MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF NOVEL POCULUM FUNGI ISOLATED FROM TURFGRASS IN FLORIDA

By

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To Aimee. My best friend and companion in my work.

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Abstract of Dissertation Presented to the Graduate School
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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF NOVEL POCULUM FUNGI ISOLATED FROM TURFGRASS IN FLORIDA

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Novel sterile fungi were isolated from bermudagrass (*Cynodon dactylon* (L.) Pers.) and seashore paspalum (*Paspalum vaginatum* Swartz) turfgrasses with symptoms similar to dollar spot in Florida between 2004 and 2009. Symptoms included small patches of discolored turfgrass that rarely exceeded 9 cm in diameter. Affected plants were blighted and turned nearly white over time. Phylogenies were constructed from sequences of the internal transcribed spacers, beta-tubulin, and elongation factor 1-alpha loci for these fungi and their closest relatives in the Rutstroemiaceae including *Poculum henningsianum* and the dollar spot pathogen *Sclerotinia homoeocarpa*. A distinct morphological difference between the novel fungi and *S. homoeocarpa* included discreet substratal stromata. The novel fungi displayed abnormal growth on nutrient rich potato dextrose agar (PDA) but were reliably isolated and maintained on one-fifth-strength PDA. On this medium, mycelia of the novel fungi were similar to *S. homoeocarpa*. The pathogenicity of these isolates was evaluated; however, the white patch symptom was never observed following inoculation. No tissue necrosis was observed on 'Penncross' creeping bentgrass (*Agrostis stolonifera* L.), 'Sea Isle Supreme' seashore paspalum, or 'Champion' and 'Sonesta' bermudagrass, but all

isolates were recovered from surface-sterilized leaf blades of inoculated grass. Inoculations of detached St. Augustinegrass leaf blades with the novel fungi produced similar results, and provided further distinction from the necrotrophic dollar spot pathogen. It remains unclear whether the novel fungi are endophytic and saprophytic colonizers, or whether specific environmental conditions, not elucidated in our experimentation, are required for symptom production. An assay to distinguish between the novel fungi and *S. homoeocarpa* was developed utilizing restriction fragment length polymorphisms observed when conserved ITS sequences were digested with restriction enzyme CviQi. Our data suggest the novel fungi described here represent sister species to *P. henningsianum* and *S. homoeocarpa* within the Rutstroemiaceae. The characterization of these sister species provides further evidence supporting the taxonomic reclassification of the dollar spot pathogen to the *Poculum* genus. Additional effort beyond this dissertation project will be required to fully describe and name the novel fungi and to fully understand their ecological role in the turfgrass environment.

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CHAPTER 1
LITERATURE REVIEW

Plant Pathology

A popular plant pathology text book written by the late chair of my department, Dr. George Agrios, chronicles the contributions of many scientists upon which the modern discipline of plant pathology was built. Antonious van Leeuwenhook's advancements in the 1670s provided one of the first glimpses of microbes through his optical systems that became the first microscopes. Observations and experimentation with microbes led Anton deBary to determine the cause of the Irish potato famine, and Louis Pasteur to develop the germ theory of disease in the 1860s (Agrios, 2005). Heinrich Hermann Robert Koch had advanced the theory by the late 1870s, and the basic principles at the core of his postulates are still used to demonstrate pathogenicity by modern plant pathologists.

Koch researched anthrax and later tuberculosis diseases primarily of livestock. Koch was able to isolate the causal agent of anthrax in pure culture, and showed anthrax symptoms were reproduced in animals inoculated with subcultures of the bacterium. He then reisolated the pathogen from the inoculated animals (Totora et al., 2002; Agrios, 2005).

From these observations and data, Koch developed a set of postulates as follows:

1. The organism suspected of causing the disease symptoms must be present in each suspected case of disease.
2. The suspected organism must be isolated from a diseased host and grown into pure culture.
3. The same disease symptoms must be reproduced on a healthy host when inoculated with the pure culture of the suspected organism.

