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Switchgrass Extractives Have Potential as a Value-added Antimicrobial Against Plant Pathogens and Foodborne Pathogens

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I am submitting herewith a thesis written by Alexander Ian Bruce entitled "Switchgrass Extractives Have Potential as a Value-added Antimicrobial Against Plant Pathogens and Foodborne Pathogens." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Bonnie H. Ownley, Major Professor

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**Switchgrass Extractives Have Potential as a Value-added
Antimicrobial against Plant Pathogens and Foodborne
Pathogens**

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Alexander Ian Bruce
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ABSTRACT

Panicum virgatum (switchgrass), a perennial grass native to North America, is a leading biomass feedstock candidate for the manufacture of cellulosic ethanol. Switchgrass is considered a viable option for biofuel production due to its cheap production cost and ability to grow on marginal land. Biofuel derived from switchgrass has been shown to be very energy efficient, producing 540% more renewable energy versus nonrenewable energy expended. Switchgrass-derived biofuel is also estimated to have greenhouse gas emissions that are 94% lower than emissions from gasoline (Schmer et al 2008). Biofuels are created through biochemical processes that utilize various enzymes and microorganisms for conversion of cellulose to ethanol. During this process, switchgrass extractives can be eliminated to allow for optimum enzyme activity, and improved efficiency of the conversion process (Thammasouk et al. 1997). Switchgrass extractives removed during this process are rich in phenolic compounds that are known to have antibacterial and antifungal properties. These compounds are concomitant with the induction of the systemic resistance response exhibited by stressed or diseased plants (Chen et al. 2010). If the extractives are not removed during the biofuel production process, the phenolic compounds will inhibit microbial and enzymatic function and decrease ethanol yield. The goal of this research was to assess the efficacy of switchgrass extractives as an antimicrobial for use as a commercial value-added product to switchgrass-derived biofuels. Specifically, the objectives of this research were to test for antimicrobial activity of concentrated switchgrass extractives against bacterial plant pathogens, fungal plant pathogens, and bacterial foodborne pathogens.

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CHAPTER 1

INTRODUCTION

Biofuel production will likely play a key role in the move away from fossil fuels to more sustainable energy sources. The United States is currently one of the world's largest manufacturers of biofuel produced from sugars and starch, known as first-generation biofuels (BP, 2016). This is in contrast to traditional fuels, such as petroleum, produced through geological mining methods. First-generation biofuels produced in the U.S. are commonly ethanol produced via anaerobic digestion of the starch from agricultural crops such as corn. Competition between food and livestock feed prices, as well as the agricultural inputs required to grow corn, are disadvantages to first-generation biofuels that have raised concern (Rathmann et al., 2010). Biomass crops rich in lignocellulose (carbohydrate polymers cellulose and hemicellulose in conjunction with the aromatic polymer, lignin) are one option for biofuel production. Biofuels produced from lignocellulose rich plants are known as second-generation biofuels (Naik et al., 2010).

One prominent feedstock option for lignocellulosic biofuel production is *Panicum virgatum*, known commonly as switchgrass. *Panicum virgatum* is a perennial grass native to North America with advantageous qualities for biofuel production. Switchgrass has C₄ carbon fixation and deeply penetrating roots that prevent soil erosion. The extensive root system of switchgrass improves soil structure and sequesters carbon in the soil (Liebig et al., 2005). Switchgrass is adapted to grow on soils with variable nutrient and water availability, and thus can grow on marginal soils that require minimal agricultural inputs (Follett et al., 2012).

Low-molecular weight phenolic compounds found in the non-structural components of switchgrass can hinder the biochemical conversion process. This non-structural portion of switchgrass is known as extractives and can inhibit the enzymes and microorganisms utilized in biofuel production (Anderson & Akin, 2008). Removal of phenolic compounds from biomass has been shown to increase ethanol yields (Klinke et al., 2004). Phenolic compounds found in plant extractives, such as p-coumaric acid and ferulic acid, are associated with disease resistance in plants (Nicholson & Hammerschmidt, 2003). Developing phenolic-rich switchgrass extractives as a value-added disinfectant against plant and foodborne pathogens could improve the economic feasibility of switchgrass as a biofuel feedstock.

Switchgrass extractives were collected from an Organosolv bioreactor in 95% ethanol. Extractives were analyzed by ultra-performance liquid chromatography with a UV detector and UPLC-mass spectrometer for antimicrobial components. Extractives were also analyzed for total phenolic concentration and sugar content using Folin-Ciocalteu reagent and high-performance liquid chromatography, respectively.

Pathogens

The pathogens chosen to test for susceptibility against switchgrass extractives included bacterial plant pathogens, fungal plant pathogens, and foodborne bacterial pathogens. Plant pathogens were chosen due to their economic importance and threat to national food security. Human health risk was the leading factor in the choice of foodborne pathogens tested.

Fungal Plant Pathogens

Fusarium oxysporum is a hemi-biotrophic ascomycete found ubiquitously in nature. While *F. oxysporum* is pathogenic on a wide variety of hosts, it is commonly associated with cucurbits (Gordon, 1997). The economic importance of *F. oxysporum* is high; vascular wilt disease caused by this fungus leads to severe losses of field crops, vegetables, and flowers. Disease symptoms include wilting, stunted growth, yellowing of lower leaves, and death of the plant. Few control methods prevent *F. oxysporum* vascular wilt disease. The usage of resistant crop varieties is the most common approach for controlling disease caused by *F. oxysporum* (Di Pietro et al., 2003).

Fusarium graminearum is a hemi-biotrophic ascomycete that causes disease of cereal crops. *Fusarium graminearum* reduces grain yield and quality, while also producing a mycotoxin that renders the grain unsafe for human and livestock consumption. Fusarium head blight, the disease caused by *F. graminearum*, can also occur when storage conditions are too moist (Dean et al., 2012).

The fungal plant pathogen, *Botrytis cinerea*, is a necrotrophic ascomycete with a host range of over 200 plant species. *Botrytis cinerea* is of high economic importance due to its ability to cause grey mold disease on crops at various stages, including post-harvest (Dean et al., 2012). Control measures for *B. cinerea* typically involve fungicide application, although development of resistance to fungicide among populations of *B. cinerea* have been reported (Leroch et al., 2011).

The necrotrophic ascomycete, *Bipolaris oryzae* is the causal agent of brown leaf spot on cereal crops such as rice, a staple crop in many countries. Brown leaf spot

causes high economic losses in warm, humid climates where the disease is prevalent, and was the cause of famine in the Bengal province in 1942 (Kumar et al., 2011).

Bipolaris oryzae has also been identified as a pathogen of switchgrass (Vu, 2011).

Alternaria alternata is a necrotrophic ascomycete that is the causal agent of stem canker and black mold disease on tomato. While black mold only affects ripe tomato fruit and is a serious post-harvest concern, stem canker disease can cause devastating effects to the vascular tissue, leaves, and fruit. *Alternaria alternata* will colonize the vascular tissue and girdle the plant, infected leaves will form dark colored lesions that are predominantly interveinal, and dark, circular lesions will form on unripe fruit (Koike et al., 2006).

Bacterial Plant Pathogens

Xanthomonas perforans is an aerobic, Gram-negative bacterium that is the causal agent of bacterial spot disease, an aggressive disease of pepper and tomato plants (Koike et al., 2006). Bacterial spot disease is most prevalent in warm, humid environments. Symptoms of plants infected with *X. perforans* include lesions on leaves and fruits, which often lead to an unmarketable crop (Ritchie, 2000). Control of *X. perforans* is difficult due to copper- and streptomycin-resistant strains (Ritchie, 1991).

Xanthomonas axonopodis pv. *malvacearum* is an aerobic, Gram-negative bacterium that causes bacterial blight in cotton, a disease that is prevalent in cotton growing areas around the world, and has significant effects on yield and crop quality (Hussain & Tahir, 1993). Control of *X. axonopodis* pv. *malvacearum* is difficult due to

the seed-borne nature of the pathogen and the systemic infection it causes. Cotton from infected plants is non-marketable due to the stained lint caused by the pathogen (Eddin et al., 2007).

Crown gall disease, caused by the aerobic, Gram-negative bacterium *Agrobacterium tumefaciens*, can infect plants from over 93 different families. Infection and gall formation occur when *A. tumefaciens* is introduced into injured plant tissue. Gall formation in the trunk and root system can lead to water stress and a decline in foliage, resulting in yield loss (Kado, 2002).

Erwinia amylovora is the causal agent of fire blight disease in many fruit crops (blackberry, apple, pear, to name a few) and ornamentals in the Rosaceae family. Fire blight can be devastating, and can be lethal to young fruit trees. *Erwinia amylovora* is a Gram-negative, facultative anaerobe (Mansfield et al., 2012).

The aerobic, Gram-negative bacteria *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *tabaci* are the causal agents of bacterial speck on tomato and wildfire disease of tobacco, respectively. Bacterial speck, while a relatively minor tomato disease, can cause significant economic damage due to dark lesions that form on foliage and fruit (Koike et al., 2006). Wildfire disease occurs on a wide range of hosts (tobacco, soybean, oats, and more). Wildfire causes circular, chlorotic areas on the foliage and spreads rapidly between hosts (thus the name wildfire). The aggressive nature of the pathogen sometimes results in the loss of an entire crop. Introduction of wildfire disease to soybean producing countries is a recent and economically important problem (Myung et al., 2009).

The aerobic, Gram-positive bacteria *Clavibacter michiganensis* subsp. *michiganensis* and *Clavibacter michiganensis* subsp. *nebraskensis* are the causal agents of bacterial canker on tomato and Goss' wilt of corn, respectively. Bacterial canker disease of tomato causes wilting and chlorosis of leaves due to systemic infection. As the disease progresses, vascular tissue begins to turn shades of brown. Secondary infections of the leaves and fruit cause raised, cream colored lesions (Koike et al., 2006).

Foodborne Pathogens

Shiga-toxin producing *Escherichia coli* (STEC) are a group of Gram-negative bacteria, pathogenic to humans. The most common source of STECs is ruminant animals, where the bacteria are non-pathogenic. *Escherichia coli* O157 is a STEC that was first identified in 1982 and can cause gastroenteritis and hemolytic uremic syndrome in humans. There have been several notable outbreaks of *E. coli* O157 in the U.S. involving unpasteurized apple cider, and beef from the food chain 'Jack in the Box' (Knight et al., 2007; Besser, 1993). While hemolytic uremic syndrome is rare, it can develop in young children, and the elderly infected with STEC, and in some cases, it can result in death. Most STEC research comes from studies involving *E. coli* O157, however non-O157 STECs are often the cause of food-related illnesses in the U.S. Serotypes O26, O111, and O45 are non-O157 STECs that have commonly caused illness but have been largely under documented due to insufficient surveillance (Brooks

et al., 2005). Estimates of illnesses caused by STECs are approximately 265,000 annual cases in the U.S., including upwards of 30 deaths (Scallan et al., 2011).

Salmonellosis, the disease caused by the Gram-negative, human pathogenic bacterium *Salmonella enterica*, is the cause of an estimated 1.2 million illnesses and 450 deaths, annually. In some cases, salmonellosis can be mild (gastroenteritis) and severe in others, even leading to invasive, life-threatening infections. In the U.S., the most common serovars linked to salmonellosis are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Scallan et al., 2011)

Staphylococcal food poisoning is an illness of the gastrointestinal tract in humans caused by several toxins produced by the Gram-positive bacterium *Staphylococcus aureus*. *Staphylococcus aureus* is carried in approximately 25% of healthy people on the skin and nose. Illness usually will not occur in healthy carriers of *S. aureus* until it is transmitted to food. Food poisoning occurs when food workers carrying *S. aureus* use unhygienic practices when preparing food (Evenson, 1988). One of the most notable strains of *S. aureus* is methicillin-resistant *S. aureus* (MRSA), which has developed resistance to penicillin and cephalosporin classes of antibiotics. Due to the inability to treat MRSA with a wide range of antibiotics, infections can cause devastating effects in hospital settings where groups of immune-compromised patients are found in close proximity (Enright et al., 2002). Approximately 463,017 hospitalizations occurred in 2009 due to MRSA-associated illnesses in the U.S. (Klein, et al., 2013).

Extractives

Antimicrobial, phenolic compounds are found ubiquitously in all plants (Nicholson et al., 2003). Switchgrass extractives are defined here as the phenolic-rich, non-structural components of switchgrass produced as a byproduct during the biofuel production process. *Panicum virgatum*, commonly known as switchgrass, is a feedstock used in the production of second-generation biofuel production. Switchgrass is an attractive choice for cellulosic biofuel production due to its inexpensive production cost and high energy yields. Biofuel produced from switchgrass yields a 540% increase in renewable energy versus non-renewable energy consumed (Schmer et al., 2008). As part of a plant's defense system, phenolic compounds are used to inhibit cellulase enzymes (Mandels & Reese, 1965). Cellulases are utilized by many plant pathogens to increase penetration into plant cells, but are also play an important role in the biochemical conversion process for biofuel production. Removal of switchgrass extractives allows the enzymes and microorganisms used in hydrolysis and fermentation to perform more efficiently. Pre-treatment options exist to mitigate the inhibition of enzymes and microorganisms caused by phenolic compounds in switchgrass, such as the use of phenolic acid esterases. Another option is the separation and collection of the phenolic compounds to be developed as a value-added co-product, potentially making switchgrass a more economically viable option for lignocellulosic biofuel production (Anderson & Akin, 2008).

The use of plants for medicinal and antimicrobial purposes is a practice that has existed for thousands of years (Evans & Cowan, 2006). With increasing concerns about the environmental safety of conventional pesticides, plant extract-based biopesticides have been gaining more recognition as an ecologically friendly alternative. Commercial biopesticide products such as Regalia®, an extract of *Reynoutria sachalinensis*, have been shown to control diseases such as powdery mildew on cucurbits and are widely used in commercial crop production (Su, 2012). Regalia® has been shown to have direct antimicrobial effects *in vitro*, as well as acting as a primer to enhance the plant's natural defense system (Conrath et al., 2015). Natural plant products also have their use in the control of human pathogenic bacteria found in food products. Plant extracts are commonly used in the food industry and are generally recognized as safe. Extracts of strawberry have been shown to have antimicrobial activity towards foodborne pathogens such as *S. enterica* Typhimurium when tested in chocolate (Kotzekidou et al., 2008). The antimicrobial activity of phenolic compounds found within plant extracts against bacteria and fungi is likely due to cellulase inhibition and bacterial membrane disruption (Mason & Wasserman, 1987; Cueva et al., 2010). The lipophilic character of small phenolic compounds found within plant extracts have been shown to accumulate in bacterial membranes and cause energy diminution (Conner, 1993).

Research Objectives

The purpose of this research was to assess switchgrass extractives for their potential as a value-added biopesticide and disinfectant. Specifically, the objectives of this research were: 1) to test switchgrass extractives for antimicrobial activity against

bacterial plant pathogens; 2) to test switchgrass extractives for antimicrobial activity against fungal plant pathogens; and 3) to test switchgrass extractives for antimicrobial activity against foodborne bacterial pathogens.

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CHAPTER 2
ANTIMICROBIAL ACTIVITY OF EXTRACTIVES AGAINST BACTERIAL
PLANT PATHOGENS

Abstract

Panicum virgatum (switchgrass) is a prominent biomass feedstock for lignocellulosic biofuel production. The phenolic-rich portion of switchgrass, known as extractives, inhibits the enzymes and microorganisms used in downstream processes and can be removed to achieve a higher biofuel yield. Several pre-treatment options exist to lessen the inhibitory phenolic compounds, such as pre-treatment with phenolic acid esterase, but another viable option is the separation and removal of the extractives to develop a value-added biopesticide. Extractives from three batches of switchgrass, each processed in a Organosolv bioreactor and concentrated three-fold, were further concentrated and assessed for antimicrobial activity against eight bacterial plant pathogens: *Xanthomonas perforans*, *X. axonopodis* pv. *malvacearum*, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *tabaci*, *Clavibacter michiganensis* subsp. *michiganensis*, and *C. michiganensis* subsp. *nebraskensis*. Assessment of antimicrobial activity of all bacterial plant pathogens was done using disk diffusion assays in a laboratory setting. Furthermore, *in planta* assays were conducted to control disease caused by *X. perforans*, *C. michiganensis* subsp. *michiganensis*, and *P. syringae* pv. *tomato* on 'Mountain Spring' tomato in a greenhouse. Antimicrobial activity of all pathogens was observed. In disk diffusion assays, *X. perforans* and *X. axonopodis* were most sensitive to extractives, while *A. tumefaciens* and *P. syringae* pv. *tomato* were the least sensitive. Based on *in planta* assays, pre-treatment of tomato foliage with extractives was effective for control of *C. michiganensis* subsp. *michiganensis*. Post-treatment with extractives was effective for

control of *P. syringae* pv. *tomato*. While treatment with extractives was effective in controlling *X. perforans*, and there was no difference in efficacy between post- and pre-treatment.

