B (mean = 0.80 g/pot) and Germplasm A (mean = 0.31 g/pot), respectively, and both isolates had no effect on Germplasm C and 'Summer' biomass yield when compared to the control with a mean range of 0.15 to 0.43 g/pot. On other germplasms, infection with JA12 caused 0.19 to 0.69 g (49.3 to 76%) biomass reduction per pot compared to the uninfected control. Infection of germplasms with isolate SK12 reduced biomass yield by 0.24 to 0.8 g/pot (32 to 71.4%). *Bipolaris sorokiniana* APCNR34 negatively impacted the biomass yield of all germplasms tested and caused 0.21 to 1.21 g/pot (64 to 90.1%) biomass reduction. The greatest impact was observed on 'Blackwell' and the least on Germplasm B (Fig 4.3).

4.5 Discussion

Nine switchgrass germplasms were screened for disease resistance against four *Bipolaris* isolates previously characterized as virulent (*B. sorokiniana* SK12), moderately virulent (*B. sorokiniana* APCNR34 and *B. victoriae* JA12), and less virulent (*B. spicifera* LA18) on 'Alamo'. None of the germplasms were resistant to all *Bipolaris* isolates. However, a low level of resistance was observed (scale = 2) to isolates *B. spicifera* LA18 and *B. oryzae* SK12. Out of the six germplasms with a moderately resistant disease reaction to *B. spicifera* LA18, four had the same grading when infected with *B. oryzae* SK12. To these moderately resistant germplasms, isolate LA18 had no effect on the plant height and biomass whereas, isolate SK12 had limited negative impact compared with the effects on other germplasms. The previously characterized highly virulent isolate, SK12, was considerably less virulent on most of the germplasms tested. One possible explanation is that the four germplasms with moderately resistant reaction possess gene(s) that confer some resistance to virulent *B. oryzae* SK12 as well as *B. spicifera* LA18. These germplasms should be further explored for sources of resistance against the *B. oryzae* and *B. spicifera* isolates. On the other hand, all germplasms tested had susceptible or highly susceptible infection responses to isolates *B. sorokiniana* APCNR34 and *B. victoriae* JA12. These isolates caused greater reduction in plant height and biomass yield compared to isolates LA18 and SK12. More switchgrass germplasms should be screened to identify resistance against *B. sorokiniana* and *B. victoriae*.

The differential response of switchgrass germplasms to *Bipolaris* species observed in this study indicated that resistance to *Bipolaris* infection is conferred by different genes or different

genotypes contained within each synthetic germplasm. Other hosts for *Bipolaris* species also exhibit differential responses to infection with different isolates. In a screen of 56 wheat varieties against spot blotch, only nine varieties with moderate resistance were recommended for breeding programs (Asad et al., 2007; Iftikhar et al., 2012). In another study where 15 wheat germplasms were screened against seedling diseases, only four germplasms were selected as tolerant (Ahmed et al., 2009). Likewise, barley genotypes exhibited differential responses to *B. sorokiniana* at seedling and mature stages of plant growth (Ghazvini, 2012; Ghazvini and Tekauz, 2007). Different resistance genes may be effective at different barley growth stages to *B. sorokiniana* (Ghazvini, 2012).

Screening switchgrass germplasms to identify potential sources of resistance against fungal pathogens is useful for breeding programs in developing varieties with biofuel-valuable traits. The germplasms Germplasm A, Germplasm B, Germplasm C, and 'Alamo' (Bamert) should be explored as potential resistance donors in plant breeding programs. Since switchgrass is grown continuously on a commercial scale, integrated disease management strategies where genetic resistance is the main component should be emphasized.

4.6 References

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Table 4.1 Analysis of variance of switchgrass germplasm disease resistance against *Bipolaris* isolates

Type 3 Test of Fixed Effects[a]					
Effect	Seedling emergence P/F- values	Post-emergence seedling death P/F- values	Diseased plant area P/F- values	Plant height P/F- values	Biomass yield P/F- values
Isolate	<0.0001/ 9.20	<0.0001/ 154.35	<0.0001/ 181.06	<0.0001/ 80.89	<0.0001/ 198.15
Germplasm	<0.0001/65.44	<0.0001/ 15.74	<0.0001/ 27.77	<0.0001/ 30.09	<0.0001/ 94.65
Isolate* Germplasm	0.0496/ 1.53	<0.0001/ 3.62	<0.0001/ 5.94	<0.0001/ 2.59	<0.0001/ 12.15

[a]DF value of analysis of variance of switchgrass germplasm disease resistance against *Bipolaris* isolates was 135.

Table 4.2 Mean disease response of nine switchgrass germplasms to *Bipolaris* infection[a]

Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Plant height (cm)	Biomass yield (g/pot)
Germplasm A	45.2 ± 2.00 D	6.3 ± 2.10 DE	27.8 ± 1.47 D	4.0 ± 0.25 E	0.22 ± 0.024 EF
Germplasm B	71.3 ± 2.00 A	8.5 ± 2.10 CDE	23.5 ± 1.47 E	5.4 ± 0.25 D	0.62 ± 0.024 B
Alamo (Bamert)	61.2 ± 2.00 B	5.2 ± 2.10 E	27.8 ± 1.47 D	6.1 ± 0.25 BC	0.87 ± 0.024 A
Blackwell	54.7 ± 2.00 C	12.8 ± 2.10 BC	30.3 ± 1.47 CD	5.7 ± 0.25 CD	0.53 ± 0.024 C
Germplasm C	44.5 ± 2.00 D	11.2 ± 2.10 BCD	28.0 ± 1.47 D	4.4 ± 0.25 E	0.29 ± 0.024 D
Cave-in-Rock	28.5 ± 2.00 E	13.2 ± 2.10 BC	33.8 ± 1.47 C	5.8 ± 0.25 CD	0.27 ± 0.024 DE
Dacotah	76.3 ± 2.00 A	12.8 ± 2.10 BC	40.5 ± 1.47 B	6.6 ± 0.25 B	0.48 ± 0.024 C
Summer	59.8 ± 2.00 BC	33.5 ± 2.10 A	47.0 ± 1.47 A	4.5 ± 0.25 E	0.17 ± 0.024 F
Trailblazer	76.3 ± 2.00 A	16.0 ± 2.10 B	40.2 ± 1.47 B	8.5 ± 0.25 A	0.64 ± 0.024 B

[a] Data reported are mean values of four isolates and four replicates ± standard error. For each measured variable, values with a common letter are not different according to an F-protected LSD at P=0.05.

Table 4.3 Means of *Bipolaris* pathogens on disease response of switchgrass germplasms[a]

Isolate	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Plant height (cm)	Biomass yield (g/pot)
Control	63.4 ± 1.49 A	1.6 ± 1.57 C	16.7 ± 1.10 E	7.8 ± 0.19 A	0.72 ± 0.19 A
B. sorokiniana APCNR34	50.7 ± 1.49 C	47.6 ± 1.57 A	54.2 ± 1.10 A	3.7 ± 0.19 D	0.13 ± 0.19 C
B. victoriae JA12	57.3 ± 1.49 B	10.0 ± 1.57 B	41.3 ± 1.10 B	5.0 ± 0.19 C	0.37 ± 0.19 B
B. spicifera LA18	58.3 ± 1.49 B	2.2 ± 1.57 C	24.0 ± 1.10 D	7.0 ± 0.19 B	0.69 ± 0.19 A
B. oryzae SK12	57.9 ± 1.49 B	5.2 ± 1.57 C	29.9 ± 1.10 C	4.9 ± 0.19 C	0.34 ± 0.19 B

[a] Data reported are mean values of four replicates ± standard error. For each measured variable, values with the same letters are not different according to an F-protected LSD at P=0.05.

