8-2012

The epidemiology of Puccinia emaculata (rust) in switchgrass and evaluation of the mycoparasite Sphaerellopsis filum as a potential biological control organism for switchgrass rust.

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I am submitting herewith a thesis written by Jonathan Allen Black entitled "The epidemiology of Puccinia emaculata (rust) in switchgrass and evaluation of the mycoparasite Sphaerellopsis filum as a potential biological control organism for switchgrass rust.." 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.

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The epidemiology of *Puccinia emaculata* (rust) in switchgrass and evaluation of the mycoparasite *Sphaerellopsis filum* as a potential biological control organism for switchgrass rust.

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

Jonathan Allen Black
August 2012

DEDICATION

I would like to dedicate this thesis to my wife, Jessica Black, and parents, Darrel and Linda Black, for their support throughout my endeavors. I would also like to dedicate this thesis to countless friends and family for their guidance.

“Tomorrow hopes we have learned something from yesterday.”

-John Wayne

“I don't measure a man's success by how high he climbs but how high he bounces when he hits bottom.”

George S. Patton

ACKNOWLEDGEMENTS

For their guidance and support, I express sincere gratitude to my graduate committee members: Dr. Mark Windham, Dr. Alan Windham, Dr. Yonghao Li, Dr. Melvin Newman, and Dr. Warren Copes. I especially would like to thank my committee chair, Dr. Mark Windham, for his guidance, camaraderie, and willingness to take me on as a graduate research assistant in this program.

I also give special thanks to Qunkang Cheng, Lisa Vito, Dr. Denita Guerry, and Jimmy Mynes for their tireless guidance, assistance, and friendships I have made.

ABSTRACT

Switchgrass (*Panicum virgatum* L.) is a warm-season, perennial grass, whose native range includes the entire United States and north into Canada, excluding areas along the pacific coast. Recently, symptoms and signs of rust disease (*Puccinia emaculata*) have been observed on agronomic switchgrass, which include chlorosis of leaf tissue, necrosis, lodging, and plant death.

To evaluate disease progress of switchgrass rust, in four fields, individual leaves of twenty-five switchgrass plants were rated once per week for fifteen weeks over two growing seasons for disease severity. Rust was first observed on Julian day 166 and 152 in 2010 and 2011, respectively. Ninety-five percent of switchgrass plants were at the 5-7 leaf growth stage before rust was first observed. Disease severity progressed logistically after detection; the rate of increase in disease severity lessened in late August to early September. The log phase of disease progression occurred from mid-June to mid-August. Leaf mortality was first observed in mid-to-late June. Greater than five percent of leaf surfaces were covered with uredia by early-to-mid October. Data collected in this study indicates when rust epidemics begin and subside on switchgrass in East Tennessee. If fungicide sprays become a viable management strategy, this data will be useful in timing those applications.

Growth and pycnidial production of *Sphaerellopsis filum* was highest on V8 juice agar, which was used to maintain cultures. To evaluate the mycoparasite's ability to impede urediospore production and viability, uredia of *P.*

emaculata on detached switchgrass leaves were inoculated with conidia of *S. filum*. Pycnidia formed in uredia at 12-14 days after inoculation. The mycoparasite significantly reduced the number of urediospores per uredium by an average of 246 spores when compared to untreated uredia.

When germination of urediospores was compared between healthy or those parasitized by *S. filum*, percent germination was 73% and 42%, respectively. Germ tubes of urediospores from healthy uredia averaged 96.9 um in length, whereas those from parasitized uredia averaged 32.3 um at three hours. As the mycoparasite reduced urediospore production, germination, and germ tube length, further investigation into its use as a potential biological control agent for *P. emaculata* is warranted.

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CHAPTER I
LITERATURE REVIEW

Switchgrass:

Switchgrass (*Panicum virgatum* L.) is a warm-season, perennial, C-4 grass in the family *Poaceae* that grows in clumps and spreads by rhizomes (7). The native range of switchgrass includes most of the United States with the exception of California and the Pacific Northwest (Figure 1.1)(30). Switchgrass can be used as a biofuel feedstock, forage crop, ornamental plant, for bank stabilization, and as a component to improve wildlife habitat (18).

Recently, agronomic switchgrass production has increased due to its usage as a crop for production of cellulosic ethanol. Screening trials were conducted from the late 1980's to early 1990's at Auburn, Purdue, Iowa State, North Dakota State, Virginia Tech, Cornell, and a company in Ohio named Geophyta, and were compiled in a report by Wright (32). Thirty-four species of potential biofuel crops were evaluated at thirty-one research sites, and switchgrass was recommended by six of the seven research institutions for further development as a biofuels crop, with sorghum or sorghum X sudangrass following in preference. Switchgrass was favored over sorghum or sorghum X sudangrass due to its relatively easy establishment and longevity of stands. Switchgrass also had higher yields than did big bluestem, tall fescue, reed canarygrass, alfalfa, birdsfoot trefoil, weeping lovegrass, and sericea lespedeza (32). Several reports were made of stand failure with rye/sorghum X sudangrass and one researcher at Oak Ridge National Laboratory reported that fertilization after stand failure should be considered since weeds fertilized at the same level

produced respectable yields (33). The United States Department of Energy (DOE) has dedicated switchgrass as an energy crop because of its potential for high fuel yield, environmental enhancement characteristics, and ability to be grown on marginal cropland without intensive fertilization or crop management (4). As a forage crop for cattle, switchgrass has the potential to be excellent, but quickly becomes tough and unpalatable as it matures and the nutritive value of the forage decreases dramatically. Toxicity issues have occurred in sheep, horses, and goats, because of chemical compounds called saponins, which can cause photosensitivity and liver damage (29). Also, switchgrass can be planted along stream banks and on steep slopes for erosion control due to its extensive root system, which helps hold the soil in place. Due to its clumping growth pattern, switchgrass provides excellent habitat for wildlife. Cover is provided for deer, quail, rabbits, and other wildlife. Passageways are present at ground level between the clumps that allow for practically undetectable movement of wildlife within the field.

Switchgrass is divided into two groups: upland and lowland types. Both upland and lowland switchgrass are deep rooted and have rhizomes. Upland grasses are better suited to well-drained soils, grow 1.5 to 1.8 meters in height, are more cold tolerant, and tend to be more vigorous than lowland switchgrass when forming rhizomes. Lowland types favor heavy soils and bottomland sites, can reach heights of 3.6 meters, and tend to be more clumped in nature (5). Due to their higher yield potential, lowland switchgrass cultivars are better suited for

biofuel production on the southeast United States. Lowland varieties, such as 'Alamo' and 'Kanlow', are recommended by the University of Tennessee as a biofuel crop (10). Switchgrass is a near obligate outcrosser, meaning that some self-pollination has been documented, but for the most part male and female plants must be present for fertilization to occur (21). Switchgrass is vegetatively clonal, and clones have been used to evaluate tiller development and growth characteristics (2). The ability to clone switchgrass cultivars is a very important characteristic when considering development of disease resistant plants.

Rust of switchgrass:

Puccinia emaculata Schw. is an obligate parasite and a member of the order *Pucciniales* (26). The uredial stage is common on switchgrass and urediospores are the primary source of inoculum for secondary disease cycles. Urediospores are single-celled, globose or oval, and are approximately 27 × 25µm. In late summer to fall, telia may be observed on infected leaves. Teliospores are two-celled; the upper cell has a thickened cell wall (22). Teliospores are 33.6 µm ± 4.8 in length and the width of apical and basal cells are 17.5 µm ± 1.2 and 15.9 µm ± 2.5 respectfully (9).

On water agar, urediospores germinated one hour after inoculation. After two to three hours of incubation, elongation and branching of hyphae occurred and appressoria were formed. When urediospores were deposited on switchgrass leaf surfaces, appressoria formed over stomata and penetrated through stomatal openings (20).

Rust can negatively impact the biomass yield, seed production, and forage quality of switchgrass (11). In July and August 2007, uredial pustules of switchgrass rust were found on the upper leaf surface of switchgrass plants located at the East Tennessee Research and Extension Center, which became the first official report of switchgrass rust in Tennessee (9). Rust has also been reported in Arkansas on 'Alamo' switchgrass, where 25% to nearly 100% of switchgrass leaves were infected by *P. emaculata* (13). Rust has also been reported on switchgrass in numerous states throughout the southeastern United States, west into Texas, and north into South Dakota. Both upland and lowland varieties of switchgrass have been reported to be infected by *Puccinia emaculata*. Jacobs and Terrell reported that five ornamental cultivars of switchgrass, Shenandoah, Northwind, Rehbraun, Warrior, and Campfire, exhibited resistance (14). However, Northwind and Shenadoah were susceptible to rust in Tennessee (M. Windham, personal communication). Gustafson et. al. examined the genetic variation of switchgrass in regards to rust resistance and found that a great deal of additive and non-additive genetic variation was present in switchgrass populations (12). Variation in pathogenicity of *P. emaculata* also exists, as isolates from ornamental switchgrass have proven to be more virulent than those collected from agronomic switchgrass (19). Ten polymorphic markers have recently been developed to further study genetic diversity within *P. emaculata* populations (31).