Introduction

The perennial bunchgrass, *Panicum virgatum* (switchgrass), is a plant native to North America that is currently being assessed as a biomass crop for lignocellulosic biofuel production. The non-structural, phenolic-rich portion recovered from biomass of switchgrass (known as extractives) contains compounds such as ferulic acid that inhibit enzymes and microorganisms used in the enzymatic hydrolysis and fermentation phases of biofuel production (Kim et. al., 2011). Phenolic compounds have long been known to be a part of the plant defense system in combatting ingress of pathogenic microorganisms (Mandels & Reese, 1965). One option for increasing the efficiency of the lignocellulosic biofuel production process is to remove and collect the extractives for development into a potentially marketable co-product, thus making switchgrass a more economically feasible biomass feedstock (Anderson & Akin, 2008).

Commercial plant extract-based products that are available reduce infection by plant pathogens both *in planta* and *in vitro*. One such product that is widely used is Regalia®, which is an extract of *Reynoutria sachalinensis*. Regalia® acts as a primer to increase the natural production of phenolics and reactive oxygen species within the plant, as well as directly inhibit the growth of pathogens on the plant (Conrath et al., 2015). Direct inhibition of bacteria by phenolic compounds found in plant extracts is

likely due to the lipophilic nature of the phenols, allowing the molecules to amass in the bacterial membrane and cause energy depletion (Conner, 1993).

Methods and Materials

Extractives Preparation

Extractives from switchgrass processed in an Organosolv bioreactor in Dr. Nicole Labbé's lab at the University of Tennessee's Center for Renewable Carbon (CRC) were extracted using an ethanol wash recycled over three batches of fresh biomass feedstock. Therefore, extractives collected in 95% ethanol from these runs had an effective concentration of 3X. Three sets of switchgrass extractives (CRC #44, CRC #25, and CRC #58) were collected and total phenolic content was determined as Gallic acid equivalents, based on reaction with Folin-Ciocalteu reagent (Fig.1; Ainsworth and Gillespie, 2007). Free sugar content of extractives was measured with high performance liquid chromatography (Table 1). Components of the extractives were analyzed with ultra-performance liquid chromatography (UPLC) with a UV detector and UPLC-mass spectrometer (Table 2). Extractives were further concentrated using a combination of Pierce Reacti-vap™ and Reacti-therm™ evaporation units. Extractives were measured volumetrically and pipetted into sterile 15-mL test tubes containing magnetic stir bars, which were then placed in the evaporation unit combination. The volume of extractives was reduced to achieve desired concentrations via evaporation under N₂ gas streamed through BD™ spinal needles connected to a 0.45-µm syringe filter. Once the desired concentration was achieved, sterile, blank antibiotic disks were saturated with

concentrated extractives. The saturated disks were placed under a laminar flow hood for 15 min to evaporate any residual ethanol that remained on the disks. Similarly, cotton-tipped applicators were saturated with concentrated extractives and dried under a laminar flow hood for 15 min to remove remaining ethanol for *in planta* assays.

Preparation of Inoculum of Bacterial Plant Pathogens

Bacterial plant pathogens (*Xanthomonas perforans*, *X. axonopodis* pv. *malvacearum*, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *tabaci*, *Clavibacter michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *nebraskensis*) were grown overnight in tryptic soy broth (TSB) on a shaker (150 rpm) at room temperature. The cultures were transferred to centrifuge tubes and centrifuged at 2000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 10 mL of phosphate buffered saline (PBS). The cultures were centrifuged a second time and the supernatant was discarded. The bacterial pellet was suspended in 10 mL of PBS in a sterile 15-mL test tube using a sterile, cotton-tipped applicator. A Biolog™ turbidimeter was used to achieve a turbidity of 50% (approximately 1×10^8 CFU per mL).

Bacterial Plant Pathogen Disk Diffusion Assay

Tryptic soy agar (TSA) plates were spread with 100 µL of bacterial solution to create a lawn of bacterial growth. Blank, sterile antibiotic disks were saturated with extractives CRC #44, CRC #58, or CRC #25. Extractive CRC#44 and CRC #25 were

used in the first assays at concentrations of 30X, 45X, 60X, and 75X. Later assays with CRC #58 were done only using concentrations 60X and 75X due to limited extractive supply. After the bacterial inoculum was spread across the surface of the agar, three extractive-treated disks and one non-extractive control disk were placed on the surface of each TSA plate. In order to diffuse the extractives from the disk onto the TSA, 40 μ L of sterile water was pipetted onto each disk. Plates were incubated at room temperature for 24 h. Zones of inhibition around each disk were measured after incubation. Inhibition zones were defined as the distance from the margin of the disk to the edge of the bacterial lawn at three different locations around each disk. The three measurements were averaged to give a single measurement per disk. Inhibition zone measurements were recorded and analyzed using mixed model ANOVA procedure in SAS 9.4.

Bacterial Plant Pathogen In Planta Assay

In order to assess the efficacy of switchgrass extractive as an antimicrobial *in planta*, extractives CRC #44, CRC #25, and CRC #58 concentrated at 60X were used as post- and pre-treatments on greenhouse tomato plants inoculated with *X. perforans*, *P. syringae* pv. *syringae*, and *C. michiganensis* subsp. *michiganensis*.

Switchgrass extractives concentrated to 60X were absorbed onto sterile, cotton-tipped swabs. The swabs were then placed under a laminar flood hood for 15 min to evaporate any residual ethanol.

Switchgrass extractives were swabbed onto three leaflets of one tomato leaf per plant. To facilitate the release of extractives, a fine mist of sterile water was sprayed

onto the leaflets before the extractive saturated swab was applied. In the pre-treatment applications, extractives were applied 1 h before inoculation with the bacterial plant pathogen. Extractives were applied 1 h after inoculation with the post-treatment.

Tomato plants were inoculated using bacterial inoculum prepared as described for the disk assay. One leaf was chosen per tomato plant, with three inoculations per leaflet. Each leaflet was punctured six times with a sterile needle to facilitate entry of the bacterial pathogens into the leaf tissue. The underside of the leaflet was supported with a rubber stopper to prevent any injuries while puncturing with the needle. Sterile cotton-tipped applicators were used to swab the leaflets with bacterial inoculum. Plastic bags, acting as humidity chambers, were placed over the leaf. Disease symptoms were allowed to manifest for 10 days, then the leaves were excised.

Percent lesion area on tomato leaves was analyzed with Assess 2.0 Image Analysis Software. Tomato leaves were photographed after being removed from the greenhouse. In order to achieve a photo for lesion area analysis, the tomato leaves needed to be as flat as possible. Non-reflective glass was placed over the top of the leaf to smooth out any ridges on the leaf. Blue poster board was used underneath the leaf to provide a uniform background color. A uniform background color allows Assess 2.0 to recognize automatically the area of the leaflet. If a uniform background color is not used, the leaflet area must be traced by hand. The leaves were photographed from a top down position using a Nikon D5200. Photographs were uploaded to Assess 2.0 to determine percent lesion area of each leaflet. Total leaflet area was determined before total leaflet lesion area. Leaflet area was calculated by using the trace function. First, a

circle was traced around the leaflet. Next, the leaf area button was used to find the leaflet area. Because a uniform color was used in the background of the photo, Assess 2.0 presets automatically find the area of the leaflet. When the program misjudged the leaflet area, the hue bar was used to make adjustments. Once the leaflet area was determined, the lesion button was used to calculate lesion area. Like the leaflet area, any adjustments that needed to be made for lesion area picked up by the program were made using the hue adjustment bar. Finally, the percent lesion button was used to calculate the percent lesion area on each leaflet.

Results and Discussion

Bacterial Plant Pathogen Disk Diffusion

Extractives from each of the switchgrass samples differed in total phenolic content (Fig. 1). Concentration of extractives had no effect on antimicrobial activity of CRC #44. Antimicrobial activity increased with an increase in concentration of extractive CRC #58, but activity decreased with an increase in concentration of extractive #25 (Fig. 2). The difference in activity in assays with CRC #58 and CRC #25 could be due to a limitation of the disk diffusion assay, i.e., there are likely limitations on the amount of extractives that can diffuse from the disks into the agar medium once an extractives concentration of 60X and higher is achieved. Alternatively, certain active components of CRC #25 could be less conducive to diffusion at higher concentrations. The eluate of the disks treated with 60X extractives will also have a relatively higher water content and could help deliver the phenolic compounds more efficiently through the bacterial

membrane than the eluate of the disks treated with 75X. The extractive preparations also differed in the reaction of plant pathogenic bacteria. Inhibition of pseudomonads and *X. perforans* was greatest for disks treated with CRC #25, but relative inhibition for the pseudomonads was not as great for extractives CRC #44 and CRC #58. In general, inhibition of *A. tumefaciens* was lower in the disk assays, relative to most other bacteria. In nature, when *A. tumefaciens* is exposed to phenolic compounds, the expression of virulence loci are induced, which could explain the lowered amount of inhibition observed (Bolton et. al.,1986) (Fig. 3). Variation in the effect of pathogen could be explained by the natural variation in phenolic compounds present in the different batches of switchgrass biomass. The extractives are not isolated compounds, but a crude mixture. Extractives from one run could contain a higher concentration of certain phenolic compounds than extractives from other runs, thus leading to variation in antimicrobial activity against the pathogens tested.

Bacterial Plant Pathogens In Planta

Disease from post- and pre-treatment with switchgrass extractive CRC #44 on tomato leaflets inoculated with *X. perforans* had no significant difference, although lesion area was significantly less than positive control leaflets inoculated with the pathogen (Fig. 4). The pre- and post-treatments were also not different from the negative controls, with no pathogen added. Overall, in the assay with CRC #44, disease pressure was low with less than 25% leaflet lesion area in the positive control. Disease pressure was higher in tests with CRC #58 and CRC #25, based on results with the

positive control (>80% leaflet lesion area). However, with both batches, pre-treatment had significantly less disease than the positive control. Pre-treatment with CRC #58 was greater than the negative control, while CRC #25 pre-treatment was not different from the negative control. Post-treatment with CRC #58 was intermediate between the positive control and the pre-treatment, but not different from either one of them. Post-treatment with either CRC #58 or CRC #25 had significantly more disease than the negative control.

In assays with tomato leaflets inoculated with *P. syringae* pv. *tomato*, leaflets pre-treated with CRC #25 and CRC #58 had significantly less lesion area than post-treated leaflets and non-extractive positive control leaflets. In assays with CRC #44, however, post-treated leaflets had significantly less lesion area than the positive control and pre-treated leaflets (Fig. 5). This suggests that CRC #25 and CRC #58 have a plant defense priming mode of action in combatting *P. syringae* pv. *tomato*, while CRC #44 acts as a direct inhibitor. It is also possible that the compounds active against *P. syringae* pv. *tomato* in the crude extract could have degraded in the hour between application of extractive CRC #44 and inoculation with *P. syringae* pv. *tomato*.

In assays with tomato leaflets inoculated with *C. michiganensis* subsp. *michiganensis*, post-treatment with CRC #44 and CRC #58 had significantly less lesion area than the positive control and the pre-treatment. While leaflets treated with CRC #25 had significantly less lesion area than the positive control, there were no differences in post- and pre-treatment. This suggests that extractive CRC #44 and CRC #58 have activity in directly inhibiting *C. michiganensis* subsp. *michiganensis* (Fig. 6).

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Appendix

Table 1. Free sugar content in dried extract as measured with high performance liquid chromatography. Values are reported as means (standard error). Data were provided by Dr. N. Labbé.

	Sucrose	Glucose	Xylose	Rhamnose	Arabinose	Fructose
CRC#25	15.4 (1.2)	43.9 (4.7)	9.4 (0.8)	6.0 (0.6)	8.6 (0.7)	42.5 (5.6)
CRC#44	13.0 (1.7)	40.0 (0.6)	15.2 (2.2)	7.8 (1.0)	8.6(0.1)	32.2 (1.3)
CRC#58	16.3 (1.6)	42.8 (7.0)	2.4 (0.5)	4.0 (0.6)	7.8 (1.6)	44.5 (3.9)

Table 2. Antimicrobial components of switchgrass extractives detected by ultra-performance liquid chromatography (UPLC) with a UV detector and UPLC-mass spectrometer. Data were provided by Dr. N. Labbé.

Chemicals	Antibacterial	Fungicide
Ascorbic acid	✓	
Caffeic acid	✓	✓
Ferulic acid	✓	✓
Furfural		✓
Gallic acid	✓	
p-Coumaric acid	✓	✓
Phenol	✓	✓
Quercetin	✓	✓
Quercitrin	✓	
Rutin	✓	
Vanillic acid	✓	✓
Vanillin		✓

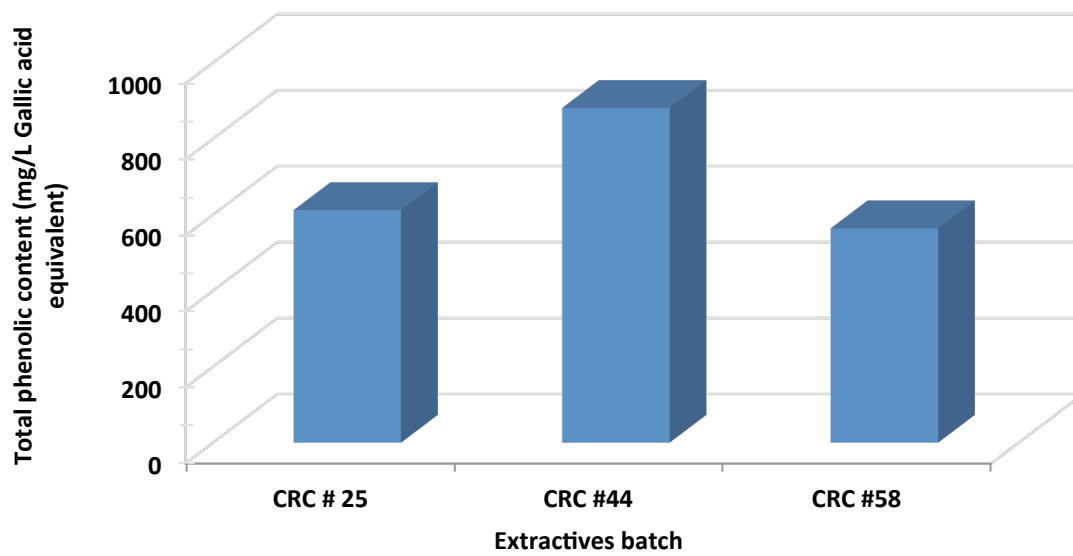


Figure 1. Switchgrass extractives batch and concentration of phenolic compounds. Phenolic content was determined in mg/L Gallic acid equivalent. Data were provided by Dr. N. Labbé.