Table 4.4 Means of the interaction of switchgrass germplasms infected with different isolates of *Bipolaris* species[a]

Isolate	Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Scale[b]	Grade[c]
B. spicifera LA18	Germplasm A	41.7 ± 4.5 P-R	0 ± 4.7 K	22.5 ± 3.3 N-S	2	MR
	Germplasm B	68.3 ± 4.5 C-I	0 ± 4.7 K	18.8 ± 3.3 P-T	2	MR
	Alamo (Bamert)	59.2 ± 4.5 H-N	0 ± 4.7 K	20.0 ± 3.3 O-T	2	MR
	Blackwell	62.5 ± 4.5 F-L	1.0 ± 4.7 K	26.3 ± 3.3 L-Q	3	S
	Germplasm C	43.3 ± 4.5 P-R	0 ± 4.7 K	22.5 ± 3.3 N-S	2	MR
	Cave-in-Rock	31.7 ± 4.5 R-T	0 ± 4.7 K	30.0 ± 3.3 J-N	3	S
	Dacotah	84.2 ± 4.5 A	2.7 ± 4.7 JK	30.0 ± 3.3 J-N	3	S
	Summer	56.7 ± 4.5 I-N	15.9 ± 4.7 HI	25.0 ± 3.3 M-R	2	MR
	Trailblazer	77.5 ± 4.5 A-D	0 ± 4.7 K	21.0 ± 3.3 N-S	2	MR

Table 4.4 Continued

Isolate	Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Scale[b]	Grade[c]
B. oryzae SK12	Germplasm A	49.2 ± 4.5 N-Q	0 ± 4.7 K	17.5 ± 3.3 Q-T	2	MR
	Germplasm B	77.5 ± 4.5 A-D	1.1 ± 4.7 JK	20.0 ± 3.3 O-T	2	MR
	Alamo (Bamert)	65.9 ± 4.5 C-J	0 ± 4.7 K	25.0 ± 3.3 M-R	2	MR
	Blackwell	48.4 ± 4.5 N-Q	0 ± 4.7 K	28.8 ± 3.3 J-O	3	S
	Germplasm C	50.9 ± 4.5 L-Q	1.5 ± 4.7 JK	21.3 ± 3.3 N-S	2	MR
	Cave-in-Rock	29.2 ± 4.5 ST	8.1± 4.7 I-K	32.5 ± 3.3 I-M	3	S
	Dacotah	65.0 ± 4.5 D-K	13.4 ± 4.7 H-J	43.8 ± 3.3 E-H	3	S
	Summer	58.4 ± 4.5 H-N	13.9 ± 4.7 H-J	36.3 ± 3.3 G-K	3	S
	Trailblazer	76.7 ± 4.5 A-D	8.7 ± 4.7 I-K	43.8 ± 3.3 E-H	3	S

Table 4.4 Continued

Isolate	Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Scale[b]	Grade[c]
B. victoriae JA12	Germplasm A	40.8 ± 4.5 Q-S	1.9 ± 4.7 JK	37.5 ± 3.3 F-J	3	S
	Germplasm B	74.2 ± 4.5 A-F	3.5 ± 4.7 I-K	27.5 ± 3.3 K-P	3	S
	Alamo (Bamert)	63.3 ± 4.5 E-L	3.7± 4.7 I-K	35.0 ± 3.3 H-L	3	S
	Blackwell	53.4 ± 4.5 K-P	8.1 ± 4.7 I-K	32.5 ± 3.3 I-M	3	S
	Germplasm C	43.4 ± 4.5 O-R	8.4 ± 4.7 I-K	35.0 ± 3.3 H-L	3	S
	Cave-in-Rock	24.2 ± 4.5 T	2.5 ± 4.7 JK	32.5 ± 3.3 I-M	3	S
	Dacotah	73.4 ± 4.5 A-G	3.6 ± 4.7 I-K	62.5 ± 3.3 BC	3	S
	Summer	66.7 ± 4.5 C-J	48.5 ± 4.7 C-E	58.8 ± 3.3 CD	4	HS
	Trailblazer	76.7 ± 4.5 A-D	9.6 ± 4.7 H-K	50.0 ± 3.3 DE	3	S

Table 4.4 Continued

Isolate	Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Scale[b]	Grade[c]
B. sorokiniana APCNR34	Germplasm A	42.5 ± 4.5 P-R	29.8 ± 4.7 FG	45.0 ± 3.3 E-G	3	S
	Germplasm B	61.7 ± 4.5 F-M	35.5 ± 4.7 E-G	40.0 ± 3.3 F-I	3	S
	Alamo (Bamert)	50.0 ± 4.5 M-Q	22.6 ± 4.7 GH	43.8 ± 3.3 E-H	3	S
	Blackwell	39.2 ± 4.5 Q-S	51.7 ± 4.7 B-D	43.8 ± 3.3 E-H	4	HS
	Germplasm C	31.7 ± 4.5 R-T	39.4 ± 4.7 D-F	46.3 ± 3.3 EF	3	S
	Cave-in-Rock	23.4 ± 4.5 T	55.5 ± 4.7 BC	57.5 ± 3.3 CD	4	HS
	Dacotah	78.3 ± 4.5 A-C	44.3 ± 4.7 C-E	50.0 ± 3.3 DE	3	S
	Summer	55.8 ± 4.5 J-O	88.0 ± 4.7 A	90.0 ± 3.3 A	5	HS
	Trailblazer	74.2 ± 4.5 A-G	61.8 ± 4.7 B	71.3 ± 3.3 B	4	HS

Table 4.4 Continued

Isolate	Switchgrass germplasm	Seedling emergence (%)	Post-emergence seedling death (%)	Diseased plant area (%)	Scale[b]	Grade[c]
Control	Germplasm A	51.7 ± 4.5 L-Q	0 ± 4.7 K	16.3 ±3.3 R-T	2	MS
	Germplasm B	75.0 ± 4.5 A-E	2.3 ± 4.7 JK	11.3 ± 3.3 T	2	MS
	Alamo (Bamert)	67.5 ± 4.5 C-J	0 ± 4.7 K	15.0 ± 3.3 ST	2	MS
	Blackwell	70.0 ± 4.5 B-H	3.5 ± 4.7 I-K	20.0 ± 3.3 O-T	2	MS
	Germplasm C	53.4 ± 4.5 K-P	6.7 ± 4.7 I-K	15.0 ± 3.3 ST	2	MS
	Cave-in-Rock	34.2 ± 4.5 R-T	0 ± 4.7 K	16.3 ± 3.3 R-T	2	MS
	Dacotah	80.8 ± 4.5 AB	0.3 ± 4.7 K	16.3 ± 3.3 R-T	2	MS
	Summer	61.7 ± 4.5 G-M	1.3 ± 4.7 JK	25.0 ± 3.3 M-R	2	MS
	Trailblazer	76.7 ± 4.5 A-D	0 ± 4.7 K	15.0 ± 3.3 ST	2	MS

[a] Data reported are mean values of four 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) = 1-25% diseased area and >80% emergence or <20% post-emergence seedling death; Scale 3: Susceptible (S) = 26-50% diseased area and >50% emergence or <40% post-emergence seedling death; Scale 4: Highly Susceptible (HS) = 51-75% diseased area and <50% emergence or >40% post-emergence seedling death; Scale 5: Highly Susceptible (HS) = 76-100% dead plants.

Table 4.5 Means of plant height and biomass yield of switchgrass germplasms infected with different isolates of *Bipolaris* species[a]

Isolate	Switchgrass germplasm	Plant height (cm)	Fresh weight biomass yield (g/pot)
B. spicifera LA18	Germplasm A	5.08 ± 0.56 K-R	0.31 ± 0.05 K-N
	Germplasm B	6.50 ± 0.56 F-K	0.75 ± 0.05 EF
	Alamo (Bamert)	8.75 ± 0.56 B-D	1.51 ± 0.05 A
	Blackwell	7.33 ± 0.56 D-H	0.90 ± 0.05 C-E
	Germplasm C	5.15 ± 0.56 K-Q	0.37 ± 0.05 I-L
	Cave-in-Rock	5.83 ± 0.56 H-N	0.49 ± 0.05 H-J
	Dacotah	7.93 ± 0.56 C-F	0.68 ± 0.05 FG
	Summer	5.18 ± 0.56 K-Q	0.25 ± 0.05 L-Q
	Trailblazer	11. 68 ± 0.56 A	1.00 ± 0.05 BC
B. oryzae SK12	Germplasm A	3.95 ± 0.56 Q-V	0.31 ± 0.05 K-M
	Germplasm B	5.00 ± 0.56 K-R	0.51 ± 0.05 HI
	Alamo (Bamert)	4.33 ± 0.56 N-U	0.58 ± 0.05 GH
	Blackwell	5.03 ± 0.56 K-R	0.35 ± 0.05 J-L
	Germplasm C	3.75 ± 0.56 Q-W	0.25 ± 0.05 L-Q
	Cave-in-Rock	5.00 ± 0.56 K-R	0.18 ± 0.05 M-R
	Dacotah	4.88 ± 0.56 L-S	0.28 ± 0.05 L-O
	Summer	4.63 ± 0.56 M-T	0.15 ± 0.05 O-R
	Trailblazer	7.10 ± 0.56 E-I	0.48 ± 0.05 H-J