Infection of the primary host (switchgrass) by *Puccinia emaculata* may

occur in one of two ways. Aeciospores are wind-blown, land on switchgrass leaves, and form dikaryotic hyphae, which penetrate through stomatal openings (M. Windham, personal communication). After ramification throughout the leaf tissue, hyphae form in the compact palisade layer where urediospores are produced (M. Windham, personal communication).

Urediospores may be blown into Tennessee from gulf coast states making dependence of primary infection from germinated aeciospores unnecessary (M. Windham, personal communication). Uredia can form in 7-10 days after infection occurs. In late summer/fall telia form and teliospores should germinate following meiosis during spring to form basidiospores which infect spurge leaves. The aecial stage is thought to occur on species of family Euphorbiacae (spurge), but the species has not been well documented (9,24). *P. emaculata* was described as being very similar to *Puccinia pamellii* Arth., so attempts were made to culture *P. emaculata* on *Euphorbia corollata*, the alternate host of *P. pamellii*, but with no success (1). The alternate host infected with *P. emaculata* has not been observed in Tennessee.

Complete management plans for switchgrass rust have yet to be developed. In several publications, it is reported that significant disease and insect problems have not been experienced in switchgrass production, but that insect and disease problems should be expected (10).

A mycoparasite of *P. emaculata*:

In 1813, *Sphaeria filum* was described to occur on two rust hosts by

Bivona-Bernadi (3). L. Castagne established the genus of *Darluca* in 1851 and used *Sphaeria filum* as the type species and the fungus was renamed *Darluca filum* (6). Spegazzini established the genus *Eudarluca* in 1908 to describe a pyrenomycete associated with uredia of rust on *Canna sp.* in Brazil (27). In 1951, Keener showed that ascospores from *Eudarluca* produced pycnidia and conidia of *Darluca filum* (15). In 1966, Eriksson renamed the mycoparasite *E. caricis* Erik. O., and this is the current designation for the teleomorphic state of *S. filum* (8). The taxonomic classification of *D. filum* was used until 1977, when the *Sphaerellopsis* Sutton genus was proposed.

Pycnidia of *Sphaerellopsis filum* are found in uredia of *P. emaculata* as well as on 369 species and 30 genera of rusts worldwide and has been found in more than 50 countries (16). Pycnidia are black, sub-globose, 90-200 µm and have ostioles where conidia are exuded in a gelatinous matrix (22). Conidia are hyaline, 1-septate, fusiform, 13-18 × 3-5 µm and have a gelatinous cap at one or both ends (20).

Several instances have been documented of *S. filum* parasitizing rusts. In 1957, Schroeder and Hassebrauk observed appressoria like structures which penetrated the urediospores of *Puccinia sp.* (25). Also, antifungal and antibacterial compounds, Darlucins A and B, have been isolated, and may be important in the mycoparasite's interaction with *P. emaculata* (35). On willow rust, *S. filum* reduced urediospore production by up to 98% (34). Urediospore collapse and disintegration in *Puccinia recondita* was observed when contact

with the mycoparasite occurred, and in some instances, spines on the urediospore walls were not present (23). In contrast, Stahle and Kranz found that there were no differences in the germination percentage of urediospores, but germ tube branching was greatly reduced after being infected with the mycoparasite (28). *S. filum* significantly reduced the number of telia as well as basidiospore production by 50-75% when *Cronartium* rust on oak in Florida was heavily infected with the mycoparasite (17).

Based on the literature reported above, we asked the following questions:

1) On what date can rust first be detected on agronomic switchgrass?
2) What growth stage are switchgrass plants at when they first become infected with *P. emaculata* in agronomic fields?
3) When during the growing season are switchgrass plants most likely to become infected with *P. emaculata*?
4) How severely are switchgrass plants infected at the time of harvest?

The following objectives have been chosen to evaluate those questions concerning the epidemiology of rust on switchgrass established in agronomic fields in Tennessee:

1) evaluate the date on which the disease can first be detected;
2) determine the growth stage of plants when disease first appears;
3) determine when the log phase begins and ends;
4) and estimate disease severity at harvest.

In order to gain a better understanding of the relationship between *S. filum* and *P. emaculata*, the following questions were asked:

1) Is *S. filum* a potentially viable biological control option for switchgrass rust?
2) What type of media is optimum for vegetative and asexual reproduction of *S. filum*?
3) What effects does *S. filum* have on urediospore production and germination?

To answer those questions, the following objectives were used:

1) determine on which medium, V-8 agar, V-8/ potato dextrose agar, or potato dextrose agar, *S. filum* has maximum growth, produces pycnidia the earliest, and the greatest number of conidia are produced;
2) determine the effects of *S. filum* on the number of urediospores produced by *P. emaculata*;
3) and evaluate the effects of *S. filum* on germ tube length of *P. emaculata* urediospores.

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Appendix

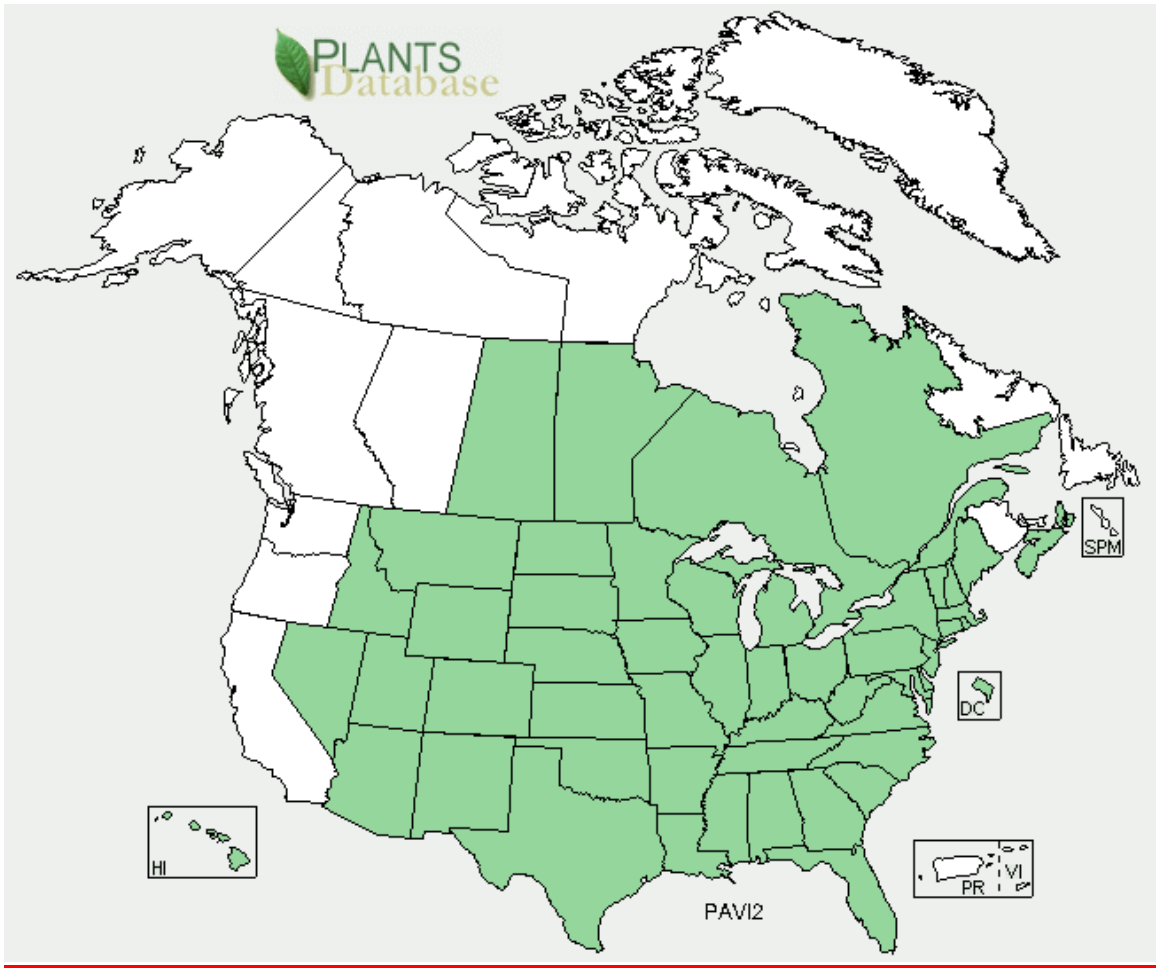

Figure 1.1. Distribution of switchgrass (*Panicum virgatum* L.) in the United States and Canada.

CHAPTER II
EPIDEMIOLOGY OF *PUCCINIA EMACULATA* IN SWITCHGRASS

A portion of this chapter was originally published by Jonathan Black[1], Qunkang Cheng[1], Mark Windham[1], Alan Windham[1], Melvin Newman[1], Yonghao Li[2], and Warren Copes[3]

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Jonathan Black, Qunkang Cheng, Mark Windham, Alan Windham, Melvin Newman, Yonghao Li, Warren Copes. 2011. Switchgrass rust epidemics (*Puccinia emaculata*) in agronomic fields in Tennessee. Phytopath. 101: S16.