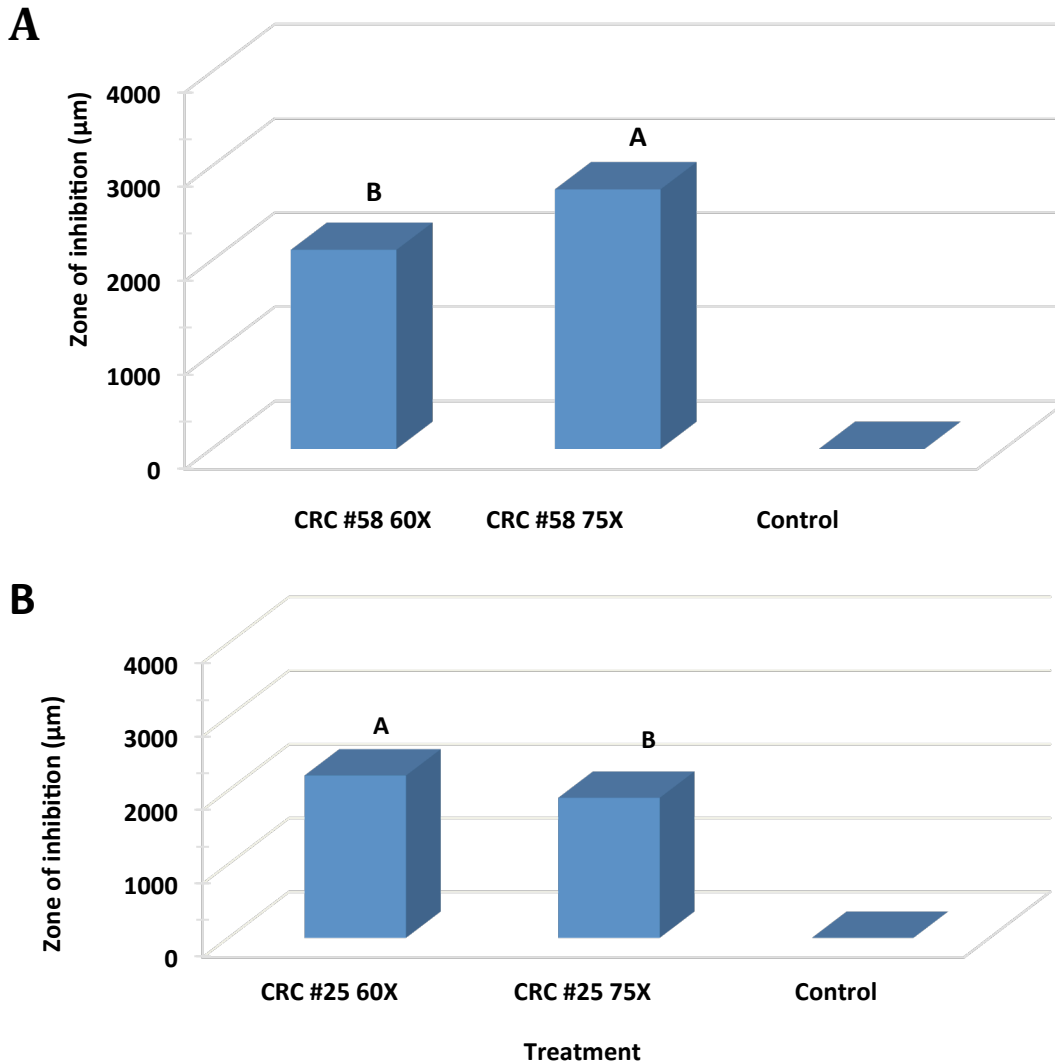


Figure 2. Effect of extractive concentration across all bacterial plant pathogens. A) Extractive = CRC #58. B) Extractive = CRC #25. Data for extractive CRC #44 not shown because the main effect of concentration was not significant. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

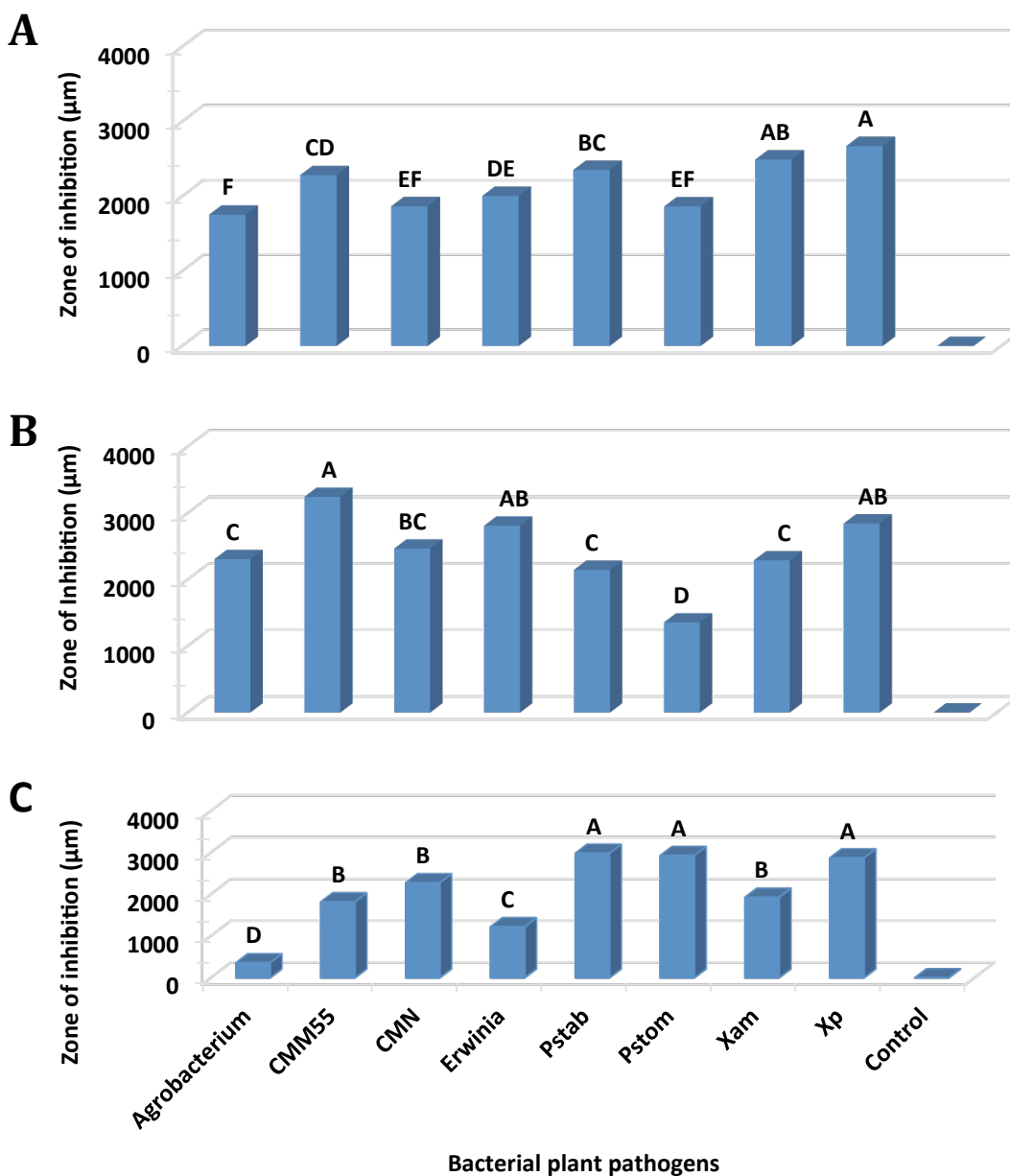


Figure 3. Effect of pathogen across different concentrations of extracts. Agrobacterium = *A. tumefaciens*, CMM55 = *Clavibacter michiganensis* subsp. *michiganensis*, CMN = *C. michiganensis* subsp. *nebraskensis*, Erwinia = *E. amylovora*. Pstab = *Pseudomonas syringae* pv. *tabaci*, Pstom = *P. syringae* pv. *tomato*, Xam = *Xanthomonas axonopodis* pv. *malvacearum*, Xp = *X. perforans*, Control = no pathogen. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44 across 30X, 45X, 60X, and 75X concentrations. **B)** Extractive = CRC #58 across 60X and 75X concentrations. **C)** Extractive = CRC #25 across 60X and 75X concentrations.

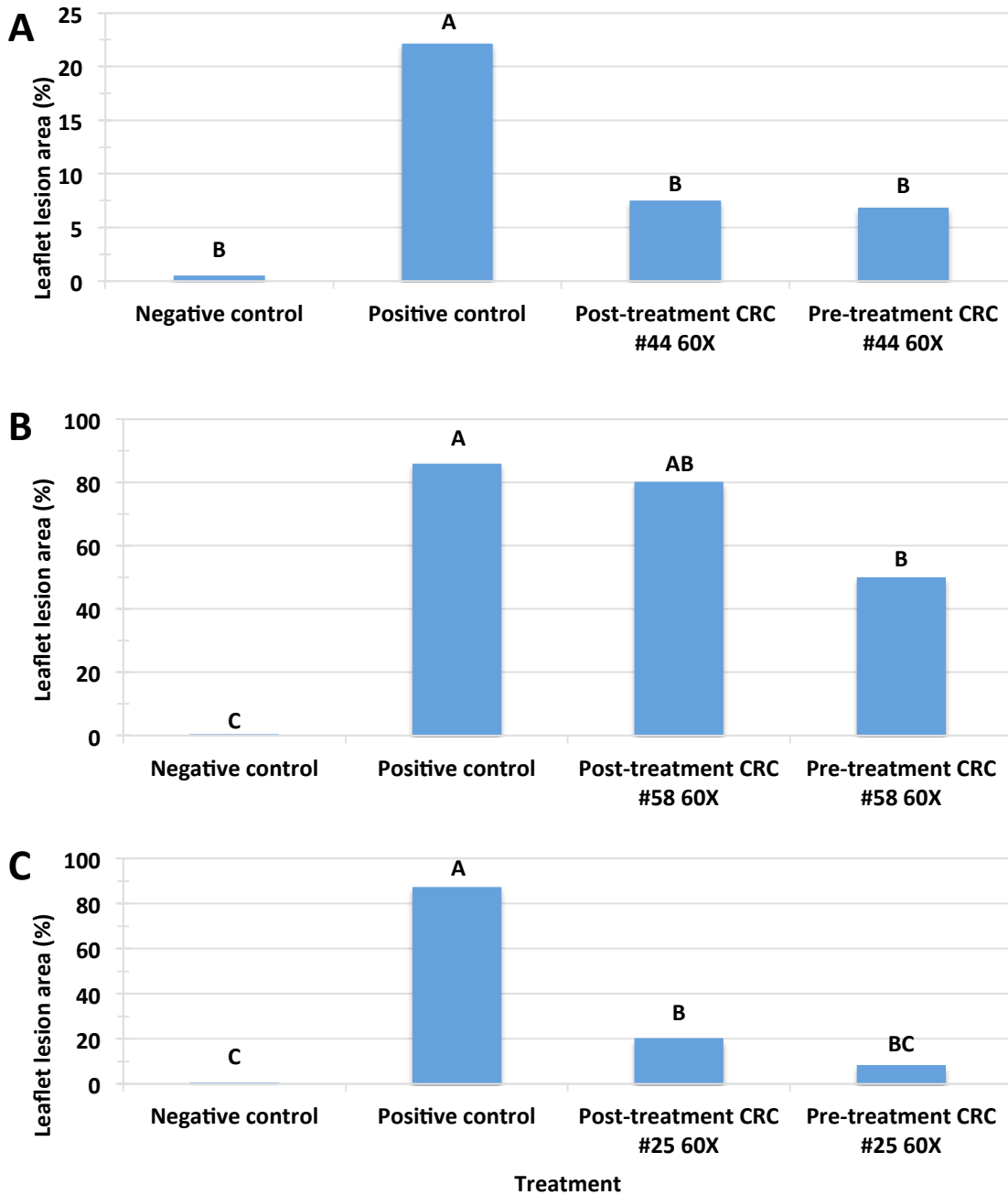


Figure 4. Effect of 60X extractive applied as post- and pre-treatment on percent lesion area of tomato leaflets inoculated with *Xanthomonas perforans*. Negative control = no pathogen applied to leaf. Positive control = pathogen applied to leaf, but no extractive applied. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **(A)** Extractive = CRC #44. **(B)** Extractive = CRC #58. **(C)** Extractive = CRC #25.

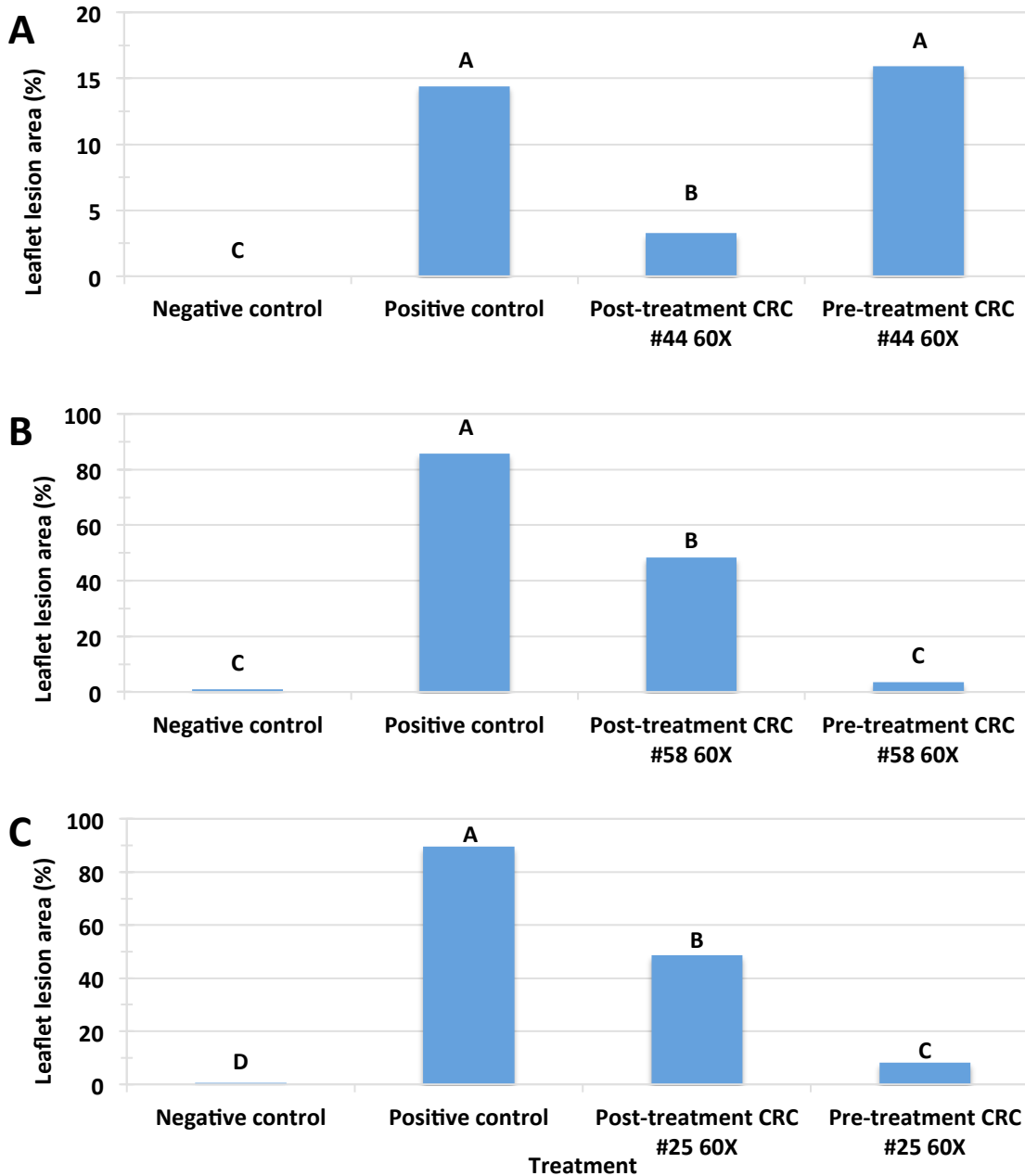


Figure 5. Effect of 60X extractive as a post- and pre-treatment on percent lesion area of tomato leaflets inoculated with *Pseudomonas syringae* pv. *tomato*. Negative control = no pathogen applied to leaf. Positive control = pathogen applied to leaf, but no extractive applied. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