Table 4.5 Continued

Isolate	Switchgrass germplasm	Plant height (cm)	Fresh weight biomass yield (g/pot)
B. victoriae JA12	Germplasm A	3.23 ± 0.56 T-W	0.11 ± 0.05 QR
	Germplasm B	5.15 ± 0.56 K-Q	0.80 ± 0.05 D-F
	Alamo (Bamert)	5.18 ± 0.56 K-Q	0.70 ± 0.05 FG
	Blackwell	5.35 ± 0.56 J-P	0.38 ± 0.05 I-L
	Germplasm C	5.00 ± 0.56 K-R	0.43 ± 0.05 H-K
	Cave-in-Rock	5.35 ± 0.56 J-P	0.12 ± 0.05 P-R
	Dacotah	5.38 ± 0.56 J-O	0.29 ± 0.05 K-O
	Summer	3.75 ± 0.56 Q-W	0.16 ± 0.05 N-R
	Trailblazer	6.43 ± 0.56 F-L	0.39 ± 0.05 I-L
B. sorokiniana APCNR34	Germplasm A	2.83 ± 0.56 U-W	0.08 ± 0.05 R
	Germplasm B	3.53 ± 0.56 R-W	0.27 ± 0.05 L-O
	Alamo (Bamert)	3.80 ± 0.56 P-W	0.17 ± 0.05 M-R
	Blackwell	3.38 ± 0.56 S-W	0.09 ± 0.05 R
	Germplasm C	2.38 ± 0.56 W	0.08 ± 0.05 R
	Cave-in-Rock	3.18 ± 0.56 T-W	0.06 ± 0.05 R
	Dacotah	5.53 ± 0.56 J-N	0.18 ± 0.05 M-R
	Summer	2.63 ± 0.56 VW	0.04 ± 0.05 R
	Trailblazer	6.00 ± 0.56 G-M	0.26 ± 0.05 L-P

Table 4.5 Continued

Isolate	Switchgrass germplasm	Plant height (cm)	Fresh weight biomass yield (g/pot)
Control	Germplasm A	4.70 ± 0.56 M-T	0.30 ± 0.05 K-O
	Germplasm B	6.83 ± 0.56 F-J	0.75 ± 0.05 F
	Alamo (Bamert)	8.53 ± 0.56 B-E	1.38 ± 0.05 A
	Blackwell	7.40 ± 0.56 D-G	0.91 ± 0.05 CD
	Germplasm C	5.63 ± 0.56 I-N	0.35 ± 0.05 J-L
	Cave-in-Rock	9.75 ± 0.56 B	0.50 ± 0.05 HI
	Dacotah	9.38 ± 0.56 BC	0.98 ± 0.05 BC
	Summer	6.38 ± 0.56 F-L	0.25 ± 0.05 L-Q
	Trailblazer	11.38 ± 0.56 A	1.08 ± 0.05 B

[a] Data reported are mean values of four replicates ± standard error. For each variable, values with the same letters are not different according to an F-protected LSD at P=0.05.



Fig. 4.1 Seedling death of cultivar ‘Summer’ caused by *Bipolaris sorokiniana* at 21 days after planting into infested soil. Infected plants (Left) and Control plants (Right).

CHAPTER 5: GENETIC CHARACTERIZATION OF *BIPOLARIS* SPECIES

5.1 Abstract

Simple sequence repeats (SSR) markers were developed from a small insert genomic library for *Bipolaris sorokiniana*, a mitosporic fungal pathogen that causes spot blotch and root rot in switchgrass. About 59% of sequenced clones (n=384) harbored various SSR motifs. After eliminating redundant sequences, 196 SSR loci were identified, of which 84.7% were dinucleotide repeats, and 9.7% and 5.6% were tri- and tetra-nucleotide repeats, respectively. Primer pairs were designed for 105 loci, and 85 successfully amplified loci. Sixteen polymorphic loci were characterized using fifteen *B. sorokiniana* isolates obtained from infected switchgrass plant materials collected from five states in the United States. These loci successfully cross-amplified at least one isolates of related species including *Bipolaris oryzae*, *Bipolaris spicifera* and *Bipolaris victoriae* that cause leaf spot on switchgrass. Haploid gene diversity per locus across all isolates studied varied from 0.633 to 0.861. Principal component analysis of SSR data clustered isolates according to their respective species. These SSR markers will be a valuable tool for genetic variability and population studies of *B. sorokiniana* and related species that are pathogenic on switchgrass and other host plants. Additionally, these markers are potential diagnostic tools for species in the genus *Bipolaris*.

5.2 Introduction

Globally, *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) is an important pathogen on a wide range of grass and cereal crops (Kumar et al., 2002) and is one of the principal pathogen on switchgrass (*Panicum virgatum* L.),causing spot blotch and root rot (Zeiders, 1984; Vu et al.,

2011a). Switchgrass is a dedicated energy crop in the U.S. (Bouton, 2008), and as the biofuel industry expands, there will be a corresponding increase in the acreage of switchgrass grown and disease risk from plant pathogens (Stewart and Cromey, 2011). This is evidenced by the recent increase in the number of reports on the incidence of *Bipolaris* species and other switchgrass pathogens from different locations in the U.S. (Farr and Rossman, 2011; Vu et al., 2011a, b; Waxman and Bergstrom, 2011). The high humidity and warm temperatures that are typical of the southeastern U.S. favor development and severity of disease caused by *B. sorokiniana* in other monocot crops (Ghazvini and Tekauz, 2007). In barley, this pathogen is capable of reducing yield by up to 33% (Valjavec-Gratian and Steffenson, 1997). *Bipolaris sorokiniana* is difficult to control chemically because it is both seedborne and soilborne; host genetic resistance is considered the most practical means of reducing economic losses caused by *B. sorokiniana* in grasses (Zeiders, 1984).

Understanding genetic diversity of *Bipolaris* species will be useful for developing and breeding durable resistant switchgrass cultivars. Previous variability studies of *B. sorokiniana* were based mainly on morphology, physiology and virulence characteristics (Valjavec-Gratian and Steffenson, 1997; Ghazvini and Tekauz, 2007; Poloni et al., 2009). Information on genetic variation within *B. sorokiniana* is limited but random amplified polymorphic DNA analysis revealed that *B. sorokiniana* isolates from wheat were genetically similar (Muller et al., 2005). Both high (Leisova-Svobodova et al., 2011) and low (Zhong and Steffenson, 2001) genetic variability have been reported in studies with amplified fragment length polymorphism analyses of *C. sativus* isolates from barley.

Simple sequence repeats (SSRs), also known as microsatellites, are repetitive two to six base pairs sequences of DNA that are abundant and highly polymorphic in many eukaryotic genomes (Karaoglu et al., 2005). In contrast to dominant markers, SSRs are codominant markers that provide more information on allelic variation per locus and distinguish between homozygote and heterozygote genotypes (Maguire et al., 2002). Simple sequence repeats have proven to be useful markers for genetic characterization of fungal species (Kaye et al., 2003; Baird et al., 2010). To our knowledge, there are no microsatellites available to study the genetic variation among *B. sorokiniana* isolates or closely related species. In this study, we describe the development and characterization of SSR markers for *B. sorokiniana*. Additionally, cross species and genus transferability of the SSR loci to *B. oryzae*, *B. spicifera*, *B. victoriae, Curvularia lunata* and *Curvularia trifolii* were investigated.