I, Jonathan Black, completed the research that follows, along with Qunkang Cheng, who assisted with data collection and statistical analyses. Dr. Mark Windham, Dr. Alan Windham, Dr. Melvin Newman, Dr. Yonghao Li, and Dr. Warren Copes each assisted with the experimental design of the project and revision of the original publication as well as the one that follows, all of them being members of my graduate committee.

Abstract

Disease onset and progession were characterized for switchgrass rust (*Puccinia emaculata*) in four agronomic fields in southeastern Tennessee. Disease severity was assessed on a scale of 0 (0% severity) to 5 (25% severity) on individual leaves of five plants per plot in five plots per field over fifteen weeks in 2010 and 2011. Rust was first observed on Julian day 166 and 152 in 2010 and 2011, respectively, when plants were at the 5-7 leaf growth stage on average in both years. Disease severity progressed in a logistics pattern. The log phase of disease progression, when disease severity developed rapidly, occurred between mid-June to mid-August. The rate of increase in disease severity lessened in late August to early September. Leaf mortality of the lower leaves occurred due to colonization by *P. emaculata*, and due to environmental factors such as insufficient rainfall or excessive shading in the lower canopy. Final disease assessments were taken in early to mid-October, at which time uredia covered an average of 3.78% and 5.10% of the leaf surface in 2010 and 2011, respectively.

Introduction

Agronomic production of switchgrass has increased recently due to its use as a cellulosic ethanol feedstock. From the late 1980's to early 1990's, thirty-four species of crops were evaluated at thirty-one research sites for suitability in biofuel production. Switchgrass was reported to have higher yields than *Andropogon gerardii* (big bluestem), *Festuca arundinacea* (tall fescue), *Phalaris arundinacea* (reed canarygrass), *Medicago sativa* (alfalfa), *Lotus corniculatus* (birdsfoot trefoil), *Eragrostis curvula* (weeping lovegrass), and *Lespedeza cuneata* (sericea lespedeza) (31). The United States Department of Energy (DOE) chose switchgrass as a dedicated energy crop because of its potential for high fuel yield, environmental enhancement characteristics, ability to be grown on marginal cropland, and low inputs required (4). Lowland switchgrass cultivars such as 'Alamo' and 'Kanlow' are well suited for biofuel production in the southeastern United States due to a higher yield potential on heavy soils, which are common in Tennessee (10).

Puccinia emaculata Schw., causal agent of switchgrass rust, was first reported in Tennessee in 2007 (9). The aecial stage reportedly found on species of family Euphorbiacae (spurge) has not been observed in Tennessee. Whether primary inoculum is by aeciospores or windborne urediospores from more southern locations is unknown. Rust has also been reported in Arkansas on 'Alamo' switchgrass, where 25% to nearly 100% of switchgrass leaves were infected by *P. emaculata* (13).

The exact impact of rust on switchgrass is unknown, but it may negatively impact the biomass yield, seed production, and forage quality of switchgrass (11). Five ornamental cultivars of switchgrass have been reported to exhibit resistance to rust, and those cultivars were Shenandoah, Northwind, Rehbraun, Warrior, and Campfire (14). Northwind and Shenadoah have proven susceptible to rust in Tennessee (M. Windham, personal communication). The objective of this research is to determine the date and plant growth stage when disease can first be visually detected, determine the temporal model that represents seasonal disease progression, and estimate disease severity at harvest.

Materials and Methods

Establishment of field plots:

Field plots were established in four fields located in Monroe County, Tennessee, where switchgrass was being grown for biofuel feedstock (Figure 2.1). In each field, a point was chosen at random on the fields outside perimeter. From that point, a visible landmark was selected on the other side of the field, such as a mountain peak, as a transect line. The center plot was marked 70m along the transect into the field with a 3m bamboo stake. The plant nearest the bamboo stake was designated the middle plant of the center plot of that field. Individual plants were tagged 1.83m to the north, south, east, and west; thus establishing five plants in the center plot. Plants were tagged on the leaf lowest to the ground. In addition, four other plots were established 30.5m to the north, south, east, and west of the center plot. Five plants were selected per plot as described for the center plot (Figures 2.2 A and B). This study was replicated in three additional fields.

Disease rating system:

A numerical rating system was used to estimate disease severity, where 0=0%, 1≤1%, 2≤5%, 3≤15%, 4≤25%, 5>25% of the leaf was covered by uredia, respectively. Plants were evaluated once per week in each field from the fourth week of May until harvest in October. All live leaves on each plant were evaluated on each observation date. Data were not recorded for dead leaves. In addition, numerous other plants were examined per field for the initial

observance of rust.

Statistical analyses:

Microsoft Excel and SAS were used to analyze data using the nonlinear model (NLIN) procedure of SAS software (Version 9.2, SAS Institute Inc., Cary, NC) was used to obtain estimates of parameters from the nonlinear form of the logistic model and differences between years or fields were compared. The Richard's equation, Y= max /(1+k*exp(-max*rate*t)), is a nonlinear logistic model, which was used to evaluate disease severity over time and estimate predictive values based on actual disease severity, apparent infection rate, and maximum disease severity at a given time in a given field. In this equation, y is the disease severity at time t; Max is the upper asymptote of the disease progress curve; k is an intercept; Rate is the apparent infection rate; and t is the time of disease assessment.

Results and Discussion

In 2010, rust was first observed in the designated plots on Julian date 166 (June 15) and was not observed in all fields until date 189 (July 8) (Figure 2.3 A). In 2011, rust was first observed on date 152 (June 1) and was finally observed in the last field on date 167 (June 16) (Figure 2.3 B). Although rust has been observed in previous years in other fields throughout the state of Tennessee, Julian date 152 is the earliest day rust has been detected since it was first recorded in the state (M. Windham, personal communication). When other plants in the field (outside the designated plots) were examined for rust, the earliest date for observing rust was 166 in 2010 and 152 in 2011 (Figures 2.3 A and B).

Disease severity values and disease estimates were plotted over the growing season for plants in each of the four fields for 2010 and 2011 (Figure 2.4 A and B). Disease severity progressed in a logistics pattern and reached the upper asymptotic value around Julian day 246 in 2010 and day 244 in 2011. Average leaf area infected with rust at the end of the growing season was approximately 3.78% in 2010 and 5.10% in 2011. There were significant differences in trends and maximum rust severity between years, but this approximation does not justify the observed amount of dried out foliage and lodged plants in each field. As plants matured, lower leaves tended to die regardless of rust severity, probably due to the natural senescence of aging leaves and shading. Among all fields, disease severity was higher on all

recorded dates in 2011 compared with 2010 (Figure 2.4 A and B). This may have been due to differences in weather patterns and primary inoculum levels. Higher initial spore concentrations could result in a larger number of foci and higher secondary inoculum levels. Weather data was not collected for comparison of weather differences per location and alternative weather stations do not exist in the region. Because of the nature of mountainous terrain, meso-scale weather conditions may not be representative of field differences.

Among all fields, the most drastic difference in disease severity occurred at the field in Vonore (Figure 2.4 A and B). This field would be characterized as a rolling hill type landscape. Disease was observed earlier in the low spots within the field. This may be due to increased humidity within the switchgrass canopy in these areas. The terrain of the field in Madisonville is similar to Vonore. Disease severity at Madisonville and Vonore (Figure 2.4 A and B) was significantly greater than the two fields in Tellico Plains (Figures 2.4 A and B) in both years, possibly due to differences in terrain. Fields in Tellico Plains are flat with more uniform microclimates. Tellico Plains is surrounded by a mountain range near Cherokee National Forest that could be a barrier to air currents, and possibly result in spores being blown over, and not actually landing in the fields many days.

Ninety-five percent of switchgrass plants in the plots were at the 5-7 leaf growth stage before rust was first observed in 2010 and 2011 (Table 2.1). We were already observing leaf senescence at the 5-7 leaf growth stage when the

disease was just beginning and infection rates were low, which is one reason that we believe rust infection is not the only contributing factor for senescence and drying out of leaves during the growing season.

The knowledge of growth stage, time of initial infection, disease progression, and maximum disease severity provides a basic understanding of switchgrass rust epidemics in eastern Tennessee climates. The information is important in designing future studies to measure yield losses in agronomic fields. Also, if fungicidal sprays can be economically justified, knowledge of disease detection dates and growth stage at initial infection will be important in timing disease onset and initial spray dates. In addition, data generated on predicted disease severity values to produce epidemic models may help switchgrass producers predict rust outbreaks in future years and implement control strategies before disease appears.