4. The same suspected organism must be recovered from the experimentally inoculated and infected host.

Completing Koch's postulates with and identifying the cause of turfgrass diseases can be difficult. Patch diseases include particularly good examples, because disease symptoms caused by a diverse group of fungi are very similar, making symptoms alone unreliable for diagnosis. Recreating symptoms such as patches observed in turfgrass swards may be impossible in controlled environment chambers due to size limitations, or due to long-term temperature and moisture requirements for disease development. Microflora that inhabit turfgrass canopies, organic layers, and rhizospheres also include aggressive saprobes and facultative parasites that can outcompete causal agents in isolation attempts. Despite difficulties that may occur in completing Koch's postulates, the cause of some turfgrass diseases may be obscured if concessions are made in the satisfaction of the requirements necessary for the completion of Koch's postulates (Clarke and Gould, 1993).

Spring dead spot disease occurs on bermudagrass (*Cynodon dactylon* (L.) Pers.) and some other warm season turfgrasses and is caused by a complex of pathogens in the *Ophiosphaerella* Speg. genus; however, one of the first fungi associated with spring dead spot is *Helminthisporium spiciferum* (Bainier) J. Nicot (= *Bipolaris spicifera* (Bainier) Subramanian). Inoculations with this fungus produced leaf spot symptoms on healthy tissue, but did not produce symptoms of spring dead spot (Wadsworth et al., 1968). Necrotic ring spot and summer patch are additional examples of the difficulty in determining the etiology of some turfgrass diseases. Twenty years of research was required before evidence was amassed that would crystalize our understanding of the

“Fusarium blight” complex of diseases that included necrotic ring spot and summer patch disease among others (Smiley and Fowler, 1984; Smiley, 1987).

The understanding of the “*Rhizoctonia*-like” fungi and the ring, blight, and patch diseases they cause also are areas of recent and current turfgrass pathology research. Kammerer et al. (2011) associated a new variety of *Waitea circinata* (var. *prodigus*) S.J. Kammerer, Burpee & P.F. Harmon as the cause of a new disease on seashore paspalum (*Paspalum vaginatum* Swartz). Koch’s postulates were completed to demonstrate that this new fungus was pathogenic to ‘Sonesta’ bermudagrass and ‘Dark Horse’ roughstalk bluegrass (*Poa trivialis* L.) as well.

de la Cerda et al. (2007) confirmed that another variety of *Waitea circinata* (var. *circinata*) Warcup & P.H.B. Talbot caused a new disease of annual bluegrass (*Poa annua* L.) distinct from yellow patch (*Ceratobasidium cereale* D.I. Murray & Burpee), but with similar symptoms. Brown ring patch was associated with *W. circinata* var. *circinata* in each occurrence of the disease, and the pathogen reproduced symptoms on five-week-old pots of annual bluegrass and ‘Pencross’ creeping bentgrass (*Agrostis stolonifera* L.) that were similar to those observed in affected turfgrass swards, but on a smaller scale and lacking the field signature of thin bright yellow rings that led them to investigate this new disease on annual bluegrass (de la Cerda et al., 2007).

Pathogenicity tests described by Baird et al. (1998), Orshinsky et al. (2012) and Lv et al. (2010) provide detailed descriptions of the methods used for spring dead spot and dollar spot pathogens. Biological differences between isolates of *Sclerotinia homoeocarpa* Bennett and their pathogenicity were recently investigated (Orshinsky et al., 2012). Detached leaf assays demonstrated that wounding can affect lesion

development on creeping bentgrass among *S. homoeocarpa* isolates infected with *Ophiostoma mitovirus* 3a (OMV3a). Lesion development on detached leaves inoculated with symptomatic isolates of *S. homoeocarpa* (isolates infected with OMV3a) developed symptoms away from the infection point compared to virulent or OMV3a-asymptomatic isolates which produced lesions at or near the point of infection. Although symptoms do appear from inoculations with OMV3a-symptomatic isolates on bentgrass, the symptoms may not be as rapid in development compared to inoculations with aggressive isolates (Orshinsky et al., 2012).