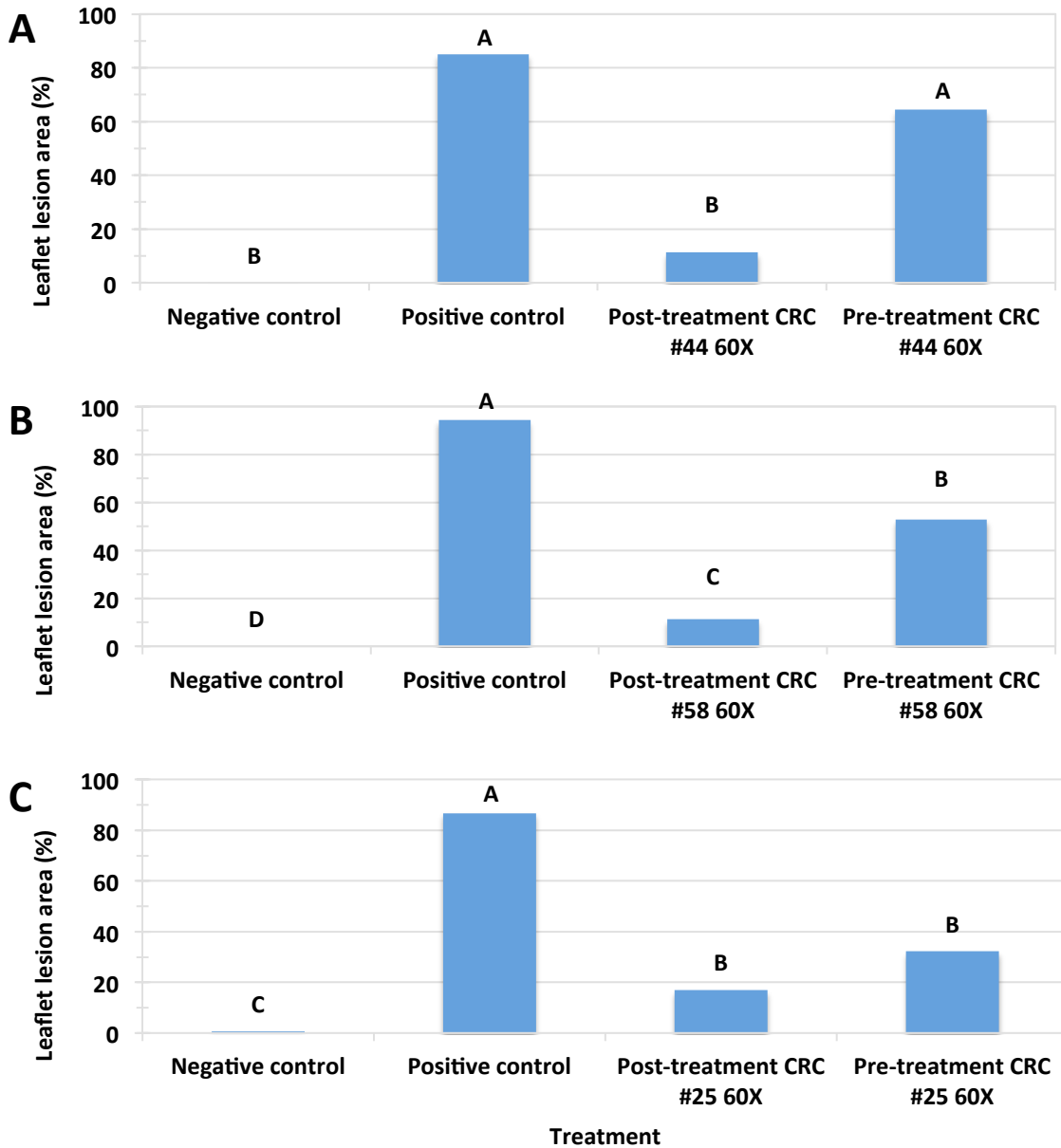


Figure 6. Effect of 60X extractive as a post- and pre-treatment on percent lesion area of tomato leaflets inoculated with *Clavibacter michiganensis* subsp. *michiganensis*. Negative control = no pathogen applied to leaf. Positive control = pathogen applied to leaf, but no extractive applied. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

CHAPTER 3
ANTIMICROBIAL ACTIVITY OF EXTRACTIVES AGAINST FUNGAL
PLANT PATHOGENS

Abstract

Value-added products are generally a waste material from an industrial process that can be developed and sold for economic gain. In production of second-generation biofuel using *Panicum virgatum* (switchgrass) lignocellulosic biomass, waste products such as lignin and the non-structural components of switchgrass are candidates for development of value-added products. Extractives from switchgrass are known to be rich in phenolic compounds that inhibit the enzymes and microorganisms used in hydrolysis and fermentation. The aim of this study was to assess antifungal effects of three batches of switchgrass extractives (CRC #44, CRC #58, and CRC #25) against *Fusarium oxysporum*, *Fusarium graminearum*, *Alternaria alternata*, *Botrytis cinerea*, and *Bipolaris oryzae* for potential development of a biopesticide. Antifungal activity was observed in disk diffusion, *in planta* and spore germination assays. Difference in colony diameter was measured in disk diffusion assays; treatments were disks treated with 60X and 75X concentrations of extracts, and 'no extract' control. *Bipolaris oryzae* was the least susceptible pathogen in disk diffusion assays with CRC #44 and CRC #58, while *A. alternata* was among the most susceptible pathogen in all disk diffusion assays. Leaflet lesion area was measured for *in planta* assays; treatments were extractives concentrated at 60X and 75X applied as post- and pre-treatments to tomato leaflets inoculated with *A. alternata* conidia. While treatment with extractives yielded significantly less lesion area in all assays *in planta*, no significant differences were observed between post- and pre-treatment with extractives CRC #44 and CRC #25. However, pre-treatment with extractive CRC #58 yielded less lesion area than the

positive control and post-treatment. Spores incubated in a water control or 10X extractives were tested for germination rates over a 4 h period. Sampling was done on water agar slides, and germ tube growth was observed microscopically. Spore germination of all fungi tested was significantly inhibited at all exposure times tested.

Introduction

Phenolic compounds are found as non-structural components of the biomass feedstock crop, *Panicum virgatum*, also known as switchgrass. These phenolic-components, known here as extractives, can be removed to increase the efficiency of the biochemical reactions used in the production of biofuel from lignocellulose (Ximenes, et. al., 2011) (Anderson & Akin, 2008). Furthermore, it is known that some phenolic compounds found in plant extracts, such as ferulic acid, have fungicidal properties (Panizzi et. al., 2002). Therefore, switchgrass extractives could conceivably be used as a fungicidal biopesticide against fungal plant pathogens. The objective of this study was to assess antifungal activity of three batches of switchgrass extractives (CRC #44, CRC #58, and CRC #25) for potential development of a biopesticide.

Switchgrass extractives collected from three different runs in an Organosolv bioreactor at the Center for Renewable Carbon (CRC) at the University of Tennessee were concentrated and tested for antifungal activity against the fungal pathogens *Alternaria alternata*, *Fusarium graminearum*, *F. oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, and *Bipolaris oryzae*. Fungicidal properties of switchgrass extractives were assessed using disk diffusion and spore germination assays. Tests using post- and pre-treatments of

concentrated extractives were conducted in greenhouse studies to survey any antifungal activity against *Alternaria alternata* on tomato plants.

Materials and Methods

Extractives Preparation

Extractives were collected from switchgrass by recycling ethanol over fresh biomass three times in an Organosolv bioreactor; therefore, the extractives had an effective concentration of 3X. Extractions were obtained from Dr. N. Labbé [University of Tennessee Center for Renewable Carbon (CRC)]. Three different extractions (referred to as 'extractive runs') were performed on different batches of switchgrass (runs CRC #44, CRC #25, and CRC #58) (Chapter 2, Fig.1). The extractives were concentrated further via evaporation of ethanol using a combination of the Pierce Reacti-vap™ and Reacti-therm™ evaporation units. Extractives were transferred to sterile 15-mL test tubes containing magnetic stir bars and reduced in volume to achieve the desired concentration. Evaporation was done under a sterile stream of N₂ gas routed through BD™ spinal needles connected to a 0.45-µm syringe filter. For disk diffusion assays, sterile blank disks were saturated with extractives and dried under a laminar flow hood for 15 min to evaporate any remaining ethanol solvent on the disks. For *in planta* assays, cotton-tipped swabs saturated with concentrated extractives were used to treat tomato leaflets. The swabs were air-dried for 15 min under a laminar flow hood to evaporate residual ethanol. Extractives used in spore germination assays were dried down from 10 to 0.5 mL (total effective concentration = 60X). Sterile water was then

added to bring the volume back up to 10 mL. A second evaporation from 10 mL (3X) to 1.5 mL (20X) was done to ensure the removal of any residual ethanol. A final concentration of 10X was achieved by mixing conidia suspended in water with 20X extractives, in water at a ratio of 1:1.

Fungal in Planta Assay

Conidia from *Alternaria alternata* were harvested from cultures grown on *Alternaria* sporulation medium (ASM; Shahin and Shephard, 1978) using sterile water and a rubber policeman. The conidial solution was adjusted to a concentration of 8×10^4 conidia per mL using a hemocytometer. The conidia were pipetted on three leaflets per tomato leaf at a rate of 10 μ L per leaflet. One droplet of conidial solution was pipetted directly onto the central vein of the leaflet. Unlike the *in planta* bacterial plant pathogen assay (Chapter 2), no puncture wounds were needed on the leaflets. Many fungal plant pathogens, like *A. alternata*, have specialized cells called appressoria that are used to generate high amounts of pressure to drive a peg of mycelium into the epidermal cells of the leaf. Switchgrass extractives concentrated at 60X were applied onto the leaflets as post- and pre-treatments 1 h before or after the conidia were added. Extractives were absorbed onto sterile cotton-tipped applicators and allowed to air dry for 30 min (to allow residual ethanol to evaporate). Non-extractive controls as well as non-pathogen controls were included. Plastic bags were placed over the leaf to increase relative humidity and promote disease. After 10 days, leaves were excised from the plant and photographed for image analysis of disease lesions. Three leaflets per plant

were analyzed for percent leaflet lesion area in Assess 2.0 and measurements were averaged per replicate. Plants were arranged in a randomized complete block design in the greenhouse. Analysis was done with mixed models ANOVA in SAS 9.4. Means were compared with an F-protected LSD at $P = 0.05$.

Spore Germination Assay

Spore germination rate was analyzed in response to exposure to switchgrass extractives concentrated at 10X. Fungal plant pathogens were propagated on potato dextrose agar (PDA) from slant collections kept in cold storage (4-8°C). After 14 days of growth on PDA, subcultures on different types of sporulation media were made.

Alternaria alternata was cultured on ASM. *Fusarium oxysporum* and *F. graminearum* were cultured on carnation leaf agar (CLA; Fisher et. al., 1982), *Bipolaris oryzae* was cultured on rabbit food agar (RFA; Hau and Rush, 1980), and *Botrytis cinerea* was cultured on PDA. Spores were harvested from the plates using 5 mL of sterile water and a rubber policeman. A hemocytometer was used to standardize the concentration of conidia to 8×10^4 conidia per mL. Water agar slides were prepared by pipetting molten water agar (0.1 mL) onto sterile microscope slides. Ten seconds after pipetting the water agar, a second microscope slide was placed over the still warm agar to flatten it out. Once the agar had fully cooled, the top microscope slide was removed and the agar slide was transferred to storage in a Parafilm® wrapped Petri dish. Centrifuge tubes (2 mL) were used to mix 20X switchgrass extractives and conidia suspended in water at a ratio of 1:1 to achieve a concentration of 10X extractives (0.5 mL of 20X extractives, 0.5

mL of conidia in water). A non-treatment control consisted of sterile water and conidia. Tubes were placed on a shaker for 4 h. Every 30 min, 10- μ L aliquots from the tubes were placed onto the water agar slides. Both the 10X extractives treatment and the non-treatment control were aliquoted in replicates of three. The spores were allowed to germinate for 24 h, after which 10 μ L of lacto-phenol cotton blue were added to each slide to halt growth and stain germ tubes for better visualization. Percent germination was calculated after visualization of each slide for both 10X extractive treatment and the non-treatment control under a compound microscope.

Fungal Disk Diffusion Assay

Blank sterile disks were treated with two concentrations of switchgrass extractives and evaluated for antifungal activity against five different fungal plant pathogens. Fungal plant pathogens *Fusarium graminearum*, *F. oxysporum* f. sp. *lycopersici*, *Alternaria alternata*, *Bipolaris oryzae*, and *Botrytis cinerea* were obtained from slant collections maintained by Dr. B. Ownley, University of Tennessee. Plugs of mycelium were transferred to PDA to propagate the cultures. After 2 weeks, subcultures were made onto water agar. A gridded background was laid underneath the water agar plate to ensure that the plug of mycelium was placed directly in the center of the plate. In order to maintain uniform size, a sterile cork borer was used to excise plugs (5-mm diameter). Cultures were allowed to grow for 3 days, after which four holes were bored into the surface of the water agar using a sterile cork borer (diameter of 5 mm). The background grid was again placed underneath the plate to ensure that the holes were made equidistant from the plug in the center (distance from plug to hole = 3.5 cm).

Molten water agar was pipetted into the holes (40 μ L) in order to seal the holes from the underside of the plate. Once the molten water agar had cooled, two disks treated with extractives and two non-extractive control disks were placed in holes opposite of one another. Sterile water (100 μ L) was added to each disk every 24 h for 3 days. The 5-mm wells dug into the plate allow for the water to rest on top of the disk and slowly diffuse the extractives from the disk into the agar. After 3 days of incubation at room temperature, the colony diameter was measured between the two extractive-treated disks and the two non-extractive control disks. Difference in diameter between treated and control disks were calculated for each replicate. Colony diameter difference was analyzed for significance with mixed models ANOVA (PC SAS, Cary, NC).

Results and Discussion

Fungal Disk Diffusion Assay

Antifungal activity of extractives CRC #44, CRC #58, and CRC #25 was assessed using disk diffusion assays against fungal plant pathogens *A. alternata*, *F. oxysporum* f. sp. *lycopersici*, *F. graminearum*, *B. oryzae*, and *B. cinerea*.

Data were pooled from two trials testing antifungal properties of extractive CRC #44 against fungal plant pathogens for four replications. Shapiro-Wilks test showed the data were normally distributed, and Levene's test showed equal variances. The effect of interaction between extractives concentration and pathogen were significant (Fig. 7). The effect of extractives concentration was not significant, representing no difference between the effectiveness of 60X and 75X concentrations at inhibiting fungal colony

growth. Colony growth was the same for all fungal pathogens when tested at 60X and 75X extractive concentrations, with the exception of *A. alternata*, which was significantly more inhibited at concentrations of 75X than at 60X. Difference in colony diameter was lowest with tests against *B. oryzae*, which suggests a higher level of resistance than the other pathogens tested (Fig. 7). Although an antifungal effect was observed in assays with CRC #44, it was only slight. Diffusion of extractives and retention of active compounds on the antibiotic disk after hydrating the disks could possibly account for this. The phenolic compounds that have diffused into the agar could also be losing bioactivity in the time it took for the mycelia to reach the area around disks.

Higher amounts of inhibition were observed across all fungal plant pathogens in 75X concentrations of CRC #58 when compared to 60X concentrations. No difference was observed in the interaction between 60X and 75X CRC #58 tested against *B. cinerea* and *F. oxysporum* f. sp. *lycopersici*. However, *A. alternata*, *F. graminearum*, and *B. oryzae* were more inhibited by 75X than 60X of CRC #58 (Fig. 8). This contrasts to what was observed in assays with CRC #44, where *B. oryzae* was the least susceptible. This implies that the components in the crude extracts differ and can vary in antifungal activity.

Effect of pathogen was significant in assays with 60X and 75X concentrations of CRC #25. *Alternaria alternata* and *B. oryzae* were inhibited the most, while *B. cinerea*, *F. graminearum* and *F. oxysporum* f. sp. *lycopersici* were inhibited the least (Fig. 9).

Fungal *In Planta* Assay

Alternaria alternata on greenhouse tomatoes (cultivar Mountain Spring) was tested for sensitivity against extractives CRC#44, CRC #58, and CRC #25 concentrated at 60X. Treatments were a post- and pre-treatment of the extractives, applied either before or after application of *A. alternata* conidia onto the tomato leaflets. A non-extractives control consisted of application of *A. alternata* conidia but not the extractives.