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Appendix

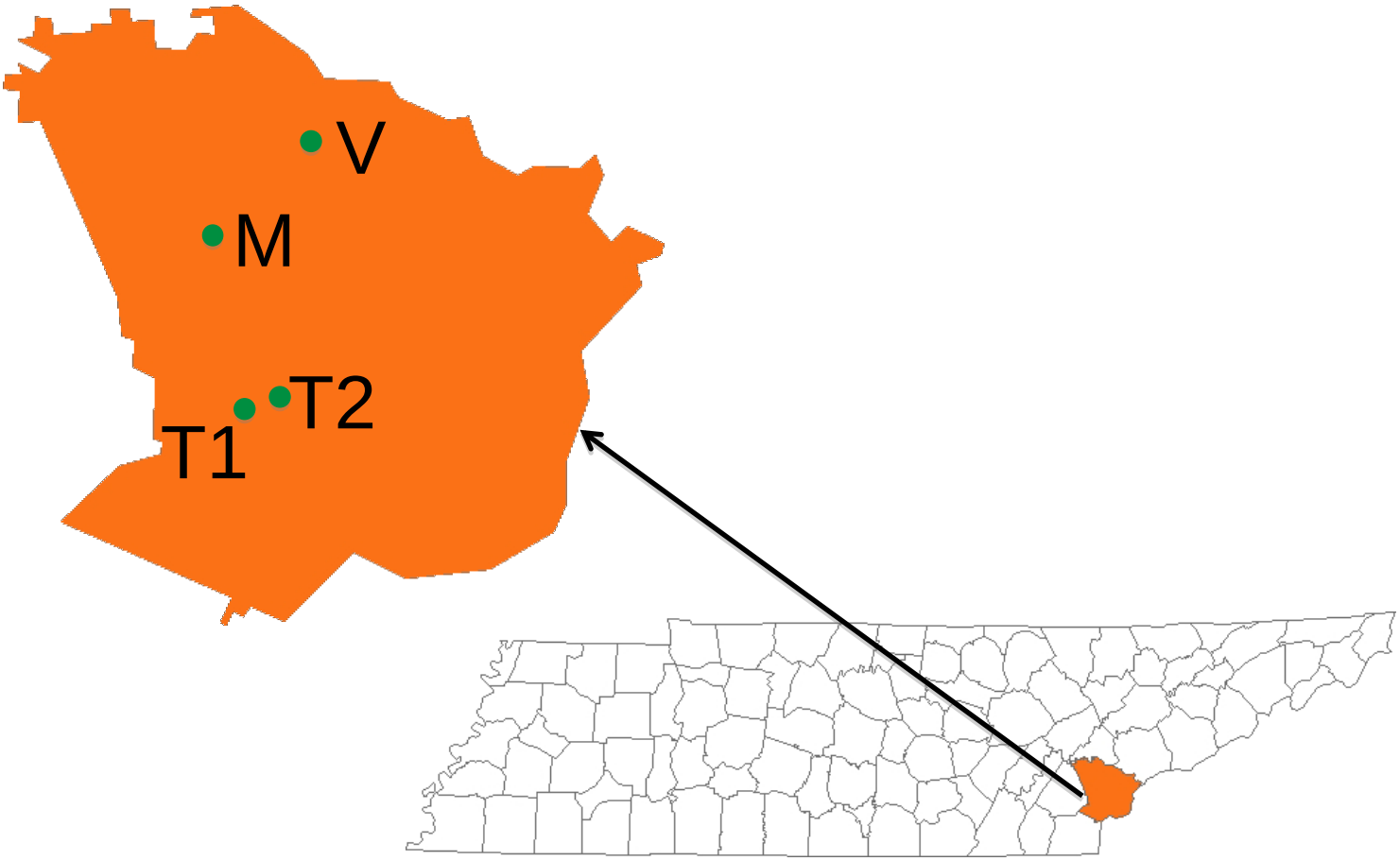
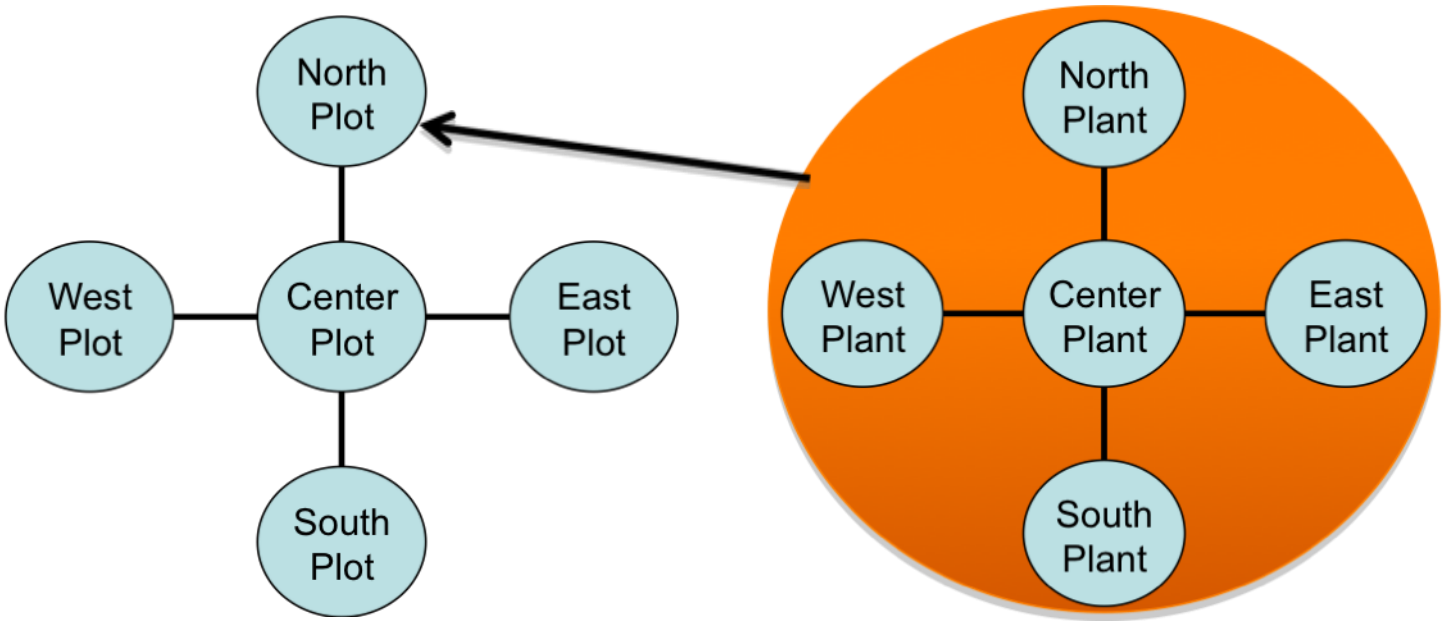


Figure 2.1. Location of fields (V=Vonore, M=Madisonville, T1=Tellico Plains Field 1, T2=Tellico Plains Field 2) in Monroe County, Tennessee.



A B

Figure 2.2. Each field contained five plots arranged in a cross pattern (A) with the center of the center plot located 60.96 m from the edge of the field and spaced 30.48 m from each other. Within each plot, five plants arranged in a cross pattern (B) at a spacing of 1.83 m apart were sampled as repeated measures.

Table 2.1. The number of infections per leaf at each leaf stage, defined as the number of fully expanded leaves per plant in 2010 and 2011.

Leaf Stage	4		5		6		7		8	
Year	10’	11’	10’	11’	10’	11’	10’	11’	10’	11’
Vonore	0	1	3	8	22	16	0	0	0	0
Madisonville	0	1	5	6	18	16	2	1	0	0
Tellico Plains 1	0	1	6	8	14	14	4	2	1	0
Tellico Plains 2	2	1	12	3	11	17	0	2	0	2