Turfgrass

Among the vast economic effects of the multi-billion dollar turfgrass industry are numerous recreational and aesthetic benefits, and less apparent functional environmental benefits that humans have been enjoying for centuries (Beard, 1973). Estimates from 2002 put the annual expenditures for the U.S. turfgrass industry at roughly $66 billion (Haydu et al., 2008). Aside from the high-quality, low-cost, recreational playing surfaces that contribute to physical health, and the aesthetic improvements that add to mental health, property values, and community pride; groundwater recharge and surface water quality, reduced soil erosion and dust prevention, heat reduction, and noise abatement are just a few of the important environmental benefits provided by maintaining high quality turfgrass (Beard and Green, 1994).

The Poaceae is comprised of some 600 genera (Beard, 1973) and over 10,000 species of grass (Christians, 2007); however, only roughly 50 species are of any importance in the turfgrass industry (Christians, 2007). Grasses are monocots (monocotyledons) which have a flag leaf, parallel veins, usually have ligules, stems with

scattered vascular bundles, and flowering parts that occur in multiples of three among a few other distinct differences compared to dicotyledonous plants (McCarty, 2005). The adaptation of turfgrass species to the environmental conditions (e.g. temperature and precipitation) in the major climatic zones of a region are primary influences on their geographic distribution (Beard, 1973).

Turfgrass species are separated into different groups according to their respective photosynthetic pathways. Photosynthesis is a process where energy from sunlight is converted to chemical energy which is then stored as carbohydrates (e.g. glucose ($C_6H_{12}O_6$)) (Karp 2002; Raven and Johnson, 2002). The basic process can be broken down into two separate events. The first part of the process (i.e. the light dependent reactions) requires light to produce the raw materials/energy sources NADPH and adenosine triphosphate (ATP). The second part of the process (i.e. the light independent reactions) does not require light, but instead uses the NADPH and ATP produced during the light dependent reactions to power the reactions needed for the synthesis of glucose (Karp, 2002).

"Cool-season" turfgrasses or C_3 grasses, have a three carbon intermediate to begin the Calvin cycle for carbon fixation. Temperature optima for these species are between 18°C and 24°C. As temperatures increase above the optimal temperature, oxygen reacts more frequently than carbon in a process called photorespiration which in turn decreases carbohydrate production (Karp, 2002). The enzyme ribulose bisphosphate carboxylase/oxygenase, or rubisco as it's called, is the site of carbon fixation but is also coincidentally the site of oxygen fixation. Rubisco's primary function is the carboxylation of RuBP; however, a second enzymatic activity exists because the

active site for carboxylation is the same as that for oxidation of RuBP. The reason is that rubisco binds to RuBP instead of CO_2 or O_2. Consequently, carboxylation and oxidation compete with each other. When temperatures are between 18° and 24°C carboxylation activity is four times as likely compared to oxidation activity (Raven and Johnson, 2002). To overcome the loss of carboxylation activity and the increase in photorespiration in warmer temperatures, plants like warm-season turfgrass evolved an alternate metabolic pathway and a specialized anatomy that allows them to fix carbon at relatively low CO_2 levels compared to plants that perform C_3 photosynthesis (Hatch, 2002). Cool-season C_3 turfgrasses can stay green through the winter and include species such as bluegrasses, bentgrasses, fescues (*Festuca* L.), and ryegrasses (*Lolium* L.) (Christians, 2007). Cool-season turfgrasses have been used in Florida to provide green putting surfaces during winter months, but disease pressure can be a problem (Dudeck, 1991; Stiles et al., 2007; Harmon et al., 2011).

Bluegrasses and fescues vary in appearance and growth habit. Bluegrass leaves have a boat-shaped leaf tip and folded vernation. Fescues have both the widest and the narrowest leaves among cool-season turfgrass species and are referred to as coarse and fine fescue respectively (Christians, 2007). Ryegrasses occur as annual and perennial varieties. They are best known for their rapid seed germination and establishment (Christians, 2007). Perennial ryegrasses are also known for their tolerance to salinity which can be a concern in Florida (Dai et al., 2009; Krum et al., 2011). Bentgrasses are widely used on golf course putting greens for their high quality playing surfaces but can have problems with weed infestations as a result of their management practices in some areas (Turgeon, 2012; Jeffries et al., 2013), and as a

group include the most susceptible hosts to *S. homoeocarpa* (Chakraborty et al., 2006; Orshinsky et al., 2012).