While there was no significant difference between the post- and pre-treatments with 60X CRC #44, both treatments had significantly less relative lesion area than the non-extractive positive control. Furthermore, the post-treatment was not significantly different from the non-extractive, non-pathogen plants. Leaflets pre-treated with 60X CRC #58 had significantly less lesion area than the non-extractives control and leaflets post-treated with CRC #58. Post-treated leaflets were not significantly different from the positive control, suggesting that post-treatment with 60X CRC #58 had no antifungal activity. In assays with 60X CRC #25, pre-treated leaflets had significantly less lesion area than the positive control, and were not significantly different than the post-treated leaflets. Leaflet lesion area of post-treated leaflets was not significantly different from either the positive control or the pre-treated leaflets (Fig. 9).

Pre-treatment with switchgrass extractives works better as a control against *A. alternata*. Results from pre-treated leaflets could be due to a priming response from the plant, allowing the plant to have a head start in combatting pathogen ingress that it would not have in post-treatment.

Spore Germination Assay

Conidia of fungal plant pathogens *A. alternata*, *B. oryzae*, *B. cinerea*, *F. graminearum*, and *F. oxysporum* f. sp. *lycopersici* were tested for sensitivity to extractives CRC #44, CRC #58, and CRC #25 concentrated at 10X by measuring germination rate at different exposure times. A non-extractive control consisted of sterile water and conidia. Twenty-four h after conidia were applied to the water agar slides, lactophenol cotton blue was applied to the slides to halt growth and visualize germination. Counts of germinating and non-germinating conidia were made microscopically. Agar slides were replicated three times and percent germination was determined for each slide. Data were analyzed with mixed models ANOVA in SAS 9.4.

Conidia of *A. alternata* had lower germination rates at every exposure time when tested against CRC #44, CRC #58, and CRC # 25 when compared to the non-extractive control. Maximum inhibition was observed at exposure times of either 2.5 or 3 h (Fig. 10).

Bipolaris oryzae conidia had lower germination rates at every exposure time when tested against CRC #44, CRC #58, and CRC #25 when compared to the non-extractive control. In assays with CRC #25, maximum germination inhibition was observed at exposure times of 3 h, while maximum germination inhibition was observed at 4 h in assays with CRC #44 and CRC #58. Further studies are needed to conclude the amount of time *B. oryzae* conidia must be exposed to CRC #44 and CRC #58 to achieve maximum germination inhibition (Fig. 11).

Although germination rates were much higher than the other pathogens tested, *B. cinerea* had significantly lower rates of germination at each exposure time tested when compared to the non-extractive control. Maximum inhibition was observed at 2.5 h of exposure time in assays with CRC #58. However, germination rates dropped at 4 h exposure time in assays with CRC #44 and CRC #25. More research will need to be conducted to observe maximum inhibition (Fig. 12).

Germination rates of *F. graminearum* conidia were significantly lower at 3 and 3.5 h exposure times in assays with CRC #25 and CRC #58, respectively. Maximum inhibition, however, was not observed in CRC #44 until 4 h exposure time, suggesting trials with longer exposure times are need to observe maximum inhibition.

Germination rates of *F. oxysporum* f. sp. *lycopersici* conidia were significantly lowest at 3 h exposure times in assays with CRC #44 and CRC #58, while lowest germination rates were observed at 3.5 h exposure time in assays with CRC #25.

The effect of germination rate inhibition was very pronounced across all assays. Significantly lower germination rates were observed in all pathogens and at all exposure times in assays with all three extractives. The inhibitory effect of the extractives could be more prominent because the conidia are more susceptible than actual mycelia. Also, compared to previous assays, the pathogens are in an environment with much more direct contact with the extractives.

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Appendix

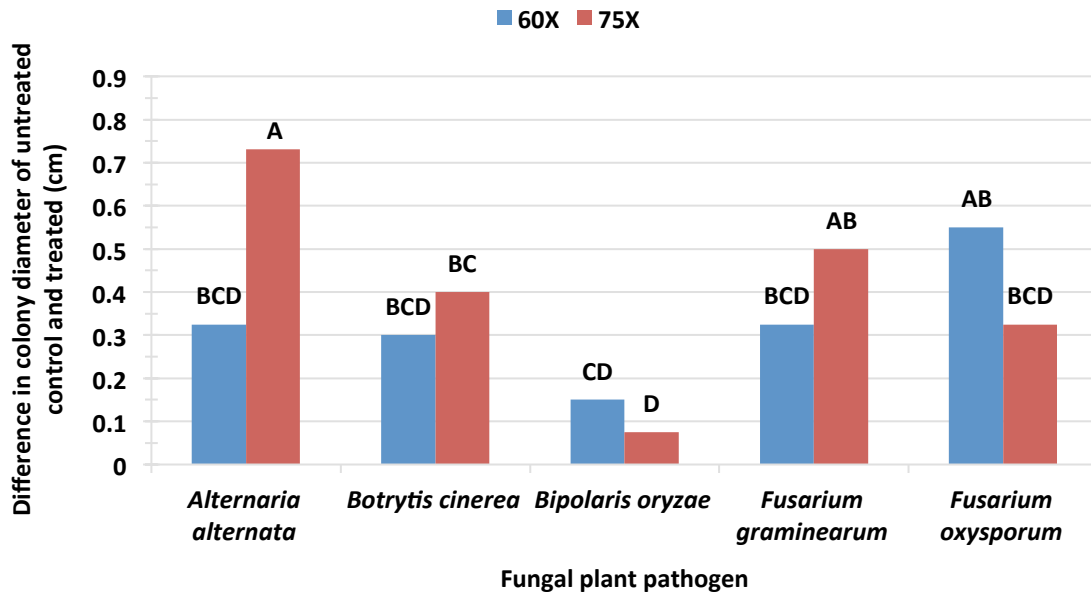


Figure 7. Effect of the interaction between fungal plant pathogens and extractive concentration of CRC #44 on fungal colony diameter. Extractive concentration rates were 60X and 75X. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

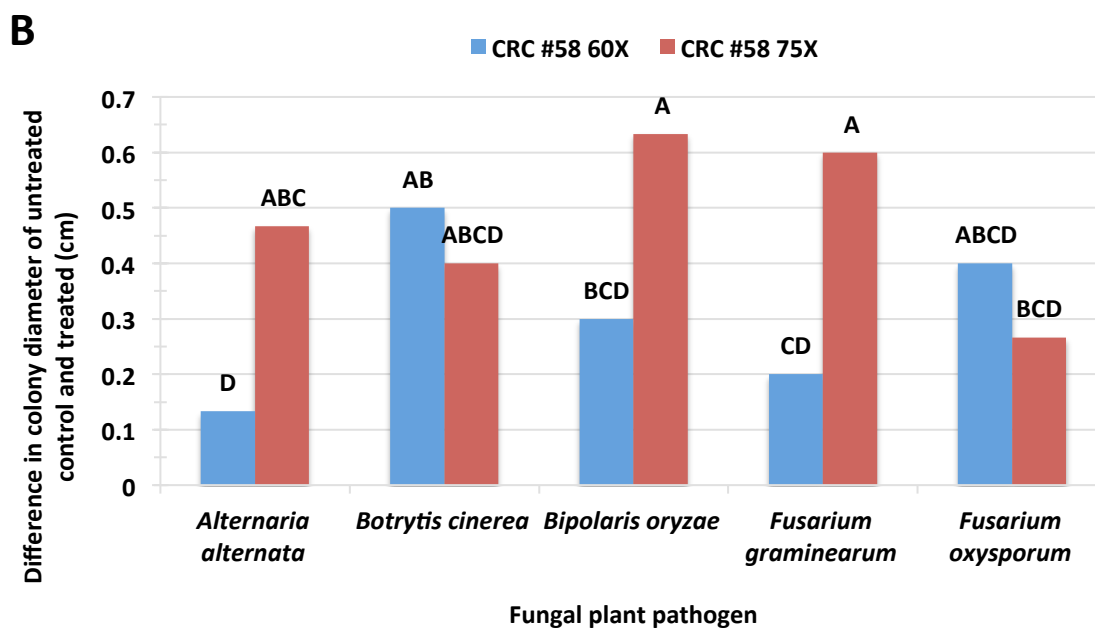
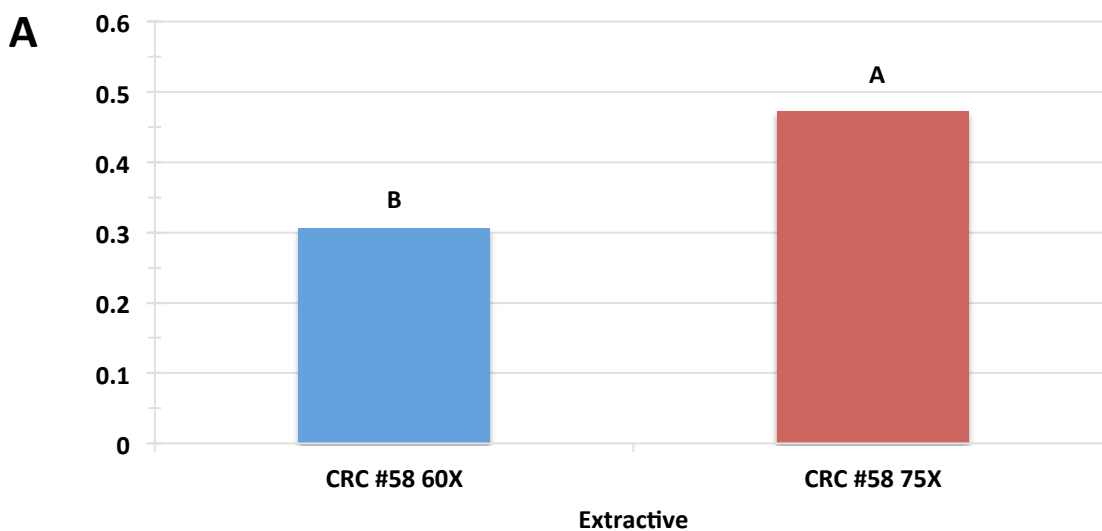


Figure 8. Effect of extractive CRC #58 on fungal colony diameter. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Effect of CRC #58 extractive concentration (60X or 75X), across all plant pathogenic fungi tested. **B)** Effect of the interaction between concentration of extractive and fungal plant pathogen.

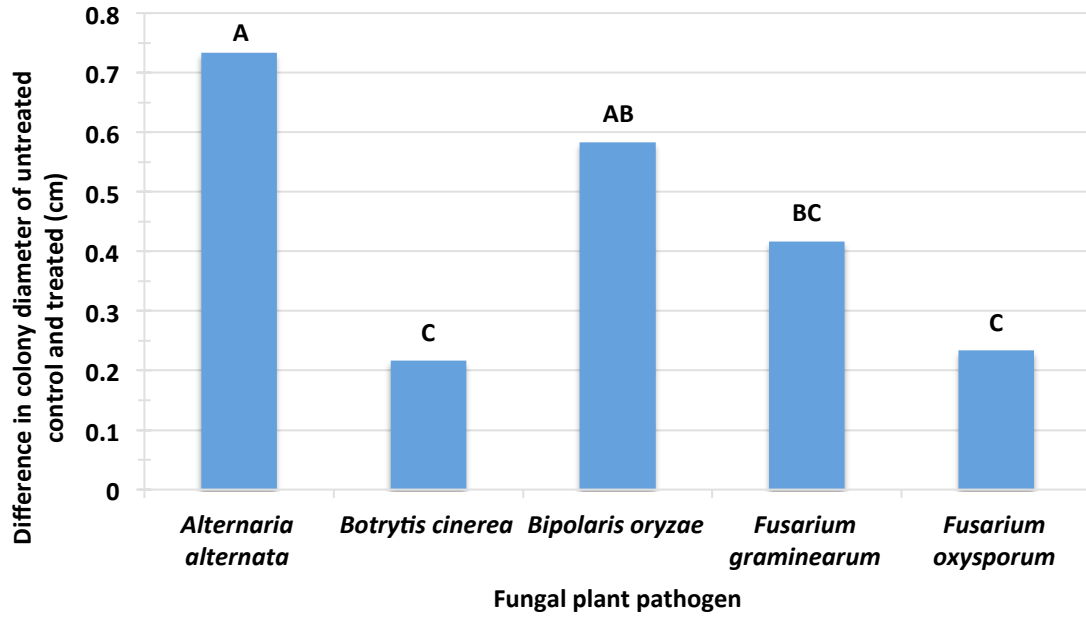


Figure 9. Effect of fungal plant pathogens across all concentrations of extractives (60X and 75X) of CRC #25 in disk diffusion assays. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$.

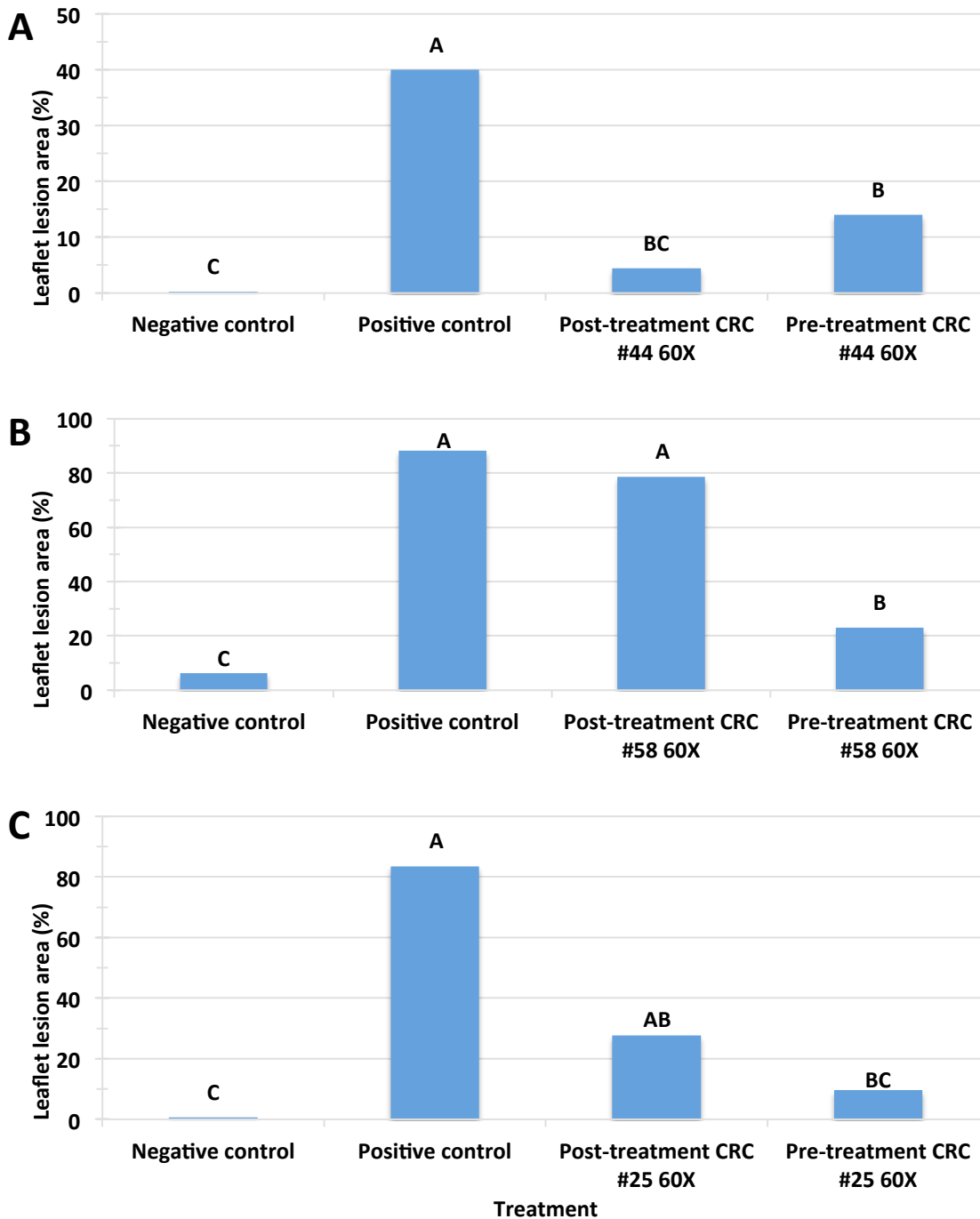


Figure 10. Effect of pre- and post-treatments with 60X concentrations of extractive on foliar lesion area caused by *Alternaria alternata*. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