5.3 Materials and methods

5.3.1 Fungal isolates and DNA extraction

Fungal isolates used in this study were obtained either from infected switchgrass seeds purchased from several commercial seed companies or from infected leaf tissue from seedlings and mature plants grown in Tennessee (Table 5.1). Species confirmation was based on colonial and conidial morphology, as well as the sequences of the internal transcribed spacer (ITS) region of 18S ribosomal DNA. The ITS regions were amplified with PCR using ITS4 and ITS5 primers (White et al., 1990) for 34 cycles at 95°C for 30 s, 58°C for 1 min and 72°C for 45 s with initial and final steps at 95°C for 3 min and 72°C for 10 min, respectively. Isolates were maintained on potato dextrose (PD) agar slants and genomic DNA was extracted from mycelia

grown in PD broth culture with a DNeasy Plant Mini Kit (Qiagen, Valencia, California), according to the manufacturer's procedure. Individual fungal samples were pulverized with a sterile mortar and pestle in liquid nitrogen prior to DNA extraction. The quantity and quality of the DNA samples were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware); DNA quality was further assessed on a 2% agarose gel stained with ethidium bromide and visualized as a distinct band with a 2000 Gel Documentation System (Bio-Rad, Hercules, California).

5.3.2 Construction and characterization of SSR-enriched library

A small insert genomic library enriched with a biotin labeled $(GT)_{12}$ motif was developed based on the method of Wang et al. (2007) with minor modifications. Briefly, genomic DNA (1.6 µg) from four isolates of *B. sorokiniana* was combined and digested with *Rsa* I at room temperature for 7 min. The resulting fragments were ligated to SNX linker adaptors (Hamilton et al., 1999) and amplified with PCR using the SNX forward primer, for 20 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 90 s, with initial and final steps of 95°C for 2 min and 72°C for 2 min, respectively. The PCR product was hybridized to $(GT)_{12}$ biotinylated oligonucleotides, and fragments containing $(CA)_n$ microsatellites were captured and recovered using Streptavidin MagneSphere® Paramagnetic Particles (Promega, Madison, Wisconsin). The recovered enriched SSR DNA fragments were ligated to *EcoR*V-cut pBluescript SK II (+) DNA at an insert to plasmid ratio of 8:1. The ligation mixture was transformed by electroporation into *Escherichia coli* TOP10 cells (Invitrogen, Grand Island, New Jersey). Transformed cells were grown at 37°C for 14–16 h on Luria-Bertani (LB) agar medium (Sambrook et al., 2001) amended with 100 µg/mL

ampicillin, the indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (x-gal), and isopropylthio-β-D-galactoside (IPTG). A total of 2496 transformed white colonies were randomly selected and each colony was grown overnight at 37°C in 200 µL LB broth amended with 100µg/mL ampicillin. The colonies were screened with PCR for inserts containing SSRs in a 10-µL reaction containing 0.01µL of *E. coli* broth culture as template DNA, 0.25 µM each of primer T7, T3 and AC_{12}, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1×GeneAmp PCR Buffer II (Applied Biosystems, Foster City, California), 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and sterile molecular grade water. Thermal cycling conditions were the following: initial step at 96°C for 2 min, 35 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final step of 72°C for 1 min. Amplified colonies that exhibited a smear following separation of the PCR product on a 2% agarose gel, were considered to contain an insert with a SSR motif and were sequenced. Plasmid DNA from positive colonies (n=384) were extracted and sequenced at the USDA-ARS Mid-South Area Genomics and Bioinformatics Research Unit (Stoneville, Mississippi) with primers T3 and T7 on an ABI 3730XL capillary electrophoresis DNA sequencer (Applied Biosystems). One hundred and ninety-six unique SSR loci (Table 5.2) were identified from the sequences when a minimum of six, four, or three repeat units of di-, tri- and tetra-nucleotides, respectively, were used as the search criterion with the program IMPERFECT SSR FINDER (Stieneke and Eujayl, 2007). Primer pairs flanking 105 SSR motifs were designed using PRIMER3 (Rozen and Skaletsky, 2000).

To characterize the SSR loci, the 105 primer pairs were initially screened for loci amplification ability using three isolates of *B. sorokiniana* (Table 5.1). Amplified products were separated on a 2% agarose gel stained with ethidium bromide and visualized with a 2000 Gel Documentation

System (Bio-Rad). Amplification was successful for 85 SSR loci, and 16 polymorphic loci were characterized using 15 *B. sorokiniana* isolates (Table 5.1). The SSRs were amplified with PCR reactions containing 4 ng genomic DNA, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1×GeneAmp PCR Buffer II (Applied Biosystems), 5% DMSO, 0.25 µM forward and reverse primers, 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and sterile molecular grade water. Thermal cycling conditions were: initial step at 94°C for 3 min, 35 cycles at 94°C for 40 s, 58°C for 40 s and 72°C for 30 s, and a final step of 72°C for 4 min. Amplified products were sized on a QIAxcel Capillary Electrophoresis System (Qiagen) using a 25-bp DNA size marker (Qiagen). These 16 loci were used to evaluate the cross species and genus transferability of SSRs from *B. sorokiniana* to *B. oryzae*, *B. spicifera*, *B. victoriae C. lunata* and *C. trifolii* (Table 5.1). The percent cross species transfer was calculated for each locus by dividing the number of isolates from other species with successful cross amplification of SSR by the total number of other isolates tested, multiplied by 100.

5.3.3 Simple sequence repeats data analysis

The program FLEXIBIN (Amos et al., 2007) was used to convert raw allele length data into allelic classes. A conservative ± 2-bp standard size error was used because the QIAxcel System (Qiagen) has a resolution of 2–5 bp for all samples analyzed. These allelic classes were used in subsequent analyses; the two *Curvularia* isolates and *B. spicifera* isolate MH12073 failed to amplify.

To measure genetic diversity, the allelic classes were used to calculate haploid diversity and the number of alleles per locus was determined (Table 5.3) using GenAlEx Version 6.1 (Peakall and

Smouse, 2006). In addition, the genetic distances were calculated and allele sharing distance matrices for all isolates were created. From these matrices, principal component analysis (PCA) was conducted to determine the relatedness (within and between species) of isolates.

5.3.4 Confirmation of SSR loci

Representative alleles were sequenced to verify that amplified loci contained the expected SSR motif and were not an artifact of PCR, and to determine if length of polymorphisms was due to differences in the SSR motif or in the flanking region. Three loci (BS051, BS070 and BS074) were amplified as described previously for nine isolates (Table 5.1). Amplified products were sized on the QIAxcel Capillary Electrophoresis System and samples of the expected size were purified with a Wizard SV Gel and PCR Clean-Up System (Promega). Purified PCR products were cloned according to the manufacturer's protocol using the pGEM®-T Easy Vector System II (Promega). Transformed *E. coli* cells were grown as previously described. Randomly selected white colonies were grown overnight at 37 C in 5 mL LB broth amended with 100 µg/mL ampicillin. Plasmid DNA purified from the broth culture using a QIAprep Spin Miniprep kit (Qiagen) was sequenced bidirectionally using primers T7 and SP6 at the Molecular Biology Resources Facility, University of Tennessee, Knoxville. Plasmid sequences were trimmed, and sequences from each locus were aligned and analyzed with SeaView (Gouy et al., 2010).

5.4 RESULTS

5.4.1 Simple sequence repeats-enriched library

All isolates were identified based on morphology and ITS region sequence, except isolates WT76 and SK202. Digestion of *B. sorokiniana* genomic DNA with *Rsa* I at room temperature for 7 min resulted in fragments ranging from 300–1000 bp, which was visualized as a smear on a 2% agarose gel. However, digestion with multiple enzymes: *Alu* I, *Rsa* I, *Hae* III and *Stu* I, at same temperature for 15 min, or with *Alu* I and *Stu* I for 2 min resulted in complete digestion, or with fragments less than 200 bp, respectively.