"Warm-season" turfgrasses are known as C_4 grasses because they have a four carbon photosynthetic intermediate produced from photosynthesis. Warm-season turfgrasses have specialized leaf anatomy, have higher temperature optima, and thrive in temperatures between 27°C and 35°C, but are less efficient at lower temperatures and levels of solar radiation (Hatch, 2002). Chlorophyll production is slowed and subsequently degraded in warm season turfgrass species when soil temperatures fall below 10°C as the plants enter dormancy. Warm-season turfgrass species include bermudagrass, zoysiagrass (*Zoysia* Willd.), St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze], and seashore paspalum (Beard, 1973; Christians, 2007).

Zoysiagrass is thought to be very tolerant of cold weather but is sensitive to large patch caused by *Rhizoctonia solani* (Okeyo et al., 2011; Obasa at al., 2013). Zoysiagrass can tolerate freezing temperatures with differences existing in cold hardiness among cultivars (Patton and Reicher, 2007). Slow growth combined with long periods of stress can make recovery time from fungal and insect pests long (Obasa, 2012; Christians, 2007; McCarty, 2005).

St. Augustinegrass is widely used as a lawn grass throughout Florida and along the Gulf Coast in Texas, Louisiana, and Mississippi. St. Augustinegrass will go dormant until soil temperatures return to 12°C to 15°C (Christians, 2007; McCarty, 2005). St. Augustinegrass does not tolerate cold stress very well, but 'Floratam' St. Augustinegrass is thought to have some drought tolerance (Busey, 2003; Steinke et al., 2010). St. Augustinegrass is prevalent in Florida and susceptible to *S. homoeocarpa.*

Detached leaves of St. Augustinegrass develop specific symptoms within 2 to 3 days when inoculated with *S. homoeocarpa* depending on the biotype (Liberti et al., 2012).

Bermudagrass is adapted for many uses including golf course fairways, tees, and putting greens, sports fields, lawns, etc. Bermudagrass is very tolerant of low mowing heights but favors high mowing heights during establishment (Wherley et al., 2011). In recent years, hybrid bermudagrasses (*Cynodon Dactylon* (L.) Pers. *x C. Transvaalensis* Burtt Davy) have been used on many golf course putting greens for their high quality and fine texture compared to other warm-season turfgrasses (McCullough et al., 2005; Christians, 2007; McCarty, 2005).

Seashore paspalum is best known for its tolerance to salinity and reduced need for nitrogen compared to bermudagrass, but is also thought to be relatively intolerant of cold temperatures (Brosnan et al., 2009; Christians 2007; Cardona et al., 1997). Seashore paspalum usually does not produce seedheads but can produce them when stressed (McCarty, 2005). Seashore paspalum is susceptible to dollar spot disease (Lv et al., 2010; Ruiz et al., 2006) and is easily propagated with the same methods used to propagate bermudagrass. *Poculum* Velen. isolates have been associated with the grass (Harmon, 2009; Ruiz et al., 2006).

Fungicide Use in the Turfgrass Industry

The popularity of golf is at an all-time high with more than 9 million people participating in the sport each year (FL DEP, 2009). The game is thought to have originated in Scotland in the mid-14th century (McCarty, 2005) and may have been around as early as the 13th or 14th century as evidenced in a 1363 writing by Thomas Rymer (Allison, 1970). Records prohibiting the sport by name are reported from the mid to late 1400s, as it was thought to affect the security of the country by adversely

affecting archery practice (Allison, 1970). Golf contributed $9.2 billion to Florida's tourism economy and provided 226,000 jobs to Floridians in 2002 (Haydu and Hodges, 2002). Golf courses in Florida average 114 acres of maintained turfgrass on more than 1300 golf courses (FL DEP, 2009). Despite turfgrass managers' best efforts; diseases of amenity turfgrass are thought to have been occurring for many years before reports began surfacing in the early 1900s (Monteith and Dahl, 1932). Effective disease control on turfgrass today requires the use of various fungicides to maintain the expected quality.