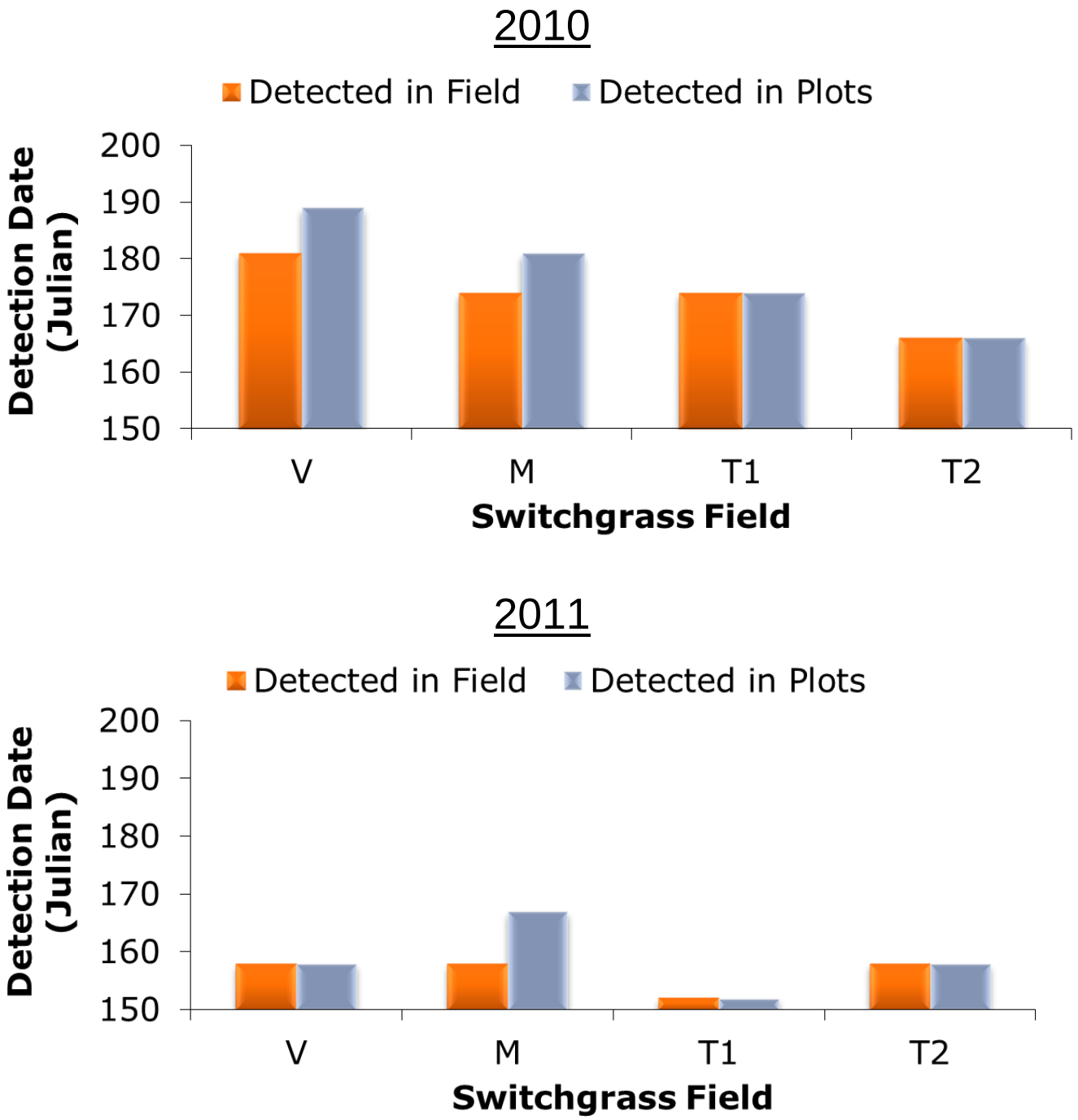


Figure 2.3 A (Top) and B (Bottom). Initial disease detection dates at the four locations (V=Vonore, M=Madisonville, T1=Tellico Plains Field 1, T2=Tellico Plains Field 2) in 2010 and 2011.

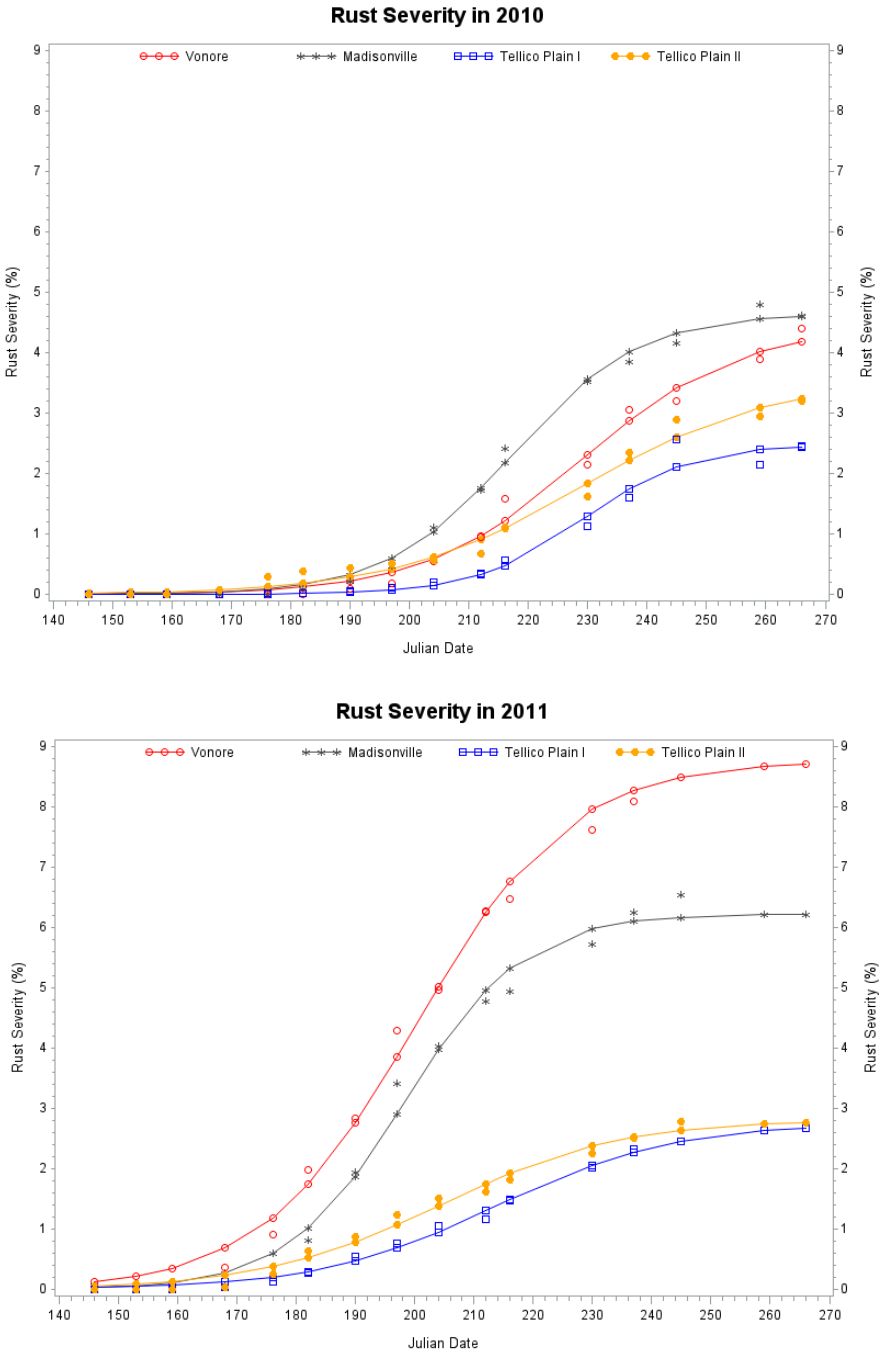


Figure 2.4. Logistical model of switchgrass rust severity for 2010 (A) and 2011 (B) growing seasons. The X axis represents percent leaf surface covered with uredia. Y axis indicates Julian date. Significant differences between trends as well as significant differences in max rust severity: 2010 - 3.78%, 2011 - 5.10% leaf surface covered with uredia.

CHAPTER III
COMPARISON OF GROWTH MEDIA FOR PYCNIDIAL AND MYCELIAL GROWTH OF *SPHAERELLOPSIS FILUM*

Abstract

A mycoparasite *Sphaerellopsis filum* was observed to be infecting rust pustules of *Puccinia emaculata* on switchgrass in 2009. This paper serves at the first report of *S. filum* being a mycoparasite of *P. emaculata* on switchgrass. The mycoparasite was identified using morphological characters, and the identity was confirmed by extracting and sequencing DNA ITS regions. Sequences confirmed with 99% identity that all isolates were isolates of *Eudarluca caricis*, the perfect stage of *S. filum*.

The mycoparasite was grown on V8 juice agar (V8), PDA and a media combining PDA and V8 juice. All media were amended with antibiotics to prevent bacterial growth. Colonies of *S. filum* grew significantly faster on V8 than on PDA during the first two weeks. However, no differences were observed between radial growth rates on the two media during weeks 3 and 4. Radial growth rates were highly variable on PDV-8+ thus the media was omitted thereafter.

Pycnidial formation differed between the three media. Pycnidia formed earliest on V8, followed by PDA+V8, and later on PDA. Pycnidia formed in 100 % of cultures grown on V8 by week two. Cultures grown on PDV8-A+ did not exhibit 100% pycnidial formation until the fourth week. Pycnidial formation was the slowest on PDA, where 12% of cultures still had not formed pycnidia by the end of the four week when the experiment was terminated.

Uredinia of *P. emaculata* on detached switchgrass leaves were inoculated

with *S. filum* conidia. Pycnidia formed 12-14 days after inoculation. Presence of the mycoparasite significantly reduced the number of urediospores per pustule by an average of 246 spores per uredium. The ability of the mycoparasite to reduce urediospore production may be important because urediospores are the inoculum that fuels secondary disease cycles of switchgrass rust.