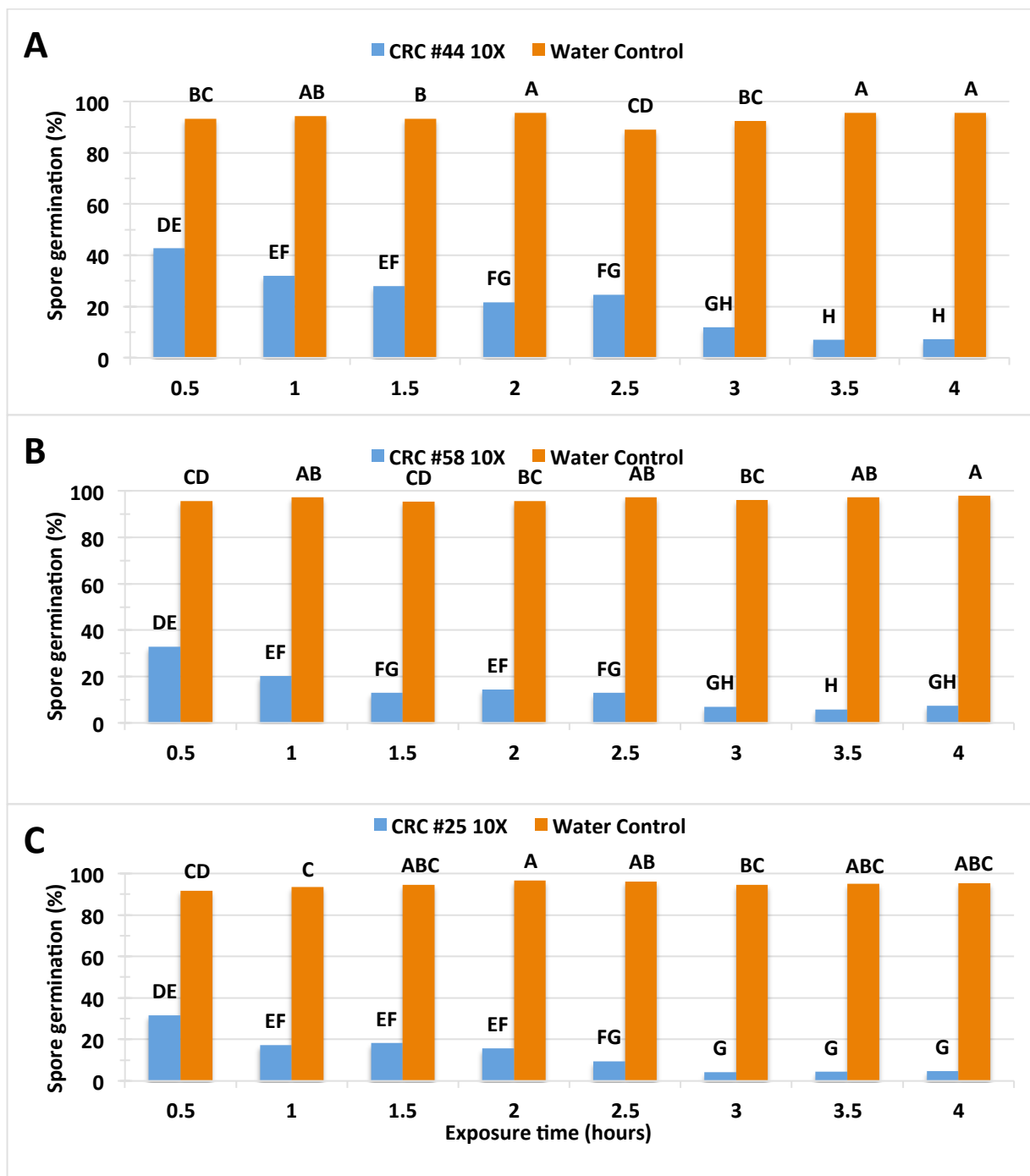


Figure 11. Effect of exposure time on spore germination of *Alternaria alternata* exposed to 10X concentration of extracts. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

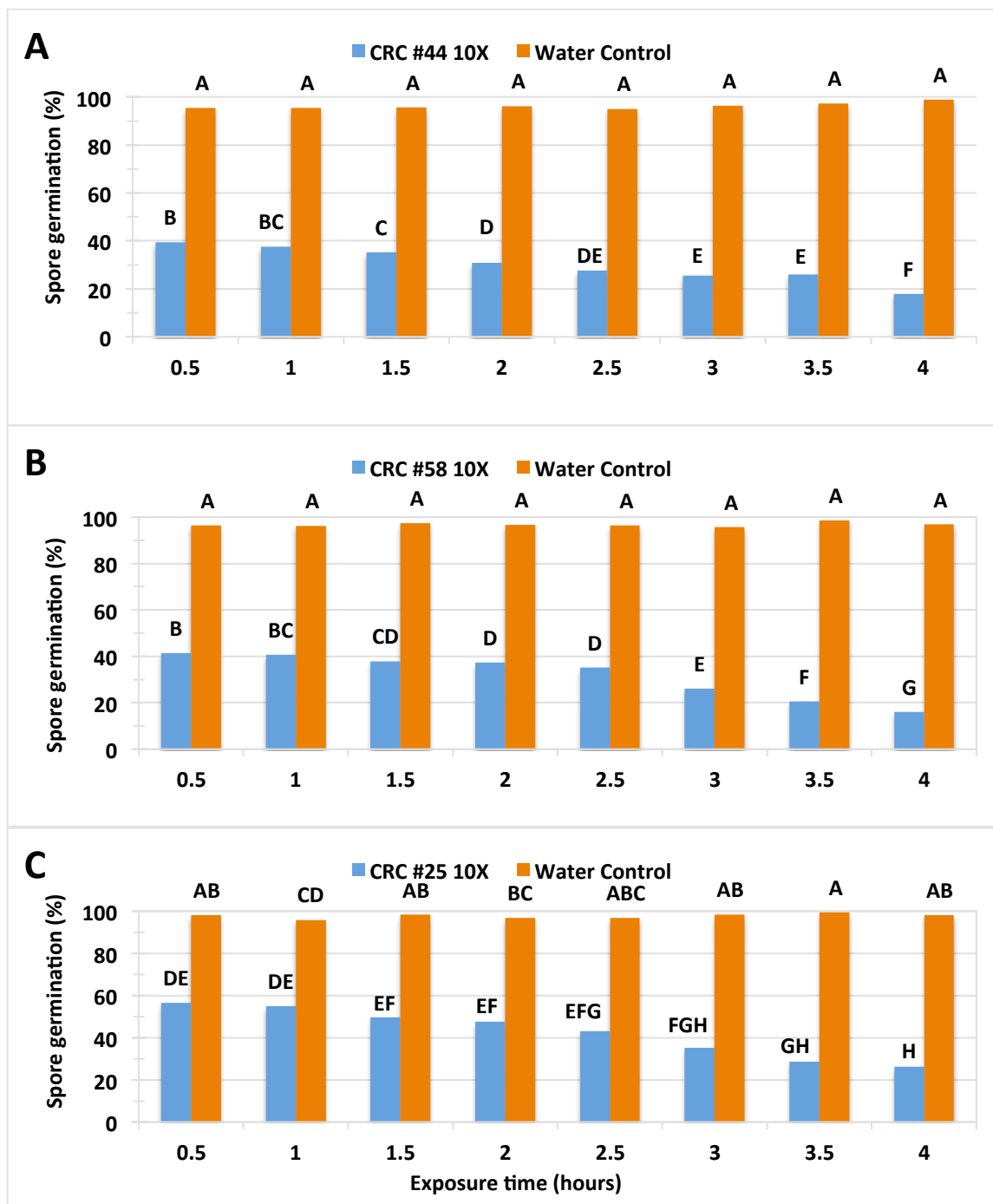


Figure 12. Effect of exposure time on spore germination of *Bipolaris oryzae* exposed to 10X concentration of extracts. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

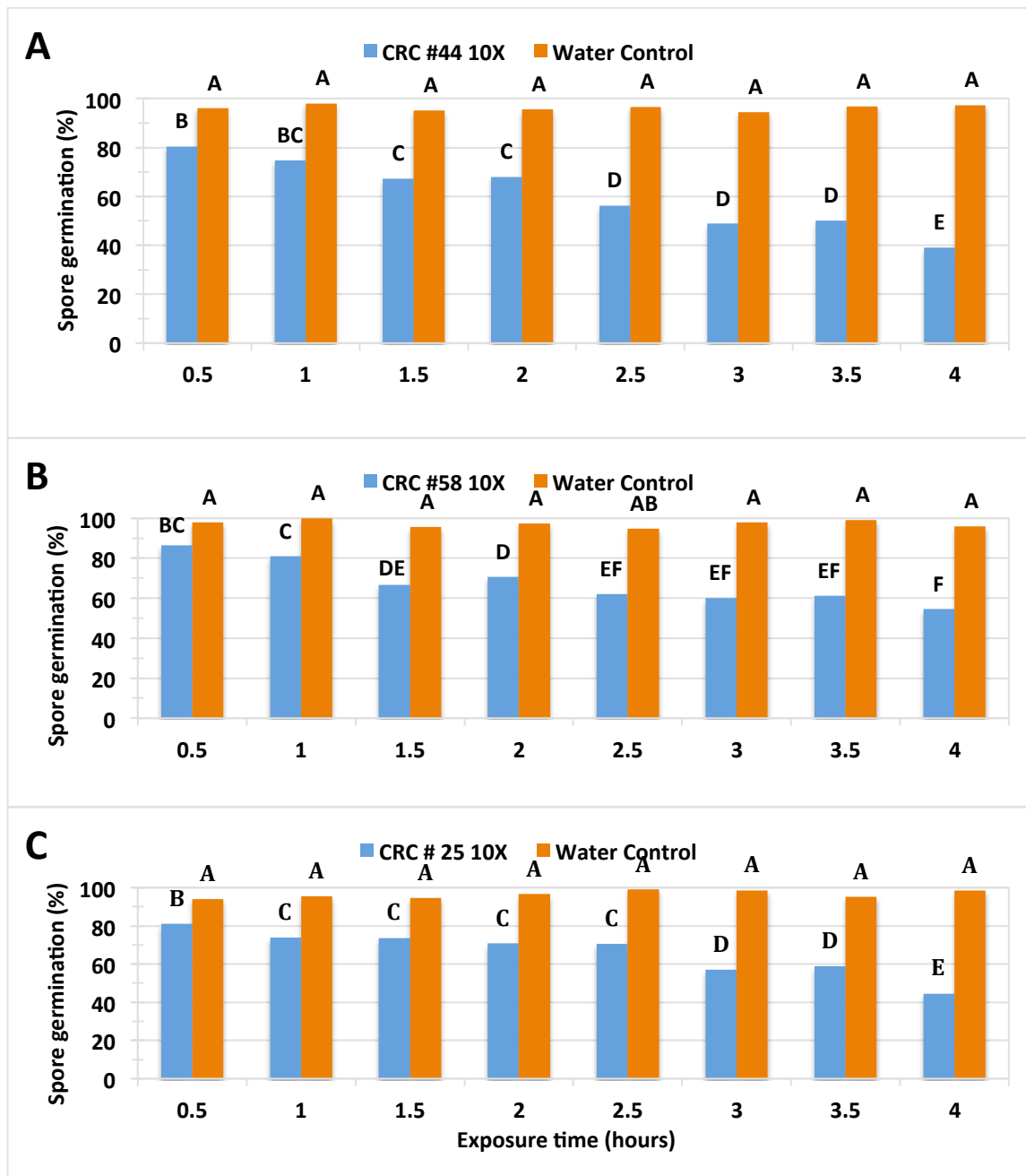


Figure 13. Effect of exposure time on spore germination of *Botrytis cinerea* exposed to 10X concentration of extractives. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

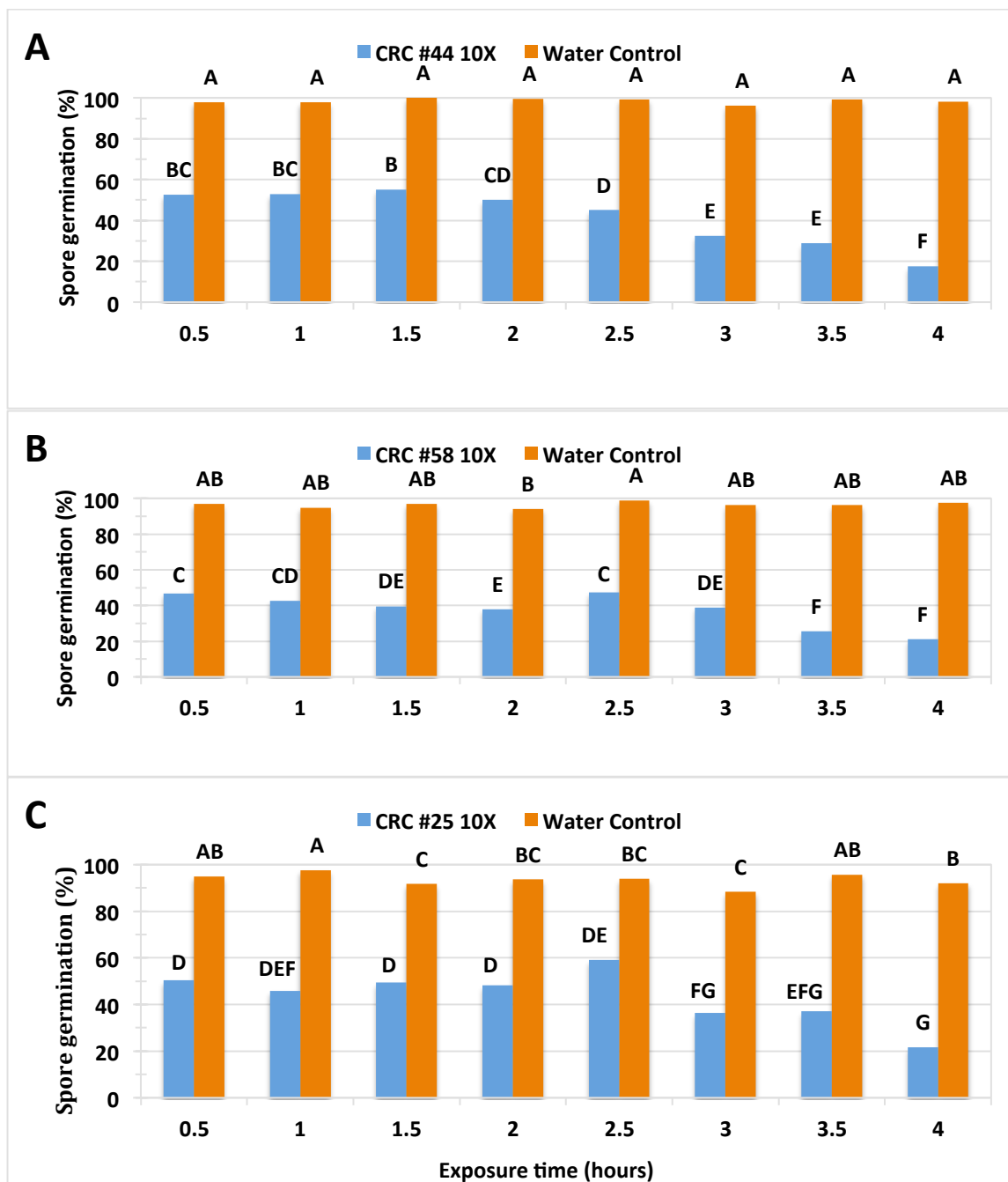


Figure 14. Effect of exposure time on spore germination of *Fusarium graminearum* exposed to 10X concentration of extractives. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