From 2496 clones screened for SSRs, 384 clones containing putative SSRs were sequenced and 226 sequences (58.9%) harbored microsatellites. However, only 196 sequences contained the desired SSR motifs. The remaining 30 sequences were either duplicated loci or a dinucleotide motif with less than six repeat units, which were considered too short and hence were not included in the present study. The 196 sequences contained 166 (84.7%) dinucleotide, 19 (9.7%) trinucleotide and, 11 (5.6%) tetranucleotide or greater repeats (Table 5.2). The dinucleotide repeats ranged from 6–102 units, whereas the majority of repeats fell between eight and 23 units; only six SSR loci had repeats length greater than 50 units. Variations in $(GT)_n$ and $(AC)_n$ motifs were the most abundant for dinucleotide repeats, with 50.0% and 42.2%, respectively, whereas the $(TA)_n$ motif was the least represented. The majority of the dinucleotide SSR loci were perfect repeats, 135 (81.3%), and only two (1.2%) were duplicated. There were nine (5.4%) interrupted and 22 (13.3%) compound repeats. Motif variations were equally represented in trinucleotide and tetranucleotide repeats without any duplication.

5.4.2 Characterization of SSR loci

Primer pairs flanking 105 unique SSR loci were designed, and following the initial screening, 85 (81%) generated PCR products when screened against three isolates of *B. sorokiniana.* A set of 32 primer pairs was randomly selected from the 85 primer pairs to test for polymorphism in the same set of isolates; 23 were polymorphic with 3–48 bp differences, whereas the remaining nine were monomorphic between alleles. Sixteen polymorphic primer pairs were used to amplify SSR loci in 15 *B. sorokiniana* isolates. Sequences containing the 16 loci were deposited in GenBank (Table 5.3).

Cross species transferability of SSRs isolated from *B. sorokiniana* to closely related species was successful at all 16 loci (Table 5.3) but there was no cross genus transfer to the *Curvularia* isolates tested. Of the 16 SSR primer pairs, 14 cross-amplified loci from at least one isolate of *B. oryzae*; the two remaining loci (BS035 and BS096) were amplified in either *B. spicifera* or *B. victoriae*. However, only four primer pairs (BS027, BS051, BS070 and BS074) amplified all 10 isolates of *B. oryzae* studied. Only two primer pairs (BS035 and BS070) produced amplicons in one out of the two *B. spicifera* isolates tested. Cross amplification of SSRs in *B. victoriae* was successful at four loci (BS074, BS096, BS098 and BS104). For all other species tested, loci BS032, BS065 and BS068 were cross-amplified only in *B. oryzae* isolate AP163. The percent cross species amplification per locus ranged from 6.25%–75.00% (Table 5.3).

5.4.3 Data analysis

Only one peak was detected for each isolate, indicating the haploid nature of the *Bipolaris* genome. The number of alleles per SSR locus for *B. sorokiniana,* and across all other species

varied from 3–9 with a mean of 5.2, and 4–12 with a mean of 6.3, respectively. Haploid gene diversity for *B. sorokiniana* ranged from 0.52–0.84 per locus with a mean of 0.68 whereas the value across all species varied from 0.63–0.86 per locus with a mean of 0.72 (Table 5.3). Where multiple isolates were available, isolates were clustered by PCA (Fig 5.1) according to species. Two principal coordinates explained 90% of the total variation for all isolates analyzed; the first coordinate explained 81% of the total variation. *Bipolaris sorokiniana* isolates were tightly clustered, whereas *B. oryzae* isolates were loosely clustered.

Sequence analysis of representative loci (BS051, BS070 and BS074) confirmed the presence of the expected microsatellite motif, except for locus BS070 that contained two different motifs. At this locus, the expected $(AC)_8$ repeat was present in *B. sorokiniana*, was missing in *B. spicifera* and was completely deleted in isolates of *B. oryzae* that were sequenced (Fig. 5.2). However, a tetranucleotide repeat $(CCCA)_3$ was conserved across all isolates. Sequence of the SSR loci of all nine isolates (Table 5.1) revealed conserved primer sites and flanking regions (Fig. 5.2), except for isolate WT62 at locus BS074, which had a 14-nucleotide deletion at the 5'end of the forward primer, but was conserved at the 3' end of the primer, which is the crucial end for successful PCR (Dieffenbach et al., 1993). At locus BS051, all isolates had the expected GT repeat, but with isolates of *B. oryzae*, the sequences contained additional GA repeats that were deleted in *B. sorokiniana* sequences.

5.5 DISCUSSION

Bipolaris sorokiniana is a potentially destructive pathogen on switchgrass. This pathogen is difficult to control, and genetic resistance is considered the most efficient means of controlling

B. sorokiniana (Zeiders, 1984). Genetic studies of *B. sorokiniana* are essential for choosing an appropriate breeding strategy to develop switchgrass cultivars resistant to this fungus.

Digestion of *B. sorokiniana* into the desired fragments needed for development of microsatellites required only one enzyme, *Rsa* I, for the minimal reaction time. Digestion with more than one enzyme resulted in complete digestion or fragments that were too small to use for library construction. From the positive colonies sequenced, only 196 (51%) contained microsatellites with the desired repeat units, whereas others either did not contain SSRs, or had too few repeat units. Similar results were reported for SSRs from *Geosmithia morbida* (Hadžiabdic et al., 2011). In general, efficiency of isolating SSRs from fungi is lower and more challenging compared to other organisms; this is due in part to short fungal SSR loci (Dutech et al., 2007). Mean haploid diversity per locus of *B. sorokiniana* was 0.6; moreover, these isolates were tightly clustered with PCA analysis. The limited genetic diversity observed among *B. sorokiniana* isolates is not unusual since this species reproduces by clonal means and sexual reproduction is rare. In addition, greater numbers of polymorphisms would be expected with longer SSRs, which were less represented in this study, and are usually rare in fungi (Karaoglu et al., 2005). Similarly, a low level of genetic variation has been reported in other plant pathogens with rare teleomorph stages (Steimel et al., 2004, Wadl et al., 2011).

The use of SSRs isolated from one species to study the genetic structure of closely related species is advantageous and cost efficient. In the present study, 100% of the primer pairs developed for *B. sorokiniana* cross amplified a locus in at least one isolate from the other three related species. This appears to contradict a report stating that cross species transfer of SSRs in

fungi is low (Dutech et al., 2007). However, a possible explanation for the apparent low transferability in the previous study is that the species included were more evolutionarily distant compared to species of *Bipolaris* tested in the present work. The inability of all the SSRs to cross amplify loci in isolates of *Curvularia* species studied implies that this genus is genetically distant from *Bipolaris*, even though some consider that these genera are either closely related or synonymous (Manamgoda et al., 2011). Cross genus transfer of SSRs in fungi is rare (Cristancho and Escobar, 2008; Baird et al., 2010). However, in the current study only one isolate each of *C. lunata* and *C. trifolii* was available, and further studies with more isolates from this genus might reveal transferability of SSRs from *B. sorokiniana*.

The genetic variation among isolates of *B. oryzae* was not correlated with geographical origin. The limited number of loci that cross-amplified all *B. oryzae* isolates tested is further evidence of the genetic variation among the *B. oryzae* isolates in this study. Investigation of additional loci might clarify the genetic variability of this species. Only single isolate of *B. victoriae* and *B. spicifera* were available for this analysis so determination of variation within species was not possible. There was a unique allelic pattern of SSRs that discriminated *B. sorokiniana* from *B. oryzae*. Loci BS051 and BS070 not only amplified all isolates of *B. sorokiniana* and *B. oryzae* studied, but revealed allele diversity that differentiated these species from each other. Moreover, two GA repeat motifs were present in addition to the expected GT motif at locus BS051 for *B*. *oryzae*. At locus BS070, the expected AC motif was present in *B. sorokiniana* but absent from the sequences of *B. spicifera* and *B. oryzae*. For all isolates sequenced at locus BS074, the expected CA motif was present. The sequencing of these loci provided additional evidence that the SSR loci in this study can be used to differentiate among species. Moreover,

these sequences revealed that differences in allele size were due to variations in the length of SSR, as well as additions or deletions of the motif. Microsatellite loci are widely distributed throughout the genome making them highly informative molecular markers for species identification (Queller et al., 1993). This is evident in the present work because our SSR data overcame the limitation of species identification based on a single region of the fungal genome. For example, species identification of isolate WT76 was problematic using the ITS region sequence. When the sequence was entered into GenBank, three possible species of *Bipolaris* were indicated with equal percentage support. In addition, identification based on morphology was challenging, since closely related *Bipolaris* species have similar conidial and colonial characteristics. Our SSR data, based on the allele pattern, PCA and loci sequences, unambiguously identified isolate WT76 as *B. sorokiniana.*

The sixteen microsatellite markers developed in this study are potential molecular markers for identification of *B. sorokiniana* and related species in epidemiological and population genetic studies. These markers are also expected to be a useful tool to formulate breeding strategies and develop resistant switchgrass cultivars that might help in the expansion of biofuel industries. This research was the first to isolate and characterize SSRs from *B. sorokiniana*, and study the cross amplification of these microsatellites in related species.