Germination rate of urediospores was compared between spores collected from healthy or infected uredia. Germination rate of spores from healthy or parasitized uredia averaged 73% and 42%, respectively. For germinated spores, the germ tube length or urediospores from unparasitized uredia was 96.9um after 3 hrs. of observation, whereas the average germ tube length for urediospores from parasitized uredia was 32.3um after 3 hrs. Since the mycoparasite reduced urediospore production, germination percentage and germ tube length of infected urediospores, further investigation into its use as a potential biological control agent is warranted.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm-season, perennial, C-4 grass in the family *Poaceae* that is native to most of the United States (21), grows in clumps, and spreads by rhizomes (4). Switchgrass can be used as a biofuel feedstock, forage crop, ornamental plant, for bank stabilization, and as a component to improve wildlife habitat (13).

Puccinia emaculata Schw. is the causal agent of switchgrass rust (6). A serious level of damage results when diseased grass stops growing prematurely and lodge because the foliage dried out. While the pathogen negatively impacts biomass yield, seed production and forage quality of switchgrass. The pathogen can be negatively impacted by a mycoparasite (8).

In 2009, a mycoparasite, *Sphaerellopsis filum*, was observed parasitizing switchgrass in Mississippi, (M. Peterson, personal communication), North Carolina (Mike Benson, personal communication) and Tennessee (Y. Li, personal communication). This mycoparasite is known to infect 369 species and 30 genera of rusts worldwide and has been found in more than 50 countries (11). Pycnidia of *S. filum* are black, sub-globose, 90-200 µm and have ostioles where conidia are exuded in a gelatinous matrix (16). Conidia are hyaline, 1-septate, fusiform, 13-18 × 3-5 µm and have a gelatinous cap at one or both ends (14). In 1957, Schroeder and Hassebrauk observed appressoria like structures, which penetrated the urediospores of *Puccinia sp*. (18). Also, antifungal and antibacterial compounds (Darlucins A and B) have been isolated, and may be

important in the mycoparasite's interaction with *P. emaculata* (24). On willow rust, *S. filum* has reduced urediniospore production by up to 98% (23). Urediospore collapse and disintegration of *Puccinia recondita* urediospores was observed from infection by the mycoparasite. In some instances, spines on the urediniospore walls were absent (17). In contrast, Stahle and Kranz found no differences in the percent germination of urediospores from infection by the mycoparasite, but germ tube branching was greatly reduced (20). *S. filum* significantly reduced the number of telia, as well as basidiospore, produced by 50-75% of *Cronartium* rust on oak in Florida (12).

The research proposed was to establish growth studies of the mycoparasite. The first objective was to derive a suitable medium for growth and pycnidial formation, so quantities of the fungus and fit spores could be produced. The second objective of the research was to investigate the effects of the mycoparasite on urediospore production and urediospore fitness by measuring percent germination. This information will be important for future studies of the ecological potential of *S. filum* to serve as a potential biological control agent for switchgrass rust.

Materials and Methods

Identification of *S. filum*:

Single spores of *S. filum* were isolated and grown in Frier's liquid media (Y. Li, personal communication). Koch's postulates were satisfied by inoculating uredia of *P. emaculata* with conidia of *S. filum*. After formation of pycnidia, conidia were isolated from cirri and grown on PDA amended with 30 mg/L of streptomycin sulfate and 30 mg/L of chlortetracycline. Spores from these cultures were compared with spores from colonies used for the initial inoculations and confirmed to be *S. filum*. Conidia of *S. filum* collected from the second set of cultures were used to inoculate fresh uredia of *P. emaculata*. Pycnidia formed in uredia, indicating that *S. filum* established a parasitic relationship on switchgrass rust.

Scanning electron microscopy (SEM) was used to observe parasitism of rust by *S. filum*. Uredia of *P. emaculata* infected with *S. filum* were excised from detached switchgrass leaves, fixed in 2.5% gluteraldehyde for 4 hours, and washed several times with distilled water. Samples were then dehydrated using a series of progressively higher concentrations of ethanol, starting with a concentration of 25% and ending at 100%. Samples were critical point dried and mounted on specimen stubs. Gold/palladium alloy was applied to the surface of the samples using a spatter coater and samples were stored in a desiccator. SEM was used to examine uredia for signs of parasitism.

To obtain genetic sequences, *S. filum* was grown on V8 agar and tissue was mascerated by crushing under liquid nitrogen. DNA was extracted using the Quiagen DNeasy Plant Mini Kit protocol for purification of total DNA (Qiagen, Valencia, CA, USA), and amplified by PCR using primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′). Gel electrophoresis was used to evaluate DNA purity by the presence of a single band of approximately 500 bp. PCR products were purified using Quiagen DNA purification kit (Qiagen, Valencia, CA, USA) and sequenced.

Culture Media Experiment for Mycoparasite *Sphaerellopsis filum*:

V-8 juice agar was prepared by adding 125 mL of clarified V8 juice, 5g of maltose, and 9 g of Bacto agar to 1L of water. PDA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was prepared by following the company's instructions. V-8/ potato dextrose agar was prepared by combining the recipes of the two previous described media with 12 g of potato dextrose used as sugar source (maltose was omitted). Antibiotics (chlorotectracycline and streptomycin sulfate, Sigma-Aldrich Corporation, St. Louis, MO, USA) were added after autoclaving to give a final concentration of 30mg of each antibiotic per liter.

To measure growth of *S. filum* on each medium, a 6mm plug of *S. filum* (grown on the same medium as the test medium) was transferred to the center of each petri dish containing 20 ml of test medium. Twenty five plates of each type of media were used for the experiment for a total of 75 plates. Cultures were grown for one week with the lid of the petri dish up and then petri dishes were

flipped upside down to prevent moisture from interrupting growth of the cultures. Surface area measurements were taken once a week. Colony size and growth were compared among test media. While data was being collected, cultures were also observed for formation of pycnidia.

Effects of *S. filum* on *P. emaculata*:

Switchgrass leaves with mature uredia *of P. emaculata* were collected from a field near Vonore, TN and examined microscopically to confirm that pycnidia of *S. filum* were absent. Leaves with uredia without pycnidia of *S. filum* were considered free of the mycoparasite. Uredia were inoculated with the mycoparasite by spraying the detached leaves with a conidial suspension of 500 x 10^3 conidiospores/mL of *S. filum*. After inoculation with the mycoparasite, leaves were examined every two days to determine when pycnidia of *S. filum* formed. Leaves with uredia that were not inoculated with *S. filum* served as controls.

To determine if *S. filum* infection influenced the number of urediospores per uredium, ten leaf disks (1cm diameter) were excised. The number of uredia per disk were counted, and the disks were then teased in 500 μL deionized water to release urediospores. The number of urediospores per 500 μL of water was determined using a hemacytometer. The spore concentration was divided by the number of uredia present on the leaf disk, giving the average number of urediospores/uredium. This experiment was repeated three times.

To determine the effects of the mycoparasite on urediniospore germ tube

length and percent germination, six switchgrass leaves cv. ‘Alamo’ were collected from the greenhouse and split across the veins. Half of each leaf was used for the control and the other half of the same leaf was used for the mycoparasite treatment. Leaf segments were inoculated with either urediospores, or urediospores followed by *S. filum* conidia once uredia were formed. Leaf segments were incubated at room temperature under florescent lighting for 14 days. Two leaf disks, 1 cm in diameter, were removed from leaves inoculated with rust urediospores (control) or urediospores and the mycoparasite conidia (treatment). Urediospores from the leaf disks were collected by sliding adaxial leaf surfaces across water agar slides to dislodge spores. Germination was assessed three hours after inoculating water agar slides. Urediospores were considered germinated when the germ tube was at least equal to the radius of the spore it originated from. To obtain germ tube lengths, 20 germ tubes were randomly measured from each slide. A total of 240 germ tubes were measured, which included 10 spores per subsample, 2 subsamples per disk, 2 disks per leaf, from 6 leaves. To determine the germination percentage, Percent germination was determined from 100 spores that were randomly selected from water agar coated slides three hours. So, a total of 12 observations were made, which included 6 leaves, 2 disks per leaf, and 1 sample per disk. A randomized complete block design with subsamplings was used to determine the mycoparasite’s effects on germ tube length and germination percentage. Data were analyzed in SAS 9.2, using the PROC GLM procedure to analyze germ

tube length and the PROC MIXED procedure to analyze germination percentage.

Results and Discussion

Identification of *Sphaerellopsis filum*

The sequence was compared with BLAST results and matched accession numbers AY572490.1, AY836373.1, AY836371.1, AY607023.1, and AY607022.1 with 99% identity (501 to 537 bp). All matching results were isolates or strains of *Eudarluca caricis*, the perfect stage of *S. filum*. To our knowledge, this is the first report of *Eudarluca caricis* as a parasite of *Puccinia emaculata*.

Morphological characteristics of *S.* filum were evaluated using light microscopy. Pycnidia were black, sub-globose, 90-200 µm and had ostioles where conidia were exuded in a gelatinous matrix. Conidia were hyaline, 1-septate, fusiform, 13-18 X 3-5 µm and had a gelatinous cap at one or both ends. The morphological identity of *S. filum* was consistent with previous reports (14,16).