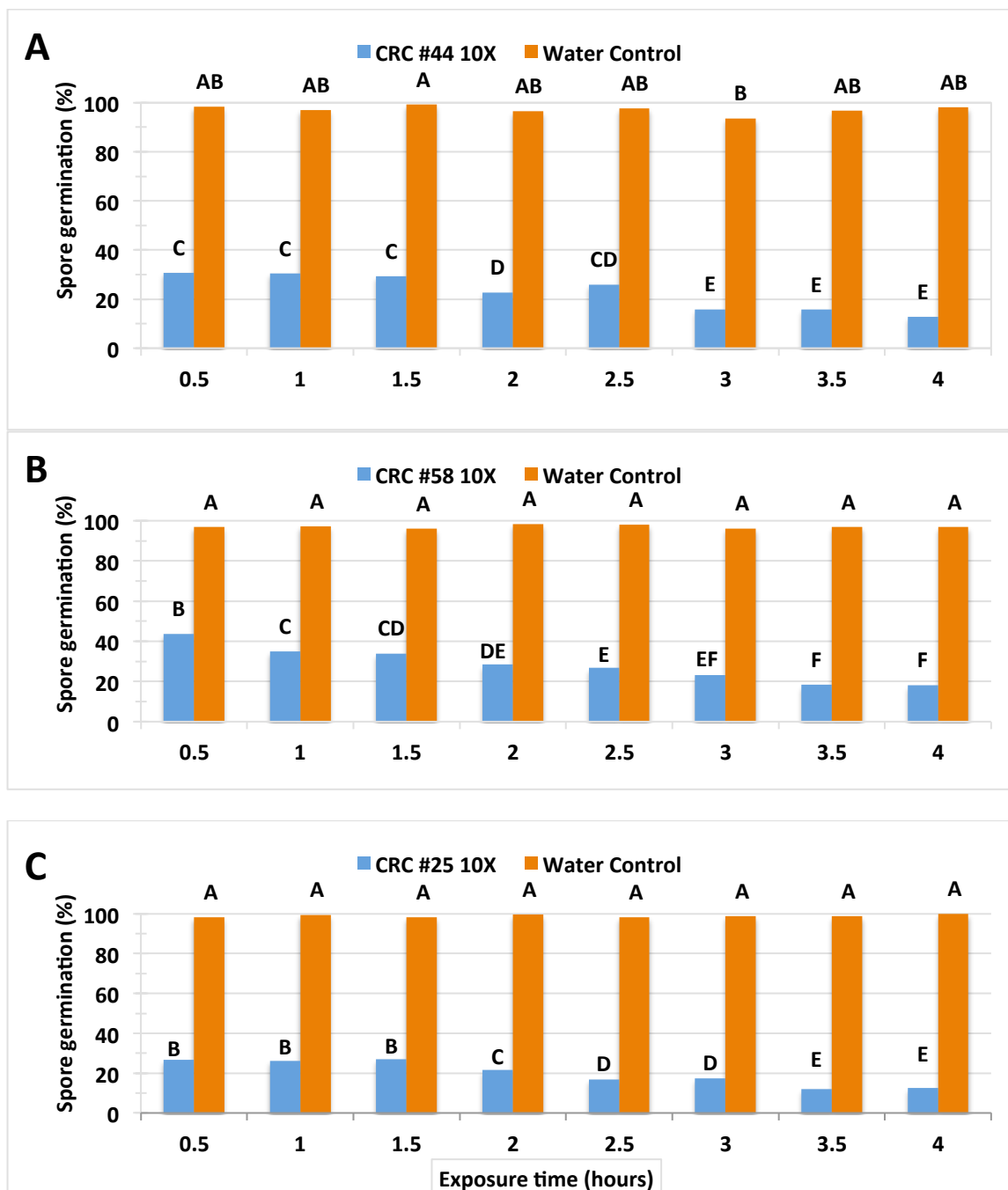


Figure 15. Effect of exposure time on spore germination of *Fusarium oxysporum* f. sp. *lycopersici* exposed to 10X concentration of extracts. Bars with different letters are significantly different according to an F-protected LSD at $P = 0.05$. **A)** Extractive = CRC #44. **B)** Extractive = CRC #58. **C)** Extractive = CRC #25.

CHAPTER 4
ANTIMICROBIAL ACTIVITY OF EXTRACTIVES AGAINST FOODBORNE
PATHOGENS

Abstract

Illnesses related to foodborne pathogens are estimated to affect around 48 million people each year in the U.S. Foodborne pathogens such as *Salmonella enterica*, *Escherichia coli*, and *Staphylococcus aureus* are a major burden on the U.S. economy and welfare of society. Switchgrass extractives, the non-structural portion of the biomass rich in phenolic compounds, are inhibitory in the fermentation and hydrolysis phases of lignocellulosic ethanol production. Phenolic compounds found in switchgrass can be inhibitory to bacteria via membrane disruption. The objective of this research was to assess efficacy of switchgrass extractives against five isolates of *E. coli*, *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, and *S. aureus* (including a methicillin-resistant *S. aureus* (MRSA) strain) for potential development of a foodwash and disinfectant. Disk diffusion assays were conducted on all foodborne pathogens included in this study. An additional fruit wash assay using switchgrass extractives as a treatment on Roma tomatoes exposed to *S. enterica* Typhimurium and *S. enterica* Enteritidis was conducted also. Extractives were active against all foodborne pathogens tested, with the exception of MRSA. Inhibition in disk diffusion assays was not significantly different among *E. coli* isolates and *S. enterica* serovars until tested at concentrations of 60X and 75X. Significantly fewer bacteria were recovered from tomatoes treated with 60X extractives compared to the non-extractive control.

Introduction

Plant extracts, such as oregano essential oil, are used in the food industry as preservatives and a control of foodborne pathogens (Lambert et al., 2001). Strawberry extract, for example, has antimicrobial effects against *E. coli* O157:H7, *S. enterica* Enteritidis, *S. enterica* Typhimurium, and *S. aureus* in chocolate (Kotzekidou et al., 2008). The antibacterial effect observed in many extracts is due in part to the phenolic compounds found within them. Phenolic compounds are found in all plants, and play an important role in combating pathogens (Nicholson & Hammerschmidt, 2003). *Panicum virgatum*, also known as switchgrass, is a biomass crop used in the production of lignocellulosic ethanol. Extractives from switchgrass are rich in phenolic compounds and can inhibit the enzymes and microorganisms used in hydrolysis and fermentation. Extractives can be removed and collected to improve the efficiency of the biofuel production process (Anderson & Akin, 2008).

Furthermore, extractives have potential to be developed into an antimicrobial for food preservation as a value-added food wash product. The aim of this research was to evaluate efficacy of concentrated switchgrass extractives for antimicrobial activity against foodborne pathogens *Salmonella enterica* (serovars Typhimurium and Enteritidis), five isolates of Shiga toxin producing *Escherichia coli* (STEC), and *Staphylococcus aureus* (including a methicillin-resistant *S. aureus* (MRSA) strain).

Materials and Methods

Extractives Preparation

Switchgrass ('Alamo') extractives were collected from an Organosolv bioreactor in Dr. N. Labbé, University of Tennessee, Center for Renewable Carbon (CRC). Extractions were performed using 95% ethanol recycled over three batches of fresh biomass to give an effective concentration of 3X. Three runs of extractives (CRC #44, CRC #25, and CRC #58) were further concentrated using the Pierce Reacti-vap™ and Reacti-therm™ evaporation units and further processed as described earlier (REF). Extractives were pipetted into sterile 15-mL test tubes containing magnetic stir bars. Test tubes were then placed into the evaporation unit combo where a stream of N₂ gas (filtered through a 0.45-μm syringe filter) connected to BD™ spinal needles evaporated the ethanol in the extractives to achieve the desired concentration. Sterile blank disks were saturated in concentrated extractives and air-dried for 15 min before use in the disk diffusion assays. Extractives used in the fruit wash assay were concentrated to 60X via evaporation from 10 to 0.5 mL. The extractives were re-suspended in (total volume = 10 mL) sterile water, then evaporated back to 0.5 mL, for a final concentration of 60X. This two-step evaporation was done to minimize the amount of ethanol solvent in the extractives.

Bacterial Foodborne Pathogens Inoculum Preparation

Bacterial foodborne pathogens *Salmonella enterica* Typhimurium, *Salmonella enterica* Enteritidis, *Escherichia coli* (non- O157 isolates O111, O26, and O45, and

O157 isolates Jack in the Box, and Cider), *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA) were grown in 15-mL test tubes containing 10 mL of tryptic soy broth at 37°C for 24 h. The cultures were transferred to 15-mL centrifuge tubes and centrifuged at 2500 rpm for 10 min. The supernatant was discarded, and the pellet was washed with phosphate buffered saline (PBS; pH = 6.8). The bacteria suspended in PBS were then centrifuged a second time and the PBS supernatant was poured off. The remaining pellet was absorbed onto a sterile cotton-tipped applicator. The cotton-tipped applicator was used to release the bacteria into a 15-mL test tube containing 10 mL of PBS until a transmittance of 35% was achieved. Transmittance was checked using a Biolog™ turbidimeter.

Disk Diffusion Assay

Extractive CRC #44, #58, and #25 was tested for antibacterial activity against *E. coli* isolates O111, O26, O45, 'Jack in Box,' and Cider, *S. enterica* serovars Typhimurium and Enteritidis, *S. aureus*, and MRSA. Assays with *S. aureus* and MRSA also included Gentamicin, a commercial antibiotic, as a standard. Sterile, blank antibiotic disks were saturated with concentrated switchgrass extractives CRC #44, CRC #25, and CRC #58. Extractive CRC #44 was tested at five concentrations (0, 30X, 45X, 60X, and 75X). Three concentrations of CRC #25 and CRC #58 (0, 60X, and 75X) were evaluated. Extractive-treated disks were dried under the sterile flow of air in a laminar flow hood to remove any remaining ethanol by evaporation. Tryptic soy agar (TSA) plates were spread with bacterial inoculum (prepared as described earlier) at a rate of 100 µL per

plate to create a bacterial lawn across the surface of the plates. Three extractive-treated disks and one non-extractive control were then placed on the surface and hydrated with 40 μ L of sterile water to facilitate the diffusion of the extractives from the disk to the bacterial lawn. Plates were then incubated at 37°C. After 24 hr, the bacteria had grown into a lawn on the plate and inhibition of growth around the disks was measured. The inhibition zones were measured by taking three measurements per disk. Each assay with each bacteria and extract were replicated thrice and carried out in duplicate. The measurements were recorded and analyzed for significance in SAS 9.4. Analysis was done with mixed models ANOVA and means were compared with an F-protected LSD at $P = 0.05$.

Salmonella Fruit Wash Assay

Salmonella enterica serovars Typhimurium and Enteritidis were tested in a fruit wash assay using Roma tomatoes and concentrated switchgrass extractive CRC #25 (60X). Organic, Roma tomatoes were purchased from Whole Foods Market (Knoxville, TN) and washed under tap water, followed by washing with autoclaved 10% trisodium phosphate (TSP) solution by submerging and stirring the tomatoes for 10 min at room temperature, followed by a 1-min wash in autoclaved deionized water. A final washing in a second container with sterile deionized water was performed to remove residual TSP left on the tomatoes. After the tomatoes had been washed and surface sanitized, they were dried for 8 h in a biological safety cabinet.

After drying, bacteria were applied onto a 3-cm diameter circle demarcated on each tomato fruit. A bacterial solution suspended in PBS was prepared as described in

previous sections for serovars Enteritidis and Typhimurium of *Salmonella enterica*. Bacteria were pipetted (50 μ L) onto the area previously circled. Tomatoes in the negative control treatment received 50 μ L of sterile water. Tomatoes were dried under the biological safety cabinet for 2 h. Once dry, 50 μ L of sterile water or 60X CRC #25 was applied to the positive and negative control groups. Each treatment remained for 1 min after application of either water or extractive then placed into a small, sterile autoclave bag containing 10 mL of tryptic soy broth (TSB). The tomatoes were then massaged by hand for one min to release any bacteria present from the surface of the tomato. Serial dilutions were performed from the respective bags into 15-mL test tubes containing TSB. The tubes were mixed using a vortex and spread plated onto TSA (100 μ L) and incubated for 24 h, at 35°C. After incubation, colonies were counted and data from three replicate treatments assayed in duplicate were analyzed for significance using mixed models ANOVA with SAS 9.4.

Results and Discussion

Disk Diffusion Assay

The effect of the interaction between CRC #44 concentration and *E. coli* isolates show that significant differences in activity against isolates occur at concentrations 60X and 75X. While inhibition was evident when *E. coli* isolates were tested against CRC #58, there was no significant difference between the isolates. In agreement with what was observed in assays with CRC #44, extractives were more active against *E. coli* isolates at concentrations of 75X than 60X in assays with CRC #25 (Fig. 15).

Salmonella enterica serovars Typhimurium and Enteritidis were not significantly different across concentrations of CRC #58 tested. However, in assays with CRC #44, serovar Typhimurium was more susceptible than serovar Enteritidis. Similar results were observed in assays with CRC #25. The interaction between serovar and concentration of extractive CRC #25 showed that serovar Typhimurium was more susceptible than Enteritidis. Also similar to what was observed in assays with *E. coli*, significant differences between pathogens were not observed until concentrations of 60X and 75X were tested (Fig. 16).

Assays with *S. aureus* yielded no difference between 75X and 60X, with the exception of CRC #44, which was significantly more active at 75X than 60X. Gentamicin disks were included as a standard. No inhibition was observed in assays with MRSA, from either switchgrass extractives or the gentamicin standard (Fig. 17).

Tomato Fruit Wash Assay

Tomato fruits surface inoculated with *S. enterica* serovars Typhimurium and Enteritidis were washed with 60X CRC #25 or sterile water (positive control). The negative control consisted of no extractive and no *S. enterica*. Colony counts of the recovered bacteria were made from dilution spread plates and analyzed with mixed models ANOVA in SAS 9.4.

Tomatoes treated with 60X CRC #25 had significantly fewer bacteria recovered than the positive control in both *S. enterica* Enteritidis and *S. enterica* Typhimurium (Fig. 18). The results imply that extractives have use as a fruit wash product. The extractives

could be applied as a stand-alone washing agent. However, further research should be conducted to gain insight as to how extractives could work in conjunction with other fruit washing agents.