Diseases of agriculture crops affected society long before the causes were well understood. Throughout history methods of disease control from praying to soaking seeds in urine have been documented (Latin, 2011). It was noticed by chance in 1882 that grapes sprayed with a mixture of copper sulfate and lime (as a theft deterrent) were healthier and more vigorous compared to plants that were not treated with the mixture (Morton and Staub, 2008; Agrios, 2005). Pierre Alexis Millardet began working on various rates of what became known as Bordeaux mixture and by 1885 the first fungicide recommendation for management of powdery mildew, a devastating disease of grapes, was available (Agrios, 2005). Additional agricultural uses were discovered, and ultimately an attempt to control turf disease was made with Bordeaux mixture when applications were made at the Arlington Turf Gardens near Washington D.C. around 1917 (Latin, 2011). Since that time, various chemical groups, formulations, and modes of action have been discovered and developed as fungicides (Morton and Staub, 2008; Latin, 2011). Target sites of some fungicides remain unknown. Others have specific

sites including cell walls, cellular membranes, cytoskeleton or microtubules, the mitochondria, nucleic acids, and/or general cellular constituents of fungi (Latin, 2011).

Dollar Spot

General Information, Signs, and Symptoms

Dollar spot historically has been one of the most costly and common fungal diseases of turfgrass in North America (Vargas, 1994; Couch, 1995). More money is spent annually on fungicides for dollar spot (caused by *Sclerotinia homoeocarpa*) control than any other turfgrass disease (Vargas, 1994). All cultivated cool-season and warm-season turfgrasses are affected worldwide (Smiley et al., 2005), and a recent study claimed that more than 70% of fungicides applied on golf courses are for management of dollar spot, brown patch, and anthracnose (Bonos, 2006).

Dollar spot is considered a serious and persistent disease of turfgrass (Smiley et al., 2005; Walsh et al., 1999). Conditions that are ideal for disease development include average temperatures around 22°C with generally wet conditions for at least two days (Hall, 1984). Infection centers are typically small, but if left untreated, may coalesce producing an unthrifty turfgrass that can be invaded by weeds (Smith et al., 1989).

Signs of the dollar spot pathogen include tufts of white mycelium with a cobweb-like appearance early in the morning before the dew evaporates from the turf (Smiley et al., 2005). Early symptoms on individual leaf blades include chlorosis and water-soaking followed by a bleached straw color. Leaf blade lesions may be bordered by a dark reddish brown band and may have a distinct hourglass shape. Lesions may also appear oblong or oval shaped on wider-bladed turfgrasses (Monteith and Dahl, 1932; Smith et al., 1989; Vargas, 1994; Smiley et al., 2005).

When infection occurs on the cut ends of leaf blades, tip dieback symptoms also may occur then progress to a leaf blade blight (Couch, 1995). Dollar spot patch characteristics may vary depending on height of cut and turfgrass type. Infection centers on fine-leaf close-mown turfgrass are small, discreet, circular patches approximately 1 to 2 cm in diameter, but on higher-mown coarse-bladed turfgrasses patches may reach, but do not typically exceed, 15 cm in diameter and are irregular in shape (Smith et al., 1989; Smiley et al., 2005).

Management and fungicide sensitivity

Repeated fungicide applications to manage dollar spot disease have led to fungicide resistance to multiple fungicide groups in some populations (Burpee, 1997; Miller et al., 2002; Putman et al., 2010). Burpee (1997) evaluated the activity of several commercial formulations during *in vitro* and field assays for control of dollar spot on creeping bentgrass. EC_{50} concentrations of a sensitive and a non-sensitive isolate were reported for the following fungicides which include, among others, some that are commonly used for dollar spot management: Benlate 50WP (benomyl), Daconil Ultex 82.5DG (chlorothalonil), Rubigan A.S. 1.0AS (fenarimol), Fluazinam 500F (fluazinam), Chipco 26019 Flo 23.3F (iprodione), Banner 1.1E (propiconazole), Systec 1998 85WDG (thiophanate-methyl), and Bayleton 25WP (triadimefon).