5.6 References

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Table 5.1 Fungal isolates studied for characterization of microsatellite loci in *Bipolaris sorokiniana* and cross transferability of SSRs to related species

Species	**Isolate ID**	**Source**[a]	**Origin**
Bipolaris sorokiniana	SLA4	Alamo	Colorado
	SLA26	Alamo	Colorado
	SLA28[b]	Alamo	Colorado
	SLA30	Alamo	Colorado
	SLA31	Alamo	Colorado
	SLA32[c]	Alamo	Colorado
	SLCO1[b]	Alamo	Colorado
	SLCO8	Alamo	Colorado
	ST2-2[b c]	Alamo	Colorado
	JA 5	Alamo	Oklahoma
	PSB3	Alamo	Tennessee
	APCNR34	Cave-in-Rock	Illinois
	APCNR150	Cave-in-Rock	Illinois
	WT65[b c]	Cave-in-Rock	Iowa
	WT76[c]	Cave-in-Rock	Iowa
Bipolaris spicifera	LA18[c]	Alamo	Oklahoma
	MH12073	Alamo	Tennessee
Bipolaris victoriae	JA12[c]	Alamo	Oklahoma

Table 5.1 Continued

Species	Isolate ID	Source[a]	Origin
Bipolaris oryzae	AP105	Cave-in-Rock	Illinois
	AP124	Cave-in-Rock	Illinois
	AP163[c]	Cave-in-Rock	Illinois
	WT27	Cave-in-Rock	Iowa
	WT30[c]	Cave-in-Rock	Iowa
	WT62[c]	Cave-in-Rock	Iowa
	WT95	Cave-in-Rock	Iowa
	SK12	Kanlow	Missouri
	SK13	Kanlow	Missouri
	SK226	Kanlow	Missouri
***Bipolaris* species**	SK202	Kanlow	Missouri
Curvulariatrifolii	APKan99	Kanlow	Oklahoma
Curvularia lunata	JC124	Cimarron	Oklahoma

[a] Switchgrass cultivars from which isolates were obtained.

[b] Isolates used for the construction of the library. With the exception of isolate SLCO1, all isolates were used for the preliminary screening of microsatellite primer pairs.

[c] Isolates sequenced to verify SSR loci.

Table 5.2 Characterization of simple sequence repeat (SSR)-enriched library for *Bipolaris sorokiniana*

	Class of Repeat[a]		
	Dinucleotide	Trinucleotide	Tetranucleotide
Number of microsatellites	166 (84.7%)	19 (9.7%)	11(5.6%)
	$(GT)_n$ or $(TG)_n$	$(CCA)_n$ or $(ACA)_n$ or $(CAC)_n$	$(GTAT)_n$ or $(GGAT)_n$
Motif	83 (50.0%)	4 (21.0%)	2 (18.2%)
	$(CA)_n$ or $(AC)_n$	$(GTG)_n$ or $(GTT)_n$ or $(TGG)_n$	$(TCTT)_n$ or $(CTCC)_n$
	70 (42.2%)	5 (26.3%)	2 (18.2%)
	$(TC)_n$ or $(CT)_n$	$(CTC)_n$ or $(CTG)_n$ or $(TGC)_n$	$(CATC)_n$ or $(ATCC)_n$
	5 (3%)	3 (15.8%)	2 (18.2%)
	$(TA)_n$	$(CAG)_n$ or $(GAC)_n$ or $(AGC)_n$	$(CAAG)_n$ or $(ACAG)_n$
	1 (0.6%)	3 (15.8%)	2 (18.2%)
	$(AG)_n$ or $(GA)_n$	$(CAT)_n$ or $(TAA)_n$ or $(TAC)_n$	$(GGGT)_n$
	5 (3%)	3 (15.8%)	1 (9%)
	$(CG)_n$	$(AGG)_n$	Various
	2 (1.2%)	1 (5.3%)	2 (18.2%)
Range of repeat (n)	6–102	4–10	3–6

Table 5.2 Continued

	Class of Repeat[a]		
	Dinucleotide	Trinucleotide	Tetranucleotide
Perfect[a]	135 (81.3%)	10 (52.6%)	4 (36.4%)
Interrupted[a]	9 (5.4%)	0	0
Compound[a]	22 (13.3%)	9 (47.4%)	7 (63.6%)
Duplication[b]	2 (1.2%)	0	0

[a] Classification of microsatellite motif as described by Gupta et al (1996).

[b] 196 unique SSRs were identified; duplicated loci not included.

Table 5.3 Characteristics of 16 microsatellite loci developed from *Bipolaris sorokiniana* and transferability to related species

Locus[a]	Primer sequences (5'–3')	Motif	Allele size	*A*[b]	h	Loci transferred (%)
S001	F: ATGAGTGATCAGAGCAGGACTTTT	$(CA)_6$	243–268	4(4)	0.637	12.50
	R:CATACAGGGTAATGTGTAGGTAGTG					
BS027	F: ACTGAAGAGACCCAGATGTCGTAG	$(TG)_6$	178–214	4(6)	0.766	68.75
	R:GGATATCCTTTGAGTTGATCTCTCC					
BS030	F: GTAGCAAACAGGCAGTAAACTTGG	$(TG)_6$	150–178	5(6)	0.720	12.50
	R: TACAATGAGGCTGCTCATATTTACA					
BS032	F: AAACTTGGTGTTCTTGATGTGTTTT	$(AG)_7(TG)_7$	227–263	4(4)	0.633	6.25
	R: ACCATTCCTACATAATGCTGTCAAT					
BS035[c]	F: GAGCAAGCCGAGTAGACCAC	$(CAGTCCAGCG)_4$	228–251	5(5)	0.633	6.25
	R: GCATGGATACACATACACACACAC					
BS036	F: ATTACCCTATGGCAGCAATCTG	$(GT)_6$	160–189	5(5)	0.653	62.50
	R: ACCCACTCTGTTCCTTTCTCATC					
BS051	F: CCACAGTAATGGTGTGTTTTTAGGT	$(GT)_6$	175–223	5(8)	0.817	68.75
	R: CCGAGTCAGTTCTTAATGCTATCAG					

Table 5.3 Continue

Locus[a]	Primer sequences (5'–3')	Motif	Allele size	*A*[b]	h	Loci transferred (%)
BS065	F: TCAATGCTAGTATTTTTCTCATTCG	$(CA)_9$	182–215	6(6)	0.781	6.25
	R: ACAATAATGACGTCACCATCTCAC					
BS068	F: GCACACATATTACTGCATACACACA	$(GT)_6$	219–248	4(4)	0.703	6.25
	R: GGGATGTAAGGTATGACAATGACC					
BS069	F: CTCTCTCCTCAGCATCCTATCATCT	$(TG)_8$	138–162	4(5)	0.726	56.25
	R: GTGGAGGGAAGCGAAAATC					
BS070[c]	F: AAACAAGAATGCTCCGAAGTTG	$(AC)_7$	219–265	3(7)	0.765	75.00
	R: CCCGTCCTCATTACCCAGTAT					
BS074[d]	F: ACGTAAGGAAAAACACCTCGAGTC	$(CA)_8$	161–202	18(2)	0.861	75.00
	R: ACTTTATCCGTGTGCATCTTCAAC					
BS096[d]	F: CACTCACTCACACATACACTACTAACT	$(ATCC)_5$	153–173	5(6)	0.672	6.25
	R: AAGGAAAATAATTGTGTCGCAGTAA					

Table 5.3 Continues

Locus[a]	Primer sequences (5′–3′)	Motif	Allele size	*A*[b]	h	Loci transferred (%)
BS098[d]	F: GGTAAGCTTTCACGCTAACAACTC	$(AC)_{23}$	132–180	9(9)	0.790	18.75
	R: ACACTTGGATAGGCGTTGAGATACT					
BS103	F: ACATATCCTTGCCCTAAACACAAT	$(ACA)_{10}$	169–210	8(8)	0.784	18.75
	R: TAGGCAGAGCTTGGATATGACTACT					
BS104[d]	F: TATGACTATAGTGTCTTGGGCACAC	$(AC)_7$	150–175	4(6)	0.626	25.00
	R: CCCACAGGCAAAGGTATATAATAAG					

[a] Shown for each locus are the forward (F) and reverse (R) primer sequence, repeat motif, allele size range (bp), allele number (A), haploid diversity (h), and percent cross species transfer to other *Bipolaris* species. Values are based on characterization of 28 isolates from four *Bipolaris* species.