Culture Media Experiment for Mycoparasite *Sphaerellopsis filum*:

Colonies of *S. filum* on V8 agar had significantly faster radial growth than those grown on PDA+ during the first two weeks (Table 3.1), with differences between means being .29 cm and .27 cm for weeks one and two in repetition one, respectively, and .75 cm and .45 cm for weeks one and two in repetition two, respectively. Nicolas and Villanueva reported that in preliminary experiments, if vitamins were added to the growth medium, initial growth of the mycoparasite was accelerated (15). This is in agreement with our results since V8 agar has a much higher concentration of nutrients than did the other two

media. In weeks three and week four, no differences were observed between treatments. Colony areas of *S. filum* grown on PDV8-A+ had a high degree of variation within replications, as well as differences in results between repetitions, and therefore results were inconsistent for the purpose of maximizing growth and conidium production .

Significant differences in pycnidial formation were evident between all treatments (Table 3.2). Pycnidia formed earliest on V8 agar followed by PDV8 agar. One hundred percent of the cultures grown on V8-A exhibited pycnidial formation by week two; cultures grown on PDV8-A did not exhibit 100% pycnidial formation until week four. Pycnidial formation was the slowest on PDA, and 12% of cultures had not formed pycnidia by the end of the fourth week.

Effects of *S. filum* on *P. emaculata*:

Formation of pycnidia occurred 12-14 days after inoculation, at which time pycnidia were so abundant that accurate counts of the number of pycnidia could not be ascertained. Presence of the mycoparasite significantly reduced the number of urediospores per pustule by 246 spores on average (Table 3.3). The ability of the mycoparasite to reduce urediospore production is very important as these spores serve as inoculum in the secondary disease cycle for switchgrass rust. Therefore, the inoculum available for fueling secondary disease cycles would be reduced significantly. These findings are in agreement with similar observations in Mississippi, North Carolina, and Tennessee (M. Peterson, Mississippi State University, M. Benson, North Carolina State University, Y. Li,

University of Tennessee). The mycoparasite significantly reduced the number of urediospores produced per pustule by 31% on average (Table 3.3).

There were significant differences between the rust spore germination percentage between the control and the mycoparasite treatment, with the germinated percentage of urediospores being 73% and 42%, respectively (Table 3.4). For germ tube length, there were significant differences between the germ tube length between the control and treatment, with average germ tube length of 96.9 µm and 32.3 µm, respectively (Table 3.5). This data is in agreement with similar observations of the mycoparasite's ability to reduce spore viability on *Cronartium* oak rust and willow rust (12, 23). If antifungal compounds are produced by the mycoparasite as reported by Zapf et. al., those compounds, along with the direct penetration of urediospores, may contribute to the reduced spore numbers, shorter germ tubes, and lower germination percentages of urediospores that are reported here (23). Parasitism of urediospores by *S. filum* by direct penetration or the role antifungal compounds play in the parasitism of urediospores is not well understood. Hyphae of the mycoparasite have been observed attached to rust germ tubes, but direct penetration of the germ tubes was not observed (18). Kranz observed that most of the hyphae mass produced by the mycoparasite was associated with rust inside the plant tissue, and that most likely, the mycoparasite was absorbing nutrients from rust urediospores (11).

In conclusion, if the mycoparasite does become a viable control option for

switchgrass rust, addition of V-8 to the growth medium should be considered for producing inoculum to infect *P.* emaculata. We observed faster radial growth in the first two weeks of the experiment and earlier pycnidial formation. Our findings of the ability of *S. filum* to significantly reduce urediospore numbers, germination percentage, and germ tube length warrant further investigation into the use of the mycoparasite as an option for biological control for rust on switchgrass.

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Appendix

Table 3.1. Means of mycelial radial growth of *Sphaerellopsis filum* on potato dextrose agar (PDA), V8 agar, and a combination of PDA and V8 agar from repeated experiments One and Two (One: N=24 for PD-A and V8-A, and N=25 for PDV8-A; Two: N=25 for all treatments). Different letters indicate significant difference.

Week	Week 1		Week 2		Week 3		Week 4	
Experiment	One	Two	One	Two	One	Two	One	Two
V8A - PDA	.29 a	.14 b	.37 a	.01 a	.39 a	.24 a	.34 a	1.05 a
V8A - PDV8A	.31 a	.27 a	.75 ab	.47 b	.58 a	.73 a	.96 a	1.08 a
PDA - PDV8A	.02 b	.12 c	.38 b	.45 c	.19 a	.49 a	.62 a	.04 a
Pr > F	.0079	.0002	.0001	.0123	.0982	.0936	.1103	.0520

Table 3.2. Pycnidial production by *Sphaerellopsis filum* on potato dextrose agar (PDA), V8 agar, and a combination of PDA and V8 agar (recipes on page 44) from repeated experiments One and Two (One: N=24 for PD-A and V8-A, and N=25 for PDV8-A; Two: N=25 for all treatments). Different letters indicate significant difference.

Week	Week 1		Week 2		Week 3		Week 4	
Experiment	One	Two	One	Two	One	Two	One	Two
PDA	0 a	3 a	4 a	18 a	12 a	18 a	21 a	22 a
V8A	22 b	23 b	24 b	25 b	24 b	25 b	24 b	25 b
PDV8A	11 c	13 c	14 c	22 c	18 c	24 b	24 b	25 b
Probabilities	<.0001,<.0001		<.0001,0.0140		.0004,.00240		0.0437,0.0439	

Table 3.3. Effects of *Sphaerellopsis* filum on urediospore production by *Puccinia* emaculata. Uredia were not infected with the mycoparasite (control) or infected with the mycoparasite (treatment) (N=20). Different letters indicate significant difference.

	Repetition 1 Pr < .0001	Repetition 2 Pr = .0001	Repetition 3 Pr = .0014
Control	**374 a**	**363 a**	**338 a**
Treatment	**96 b**	**119 b**	**122 b**

Table 3.4. Effect of *Sphaerellopsis* filum on germination percentage of *Puccinia emaculata*. Urediospores were not infected with the mycoparasite (control) or infected with the mycoparasite (treatment) (N=12). Different letters indicate significant difference.

	Repetition 1 Pr < .0001	Repetition 2 Pr = .0002
Control	**73% a**	**68% a**
Treatment	**42% b**	**40% b**

Table 3.5. Effects of *Sphaerellopsis filum* on germ tube length of urediospores of *Puccinia emaculata*. Uredia were not infected with the mycoparasite (control) or infected with the mycoparasite (treatment) (N=240). Different letters indicate significant difference.

	Repetition 1 Pr < .0001	Repetition 2 Pr < .0001
Control	**96.9 μm a**	**88.0 μm a**
Treatment	**32.3 μm b**	**32.1 μm b**

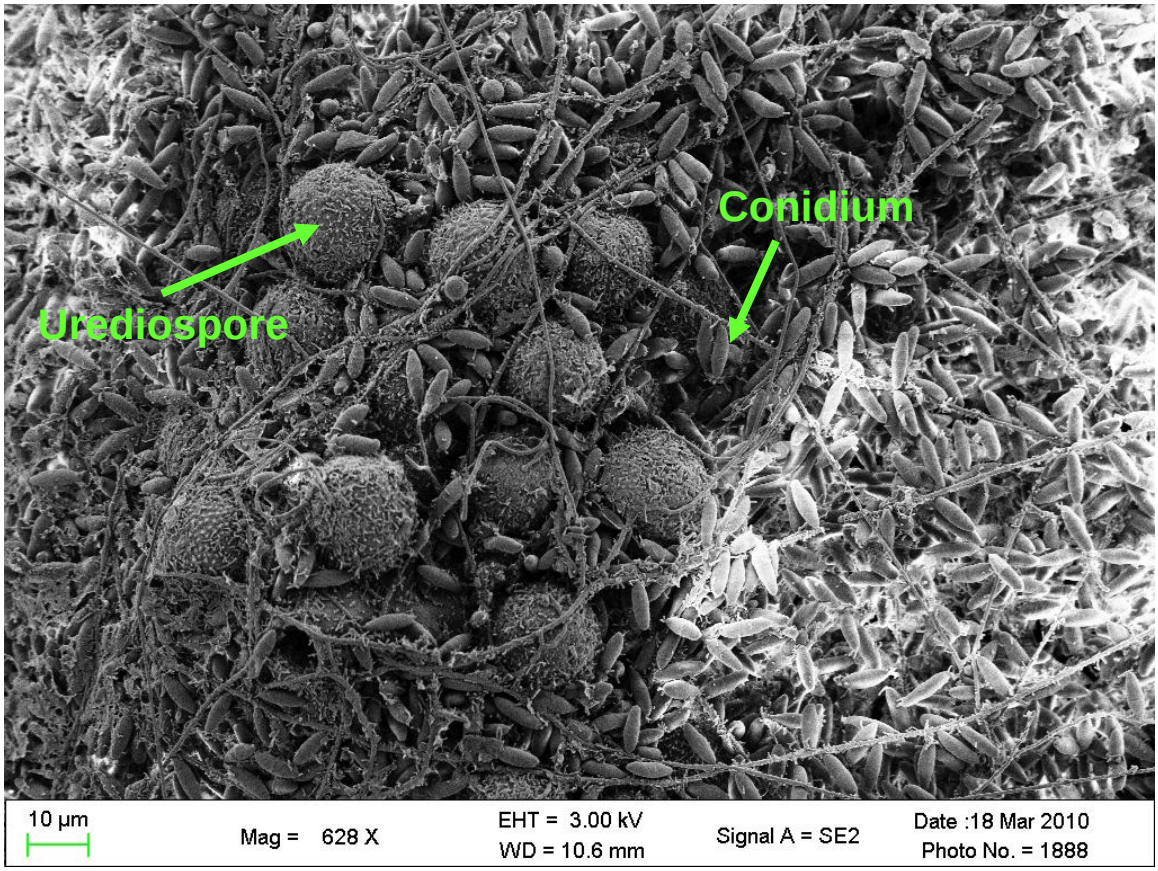

Figure 3.1. Uredium of *Puccinia emaculata* parasitized by *Sphaerellopsis filum*.