Miller et al. (2002) compared *S. homoeocarpa* sensitivities to propiconazole for six populations with over 400 isolates collected from golf courses in Georgia. At least 50 isolates were included from each location which consisted of four sites with no prior exposure to propiconazole, and two sites that had a history of propiconazole use for the previous 4 to 6 years. Baseline sensitivity was determined by estimating the EC_{50} values by linear regression of the relative growth inhibition for parts of the exposed and

unexposed populations. EC_{50} was significantly higher in populations that were exposed to demethylation inhibiting (DMI) fungicides for a few years prior. Sensitivity to propiconazole was compared at 0.02 μg a.i. ml^{-1} and 0.2 μg a.i. ml^{-1} for all collected isolates and although a significant correlation was noted between the relative growth of the exposed and unexposed populations at both concentrations, a higher correlation (r = 0.90, $P < 0.0001$) was noted at 0.02 μg a.i. ml^{-1} making it the more useful of the two discriminatory doses for distinguishing between sensitive and insensitive isolates within the population.

A recent study of sensitivity to fungicides was completed for several *S. homoeocarpa* populations. Putman et al. (2010) performed an extensive and expansive sampling effort that included a baseline population that had never been exposed to synthetic fungicides. Mean EC_{50} values for iprodione and propiconazole were determined by probit transformation, and each of the populations screened for sensitivity on fungicide amended media. In addition to assays for iprodione and propiconazole sensitivity, a qualitative discriminatory dose of 1,000 μg a.i. ml^{-1} was used to screen isolates for sensitivity to thiophanate-methyl on which the fungus either grows or not. The majority of populations that had been previously exposed to repeated propiconazole and iprodione fungicide use were less sensitive compared to the unexposed population. Sensitivity to thiophanate-methyl varied among the tested populations. The work by Putman et al. (2010) and Miller (2002) provides reliable EC_{50} concentrations for propiconazole and iprodione, and a qualitative means to assess sensitivity to thiophanate-methyl. These values can serve as a baseline to monitor shifts in fungicide sensitivity among *S. homoeocarpa* populations in the northeastern

and southeastern states. Studies that include EC_{50} concentrations for boscalid and azoxystrobin sensitivity in other fungi are available (Vincelli and Dixon, 2002; Zhang et al., 2009), and these concentrations taken with the information provided by Burpee (1997), Miller et al. (2002), and Putman (2010), provide a useful starting point for a preliminary assay to determine the sensitivity of the novel *Poculum*-clade isolates in this study to a broad group of active ingredients.

Agronomic Management Options

Cultural practices including dew removal and mowing frequency can influence dollar spot epidemics. Delvalle et al. (2011) observed reduced dollar spot pressure and improved fungicide performance in trials with increased mowing frequency; however, a similar study reported that reduced mowing frequency and the use of growth regulators (e.g. trinexapac-ethyl, paclobutrazol) also reduced dollar spot pressure (Williams et al., 1996; Delvalle et al., 2011; Putman and Kaminski, 2011). Appropriate nitrogen fertilizer rates and application frequency contribute to overall plant health which can influence dollar spot development and management (Couch and Bloom, 1960; Markland et al., 1969; Walsh et al., 1999). Silicon applications have not been found to reduce dollar spot severity (Zhang et al., 2006; Uriarte et al., 2004). Biological control of dollar spot has had very limited success through the use of endophytes and hypovirulence (Clarke et al., 2006; Deng and Boland, 2004; Zhou and Boland, 1998). Breeding for genetic resistance to *S. homoeocarpa* is an objective of long term breeding efforts (Bonos et al., 2004; Bonos, 2006). Composts and topdressings also have the potential to reduce fungicide inputs required to manage dollar spot (Boulter et al., 2002; Nelson and Craft, 1992; Goodman and Burpee, 1991).

Population Biology

Vegetative compatibility groups (VCG) have been used to study the diversity of *S. homoeocarpa* populations (Powell and Vargas, 2001; Deng et al., 2002). Powell and Vargas evaluated compatibility among 1300 isolates of *S. homoeocarpa* collected from sites in Michigan, Illinois, and Wisconsin between 1995 and 1997 in a study to determine if seasonal epidemics were caused by unique populations. Six different compatibility groups were identified during both seasonal epidemics occurring from May to July and August to October indicating some diversity within the population. Sequences of the internal transcribed spacer 1 (ITS1) were identical for selected isolates from each location for each seasonal epidemic indicating both epidemics were caused by the same species of fungus despite the differences observed within the population.