[b] Allele number per locus for *B. sorokiniana*; values in parenthesis are allele numbers per locus across all species.

[c] Loci that cross amplified *Bipolaris spicifera*.

[d] Loci that cross amplified *Bipolaris victoriae*. GenBank Accession Nos. JN903490 –JN903505.

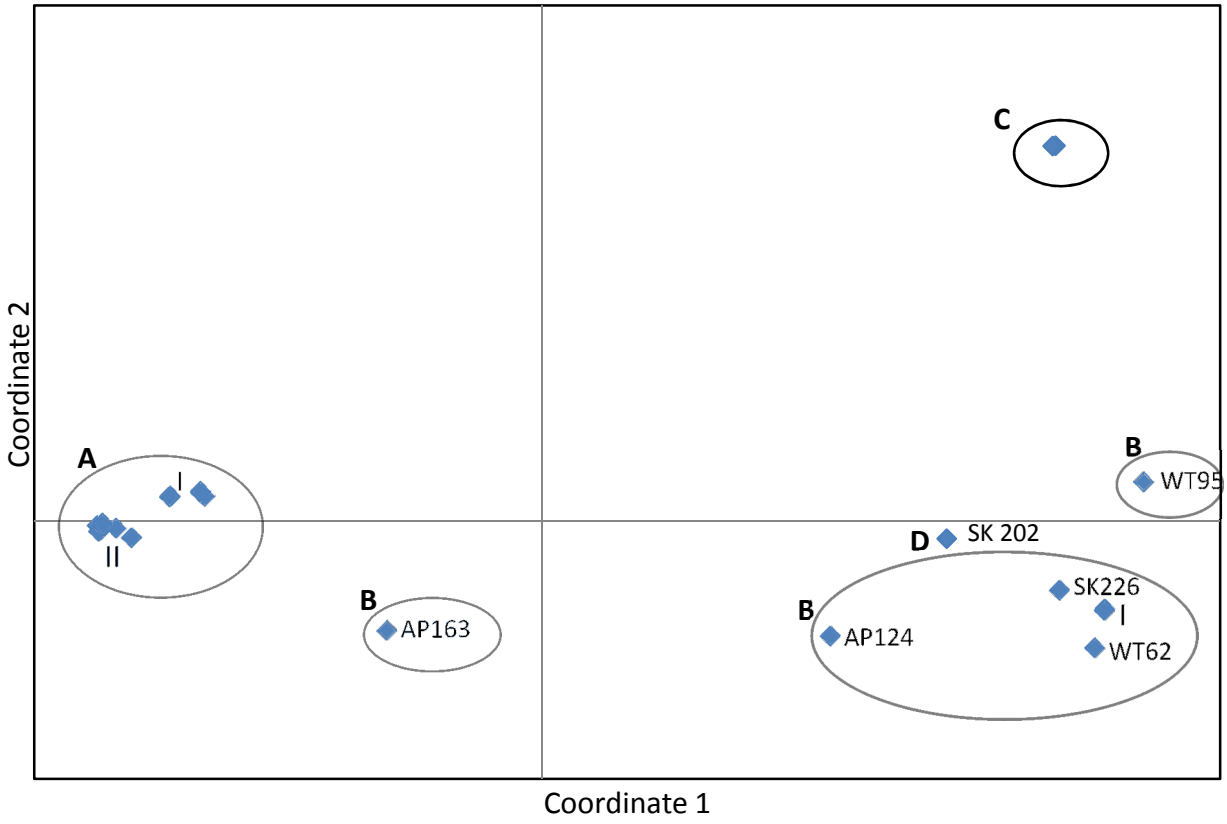

Fig. 5.1 Principal coordinate analysis based on Nei's genetic distance matrix generated from SSR data of 15 isolates of *B. sorokiniana* (A: cluster I consisted of isolates APCNR34, APCNR150, JA5, SLA28, ST2-2, and WT65,and cluster II consisted of PSB3, SLA4, SLA26, SLA30, SLA31, SLA32, SLCO1, SLCO8, and WT76); 10 isolates of *B. oryzae* (B: cluster I consisted of isolates AP105, SK12, SK13, WT27 and WT30, and five other isolates (AP124, AP163, SK226, WT62, and WT95) that are shown in the figure); one isolate each of *B. spicifera* (LA18) and *B. victoriae* (JA12) that clustered together (C); and an unidentified *Bipolaris* species (SK202) (D). Two principal coordinates explained 90% of the total variation for all isolates analyzed; however, the first coordinate explained 81% of the total variation.

Locus BS051

```
SLA32 CCACAGTAAT GGTGTGTTTT TAGGTCATGG AGAGTG.... .........A
ST2   CCACAGTAAT GGTGTGTTTT TAGGTCATGG AGAGTG.... .........A
WT65  CCACAGTAAT GGTGTGTTTT TAGGTCATGG AGAGTG.... .........A
WT76  CCACAGTAAT GGTGTGTTTT TAGGTCATGG AGAGTG.... .........A
WT62  CCACAGTAAT GGTGTGTTTT TAGGTCATAG AGAGAGAGAG A....AGTGA
WT30  CCACAGTAAT GGTGTGTTTT TAGGTCATAG AGAGAGAGA. .....AGTGA
AP163 CCACAGTAAT GGTGTGTTTT TAGGTCATAG AGAGAGAGAG AGAGAAGTGA

SLA32 CCGAGCGAGA CAAAGTG... .......... ...TGTGTGT GTGTG....C
ST2   CCGAGCGAGA CAAAGTG... .......... ...TGTGTGT GTGTG....C
WT65  CCGAGCGAGA CAAAGTG... .......... .....TGTGT GTGTG....C
WT76  CCGAGCGAGA CAAAGTGCG. .........T GTGTGTGTGT GTGTG....T
WT62  CCGAGCAAGG GAGAGAGGGA GAGAGAGAGT GGGTGTGTGT GTGTG....C
WT30  CCGAGCAAGG GAGAGAGGGA GAGAGAGAGT GGGTGTGTGT GTGTGTGTGC
AP163 CCGAGCAAGG GAGAGAGGGA GAGAGAGAGT GGGTGTGTGT GTGTG....C

SLA32 GTGTGCAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
ST2T  GTGTGCAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
WT65  GTG..CAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
WT76  GTG..CAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
WT62  GTG..CAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
WT30  GTG..CAAGA AGCGTCTGAT AGCATTAAGA ACTGACTCGG
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Locus BS070

```
SLA32 AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGACCCT
ST2   AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGACCCT
WT65  AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGACCCT
WT76  AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGACCCT
LA18  AAACAAGAAT GCTCCGAAGT TGAGGCGACA  GGC....... .......CTT
WT62  AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGGCCCT
WT30  AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGGCCCT
AP163 AAACAAGAAT GCTCCGAAGT TGAGGCGG.A  GGCTGTGGAC CTCTGGCCCT