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Appendix

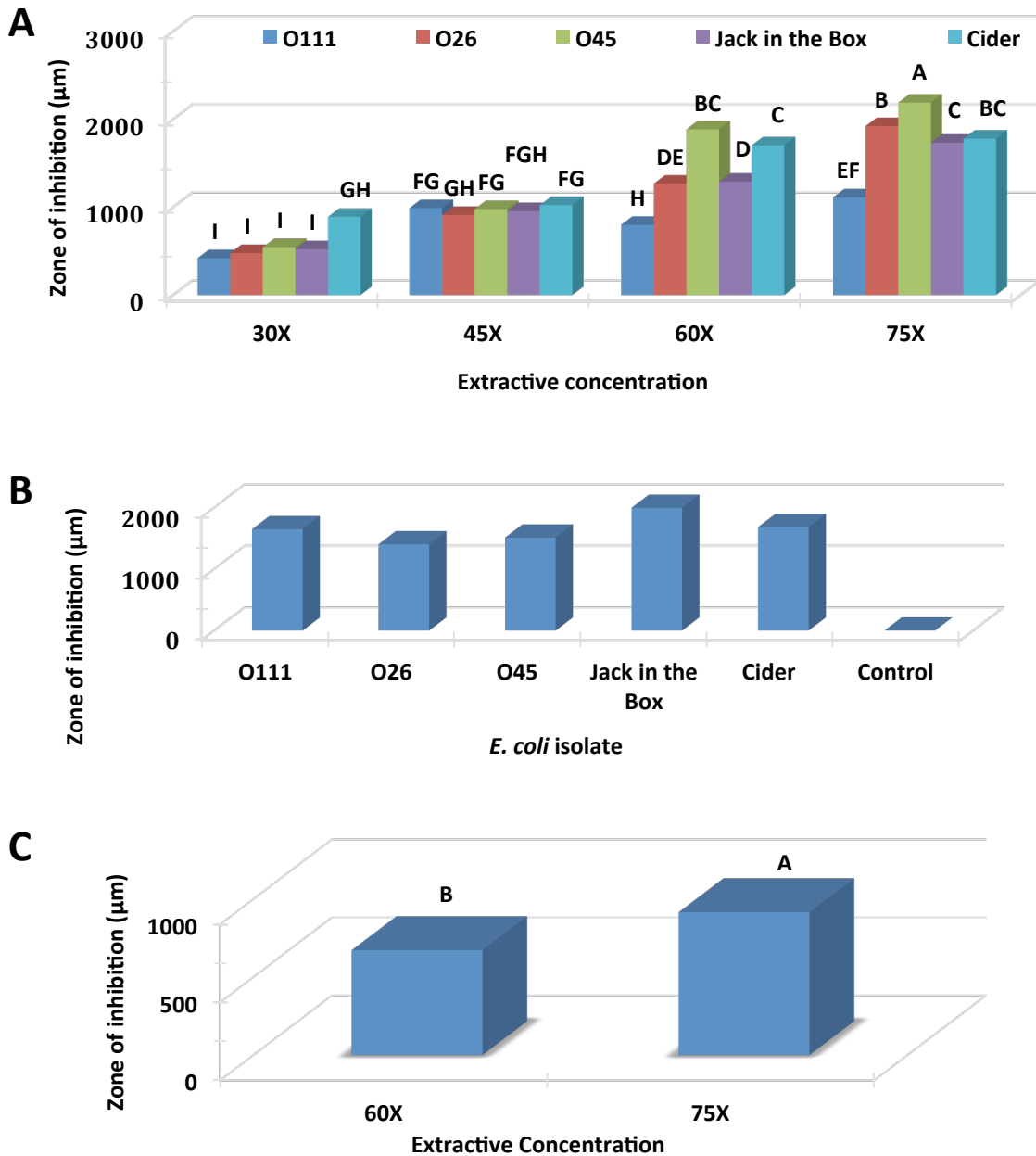


Figure 16. Effect of switchgrass extractives on inhibition of *Escherichia coli*. Bars with the same letter or no letter are not significantly different according to an F-protected LSD at $P = 0.05$. **A)** Effect of the interaction between concentration of CRC #44 and isolate of *E. coli*. **B)** Effect of *E. coli* isolate across all concentrations of CRC #58. **C)** Effect of concentration of CRC #25 across all isolates of *E. coli*.

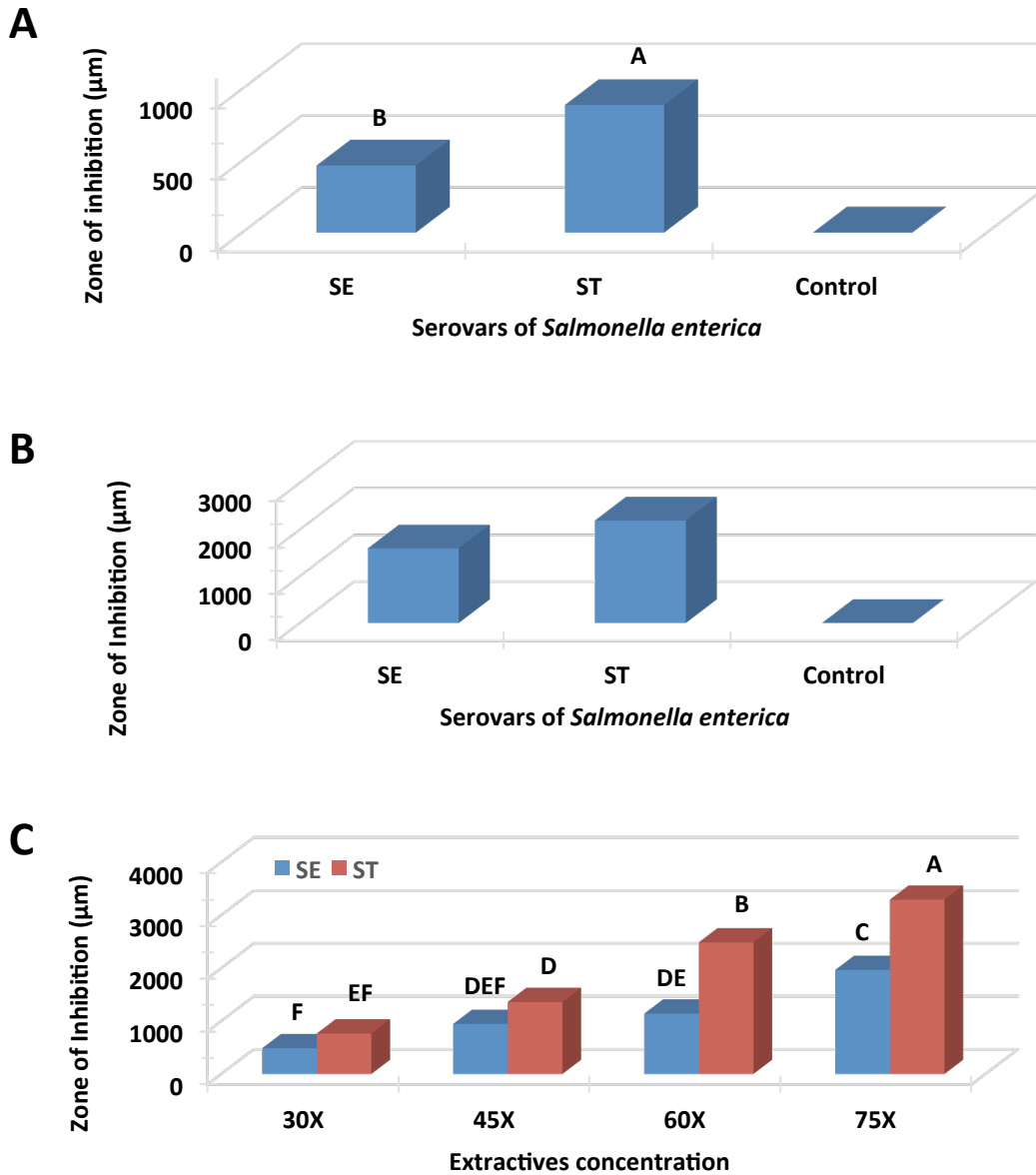


Figure 17. Effect of switchgrass extracts on inhibition of *Salmonella enterica*. Bars with the same letter are not significantly different according to an F-protected LSD at $P = 0.05$. **A)** Effect of *S. enterica* serovar across all concentrations of CRC #44. **B)** Effect of *S. enterica* serovar across all concentrations of CRC #58. **C)** Effect of interaction between concentration of CRC #25 and *S. enterica* serovar.

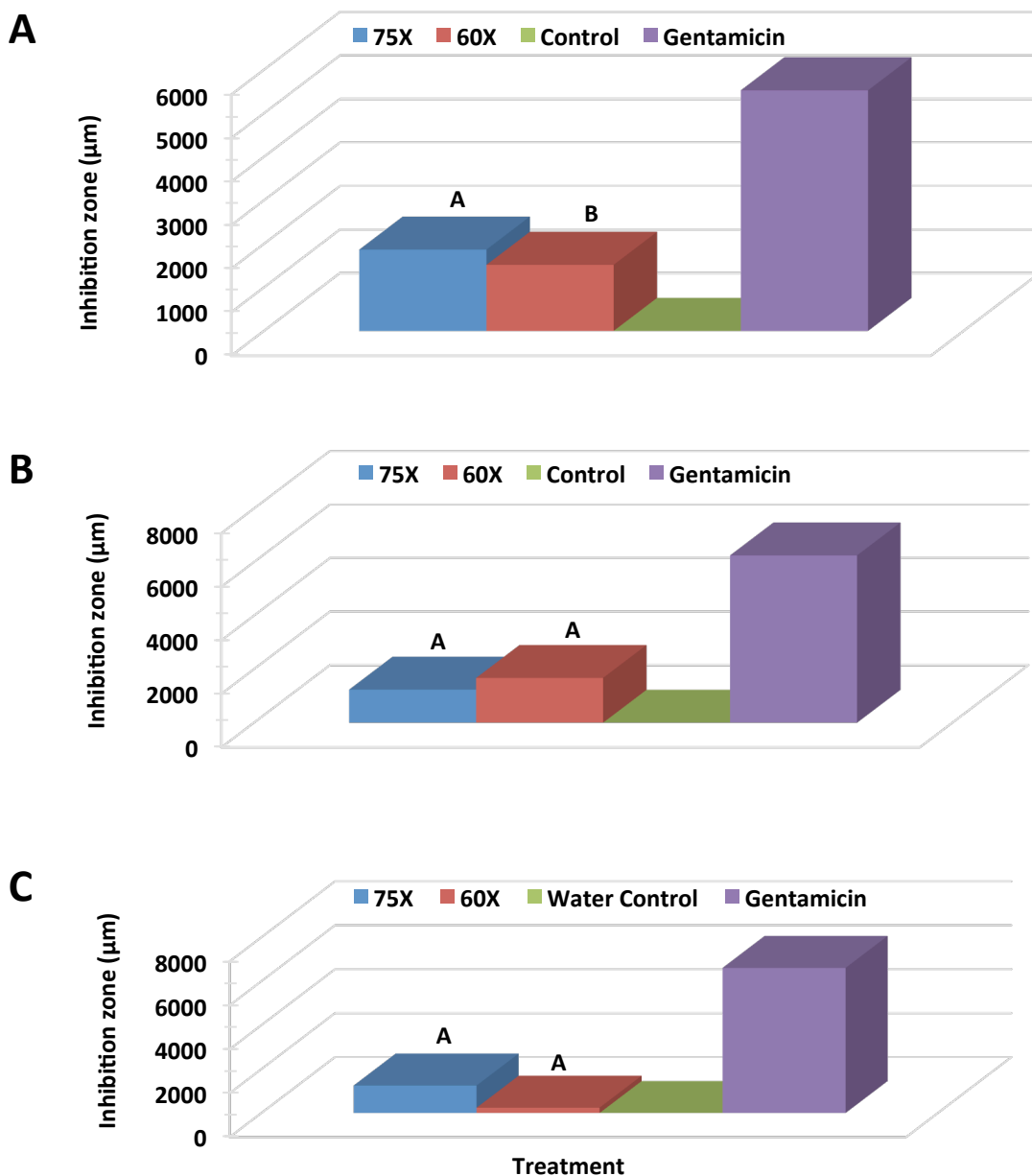


Figure 18. Effect of switchgrass extractives on inhibition of *Staphylococcus aureus*. Bars with the same letter are not significantly different according to an F-protected LSD at $P = 0.05$. Values for the control and gentamicin were included as a comparison only, and were not included in the data analysis. **A)** Effect of CRC #44 against *S. aureus*. **B)** Effect of CRC #58 against *S. aureus*. **C)** Effect of CRC #25 against *S. aureus*.

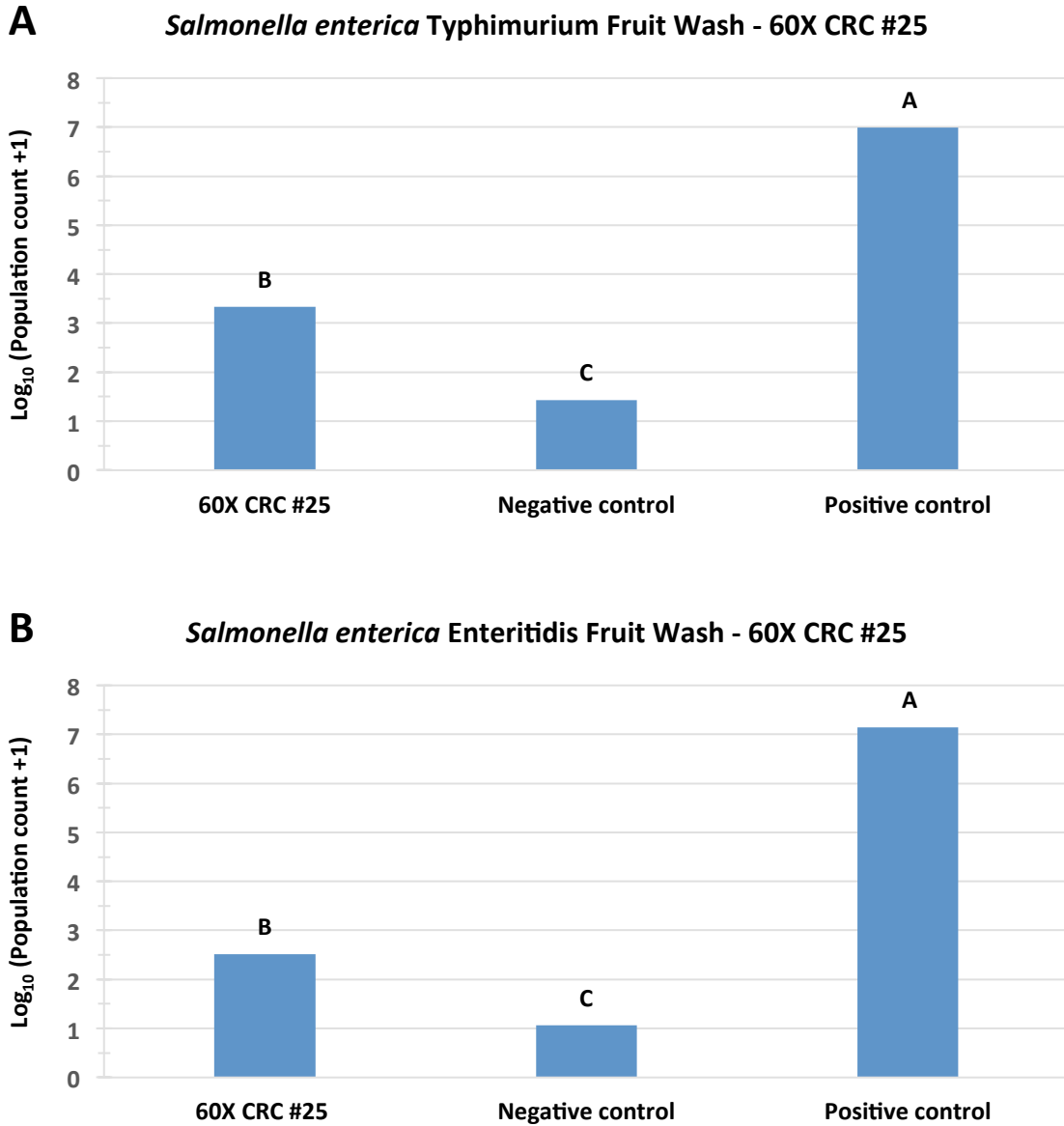


Figure 19. Effect of switchgrass extractives on population counts of *Salmonella enterica* serovars on tomato fruit. Bars with the same letter are not significantly different according to an F-protected LSD at $P = 0.05$. **A)** Population counts recovered from tomatoes swabbed with *S. enterica* Typhimurium. **B)** Population counts recovered from tomatoes swabbed with *S. enterica* Enteritidis.

CHAPTER 5
CONCLUSION

Results from this research provide evidence that switchgrass extractives have potential as a value-added commercial biopesticide and disinfectant. Extractives were antibacterial and antifungal activity against all plant pathogens tested *in vitro* and *in planta*. More research needs to be conducted to determine optimal treatment times *in planta*. Spore germination assays with longer exposure times should also be conducted to determine the time of maximum inhibition for pathogens such as *Botrytis cinerea*, *Bipolaris oryzae*, and *Fusarium graminearum*. With the exception of Methicillin-Resistant *Staphylococcus aureus*, switchgrass extractives were active against all foodborne pathogens tested. Some of the largest inhibition zones were observed in disk diffusion assays with foodborne pathogens; this suggests great potential for use in the food service industry. One possible explanation for the antibacterial activity observed in foodborne pathogen assays could be due to their evolutionary history. Plant pathogens evolved in conjunction with the plant's natural defense system, which is comprised of many of the phenolic compounds tested in this research, whereas foodborne pathogens did not. Commercial development of switchgrass extractives has large implications resource sustainability and environmental safety. Developing switchgrass extractives as a co-product to biofuel production would improve the economic feasibility of switchgrass as a biomass feedstock and provide less selection pressure towards pesticide resistant pathogens.

VITA

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