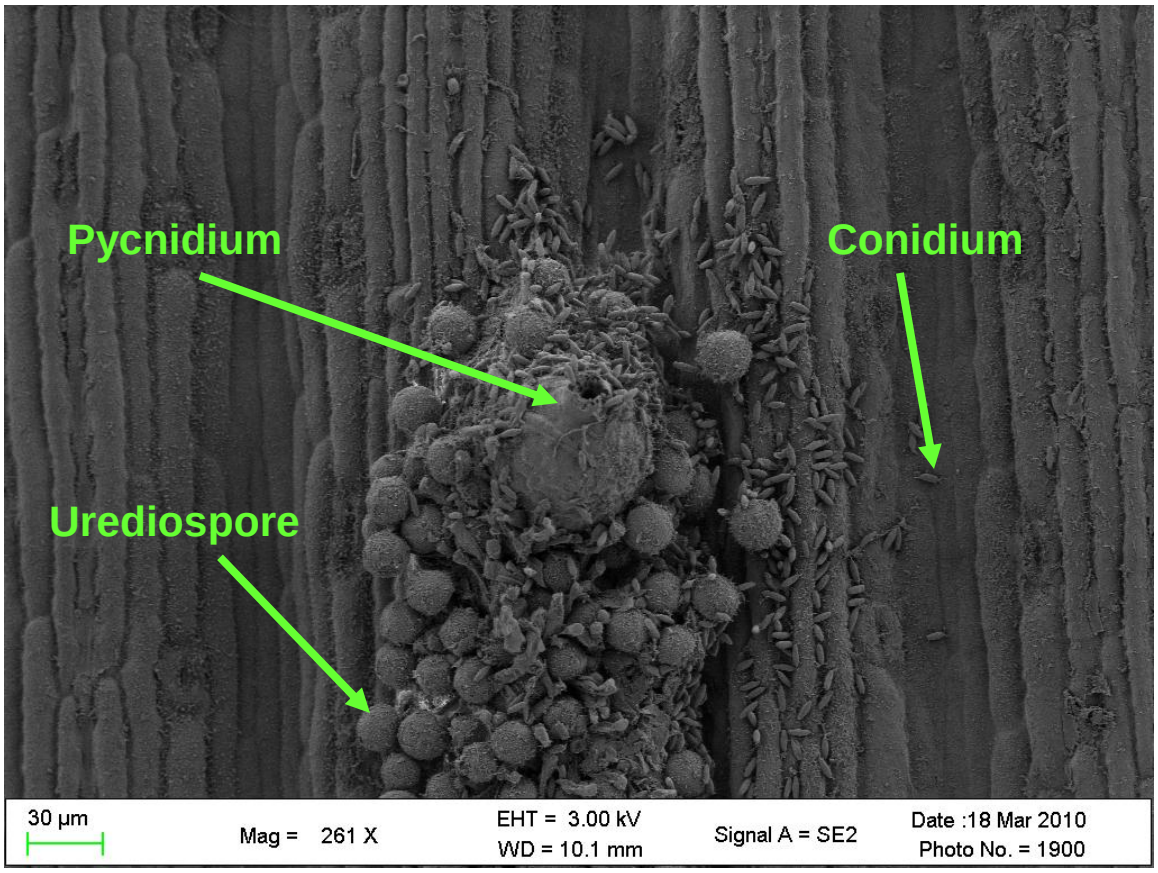

Figure 3.2. Pycnidium of *Sphaerellopsis filum* in a *Puccinia emaculata* uredium that has ruptured and dispersed conidia on the adaxial surface of switchgrass leaf.

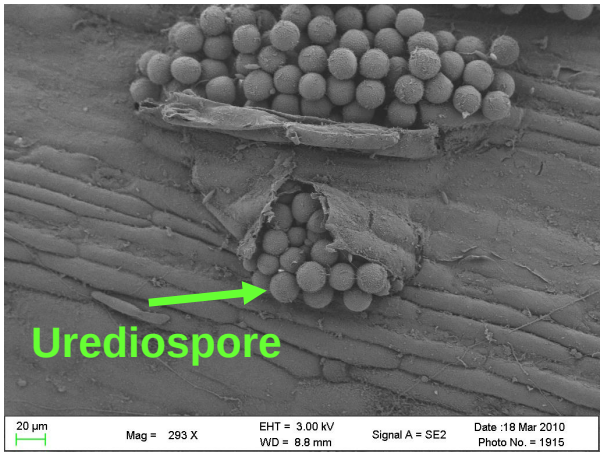

Figure 3.3. Uredium of *Puccinia emaculata* that has ruptured through the epidermis of *Panicum virgatum.*

Figure 3.4. Pycnidia of *Sphaerellopsis filum* embedded in uredia of *Puccinia emaculata* on *Panicum virgatum.*

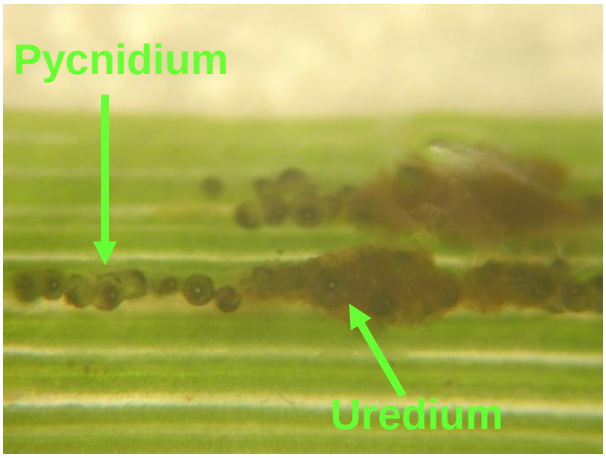

Figure 3.5. *Panicum virgatum* leaf from an agronomic field in Monroe County, TN infected with uredia of *Puccinia emaculata*.

CHAPTER IV
CONCLUSION

Epidemiology of *Puccinia emaculata*

Rust disease on switchgrass is becoming an increasing concern for agronomic producers as well as the horticultural industry due to its ability to spread rapidly and potential to cause yield losses. This thesis has identified the timing of initial detection of swithgrass rust, the rate of disease progression, and the maximum disease severity of rust in switchgrass fields in the Vonore, TN area. From this knowledge, future studies to measure yield losses can be designed to accurately reflect when and how rust epidemics will occur in swithgrass fields. In addition, the epidemic models can be utilized to predict disease severity values over time to help switchgrass producers predict when rust outbreaks are likely to occur. Knowing the timing of when the disease can first be detected and when epidemics of rust enter the log-phase is also critical for timing the application of fungicidal sprays for disease control.

Evaluation of *Sphaerellopsis filum*

Our findings of the ability of *S. filum* to significantly reduce urediospore numbers, germination percentage, and germ tube length will lead to addition research to evaluate *S. filum* as a biological control option for rust on switchgrass. If the mycoparasite does become a viable control option for switchgrass rust, the addition of V-8 juice to the growth medium should be considered since V-8 juice addition leads to faster radial growth and earlier pycnidial formation. This information will be important for future studies on the ecological potential of *S. filum* to serve as a potential biological control agent for

switchgrass rust.

VITA

Jonathan Black was born in Columbia, TN in February 22, 1986. When he was in the fourth grade, he and his family moved to Murfreesboro, TN, where he attended Cason Lane Academy. For high school, Jonathan attended Riverdale High School, where he was the Arms Officer, Rifle Team Commander, Orienteering Team Commander, and Drill Team Commander in the Junior Reserve Officer Training Program. Jonathan went to Middle Tennessee State University for his undergraduate degree in Animal Science with concentrations in Agriculture Education and Secondary Education, which he received in May of 2009. He has worked for Home Depot, Tractor Supply Company, Tennessee Farmers COOP, and Murfreesboro Parks and Recreation, and came to the University of Tennessee Knoxville in the summer of 2009 to pursue a Master's degree in Entomology and Plant Pathology under the supervision of Dr. Mark Windham. Currently, Jonathan is working as an Extension Agent for UT Extension in Williamson County, Tennessee.