SLA32 AGCGAGCAAG CAGCGTGGCG GGCCCTGTCT TGAGAGCGGA TCCGATGTGA
ST2   AGCGAGCAAG CAGCGTGGCG GGCCCTGTCT TGAGAGCGGA TCCGATGTGA
WT65  AGCGAGCAAG CAGCGCGGCG GGCCCTGTCT TGAGAGCGGA TCCGATGTGA
WT76  AGCGAGCAAG CAGCGCGGCG GGCCCTGTTT TGAGAGCGGA TCCGATGTGA
LA18  TG.GCGCGGG CACCCC..CT GGC..AACCA TGGCGATGGG ..CAATGC.A
WT62  AA.GTGCAAG CAGCGCAGCG GGCCCCGTCT TGA..GCGGA TCCGATGTGA
WT30  AA.GTGCAAG CAGCGCAGCG GGCCCCGTCT TGA..GCGGA TCCGATGTGA
AP163 AA.GTGCAAG CAGCGCAGCG GGCCCCGTCT TGA..GCGGA TCCGATGTGA
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SLA32  CCCACCCACC CACGCTTGCC CACACACAC. .........A CACACTTGCA
ST2    CCCACCCACC CACGCTTGCC CACACACAC. .........A CACACTTGCA
WT65   CCCACCCACC CACGCTTGCC CACACACACG CGCGCACATA CACACTTGCA
WT76   CCCACCCACC CACGCTTGCC CACACACA.. .......... ..CACTTGCA
LA18   CCCACCCA.. CACAGCTACT GGAAGATGGT GAAGTC..TG GCCGTGCGAA
WT62   CCCACCCACC CACGCTTGC. .......... .......... .........A
WT30   CCCACCCACC CACGCTTGC. .......... .......... .........A
AP163  CCCACCCACC CACGCTTGC. .......... .......... .........A


SLA32  GGCCGCAATA TACTGGGTAA TGAGGACGGG
ST2    GGCCGCAATA TACTGGGTAA TGAGGACGGG
WT65   GGCCGCAATA TACTGGGTAA TGAGGACGGG
WT76   GGCCGCAATA TACTGGGTAA TGAGGACGGG
LA18   AGC.GCAATA TACTGGGTAA TGAGGACGGG
WT62   GGCCGCAATA TACTGGGTAA TGAGGACGGG
WT30   GGCCGCAATA TACTGGGTAA TGAGGACGGG
AP163  GGCCGCAATA TACTGGGTAA TGAGGACGGG
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Locus BS074

```
SLA32 ACGTAAGGAA AAACACCTCG AGTCGTCCAG CCCGGGGCAC ACACACACAC
ST2   ACGTAAGGAA AAACACCTCG AGTCGTCCAG CCCGGGGCAC ACACACACAC
WT65  ACGTAAGGAA AAACACCTCG AGTCGTCCAG CCCGGGGCAC ACACACA...
WT76  ACGTAAGGAA AAACACCTCG AGTCGTCCAG CCCGGGGCAC ACACACA...
JA12  ACGTAAGGAA AAACACCTCG AGTCGTCCAG CCCGGGGCAC ACACACA...
WT62  .......... ....ACCTCG AGTC...CAG CCCGGGGCAC ACACA.....
WT30  ACGTAAGGAA AAACACCTCG AGTC...CAG CCCGGGGCAC ACACACACA.
AP163 ..GTAAGGAA AAACACCTCG AGTC...CAG CCCGGGGCAC ACACA.....

SLA32 ACACACACAC ACACACACAC AACGCCGTTG AAGATGCACA CGGATAAAGT
ST2   A......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAAGT
WT65  .......... .........C AACGCCGTTG AAGATGCACA CGGATAAAGT
WT76  .......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAAGT
JA12  .......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAA..
WT62  .......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAAGT
WT30  .......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAAGT
AP163 .......... .......CAC AACGCCGTTG AAGATGCACA CGGATAAAGT
```

Fig. 5.2 Sequence alignment of representative loci from selected isolates of *Bipolaris sorokiniana* (SLA32, ST2, WT65 and WT 76), *Bipolaris oryzae* (WT62, WT30 and AP163), *Bipolaris spicifera* (LA18) and *Bipolaris victoriae* (JA12). All conserved region including the flanking sequences are not shown. Forward and reverse primer sites are highlighted in red, expected motif in purple and additional repeats found in green.

CHAPTER 6: CONCLUSION

This study clearly showed that *Bipolaris* species play an important role in stand establishment, which is a major challenge in switchgrass production. These pathogens significantly reduced biomass yield. High-virulent isolates greatly impacted switchgrass, even at low inoculum rates. The presence of such isolates in the field can be problematic since only a few conidia are required for disease development. *Bipolaris* not only negatively affects biomass quantity but also impacts quality by reducing biomass fitness for biofuel conversion. Infection of switchgrass with *Bipolaris* resulted in a 4 to 7% reduction in the carbohydrate content of biomass. Since feedstock carbohydrate content is directly proportional to ethanol yield, it is likely that *Bipolaris* will reduce ethanol production by at least 4%. Further studies on the effect of *Bipolaris* infection on the feedstock fermentation process and yield will need to be conducted to determine the actual impact of these fungal pathogens on bioethanol production.

Six out of nine germplasms screened were moderately resistant to the isolates of *B. spicifera* and *B*. *oryzae* tested. These germplasms can further be explored as resistance donors in plant breeding programs for development of switchgrass cultivars with desirable biofuel traits. None of the germplasms were resistant to the isolates of *B. sorokiniana* and *B. victoriae* tested. More switchgrass germplasms should be screened against more *Bipolari*s isolates to identify a source of resistance against other *Bipolaris* species. Sixteen polymorphic SSR loci developed from a small insert genomic library for *B. sorokiniana* were used to characterize fourteen *B. sorokiniana* isolates. These loci successfully cross-amplified at least one isolate of *B. victoriae*, *B. spicifera* and *B. oryzae*. The co-dominant markers developed are valuable tools for genetic

characterization and epidemiological studies of *B. sorokiniana* and related species that infect switchgrass and other host plants. These markers are also expected to be useful tools to formulate breeding strategies and develop resistant switchgrass cultivars that will aid in the expansion of biofuel industries.

As the biofuel industry develops in the U.S., switchgrass will be grown continuously on a commercial scale, and a concomitant increase in disease threats to switchgrass is expected. An integrated disease management strategy with a genetic resistance component will be needed to reduce the impact of fungal pathogens, like *Bipolaris* species, on switchgrass production.

VITA

Oluseyi Lydia Fajolu (formerly Adedire) was born in Ado-Ekiti, Nigeria. She graduated from the Federal University of Technology Akure (FUTA), Nigeria with a first class honors degree in Microbiology. She was honored as the best graduating student of her class, and the best graduating female student in the College of Sciences.

In 2005, she began her career in academics as one of the pioneer lecturers in the Department of Biological Sciences, Redeemer University, Nigeria, where she taught introductory and some second-year Biology classes. At the same time, she was also studying at FUTA for a Master of Science (M.Sc.) degree in Food Microbiology. She completed all the necessary class work required to obtain the M.Sc. degree but before she could complete the laboratory work, she received an opportunity to proceed to the U.S.A. for further studies.

In the fall of 2007, she began an M.S. degree program in the Department of Entomology and Plant Pathology (majoring in Plant Pathology) at The University of Tennessee, Knoxville (UTK); U.S.A. She also started working as a Graduate Research Assistant in the Plant Virology Laboratory under the supervision of Dr. Reza Hajimorad. Her thesis research was entitled, "Genetic Variability of *Hosta Virus X* in Hostas." In the fall of 2009, she began a Ph.D. program in the same department. However, she moved to the Soilborne Plant Pathogens Laboratory to continue her work as Graduate Research Assistant under the supervision of Dr. Bonnie Ownley.

Due to her outstanding performances, Oluseyi has received several awards and recognitions. These include: Extraordinary Professional Promise – UTK Chancellor Honors Award; American Phytopathological Society Graduate Student Travel Award; ESPN Graduate Student Award; and

the Nigerian National Youth Service Corps Community Service Merit Award. She is also a member of the Honor Society of Agriculture, Gamma Sigma Delta.

Upon graduation, Oluseyi's immediate plans are to obtain a teaching position in higher education, thus realizing her long-held dream of transferring knowledge to younger generations. She also intends to continue to work on research in the field of molecular biology with an emphasis on plant-pathogen interactions.

Oluseyi is married to her college friend, Olufemi Nelson Fajolu and they are blessed with two daughters.