Deng et al. (2002) investigated the effect of vegetative incompatibility on transmission of dsRNA during their work to develop a biological control option for dollar spot. Four vegetative compatibility groups were observed among 116 isolates with the majority of those occurring in two groups similar to observations made by Powell and Vargas (2001). Transmission of dsRNA was successful in all compatible reactions but there was also some transmission observed in incompatible reactions. Deng et al. (2002) described three types of incompatible reactions: gap, line gap, and barrage. Gap reactions are characterized by a 3 to 10 mm gap at the zone of interaction with two dark lines on the bottom of the plate. Line gap reactions are 1 mm wide at the zone of interaction with a single line on the bottom of the plate, and barrage reactions are characterized by a thick layer of mycelium with no gap at the zone of interaction and a dark zone on the bottom of the plate. Gap reactions were poor at transmitting dsRNA,

but line gap reactions succeeded in transmitting the dsRNA in greater than 30% of the pairings. In barrage reactions where transmission was attempted, the results were variable but, the conclusion was that transmission of dsRNA in barrage reactions was limited.

A 2004 report on a study of 65 isolates collected from the north and southeastern states from 1972 to 2001 included the following locations: Pennsylvania, New York, New Jersey, Illinois, Florida, North Carolina, Delaware, Virginia, and Canada (Viji et al., 2004). VCG, amplified fragment length polymorphism (AFLP), and pathogenicity among VCGs were compared for these isolates. AFLP analysis resulted in two distinct groups. One group contained all the isolates except those from Florida which formed a separate group. Although not consistent among all VCGs, Viji et al. (2004) reported a relationship between VCGs and AFLP groups indicating some level of genetic diversity between VCGs.

Vegetative compatibility among several isolates of fungi thought to be *S. homoeocarpa* isolated from turfgrass in Florida with dollar spot symptoms was compared by Ruiz et al. (2006). These fungi were found to be incompatible among themselves, and all tested *S. homoeocarpa* VCGs as well. Preliminary sequence analyses of the internal transcribed spacer (ITS) from the recovered fungi were compared to sequences in Genbank and found to be most similar to *Poculum henningsianum* (Plöttn.) T. Schumach. & L.M. Kohn. These fungi associated with dollar spot symptoms appeared to include a diverse group of fungi including *S. homoeocarpa*, and previously undescribed species likely belonging to the *Poculum* genus.

Distinct morphological, genetic, vegetative, and mating-type differences were observed between isolates collected from golf courses with dollar spot symptoms in Florida and isolates from collections in North Carolina and Indiana. Sequence comparisons of the ITS and MAT loci produced two distinct groups recognized as common and Floridian type *S. homoeocarpa* that could be reliably separated with RFLP analysis of the ITS region for selected isolates (Liberti et al., 2012). Morphological differences in Floridian isolates included pigment production under intense light incubation, and stratified stroma formation compared to common isolates. These stratified stroma appeared as layers that may or may not occur in concentric rings. Results from vegetative compatibility assays generally agreed with previous studies in that very little compatibility occurred among the Floridian isolates, while the bulk of the common isolates belonged to two vegetative compatibility groups. Differences in symptom production on detached leaves of St Augustinegrass were noted between Floridian and common isolates as well (Liberti, et al., 2012).

Taxonomy and Nomenclature of Dollar Spot Fungus

Similarities in colony morphology between the "brown patch" and the "dollar spot" pathogens, and similarities in disease symptoms, initially led researchers to believe that dollar spot was caused by a *Rhizoctonia* sp. DC. (Monteith and Dahl, 1932; Bennett, 1937). While characterizing fungi collected from Great Britain, Australia, and the U.S., Bennett discovered three distinct strains of a "*Sclerotinia*" Fuckel fungus caused dollar spot symptoms (Bennett, 1937). Bennett described a "perfect strain" that produced ascospores and conidiospores, an "ascigerous strain" that produced ascospores and microconidia, and a "non-sporing strain" that produced no ascospores or conidia except for some rudimentary microconidial structures (Bennett, 1937).

Bennett's description of the stroma associated with each of the three strains is not consistent with stroma produced by *Sclerotinia*. Fungi classified in the genus *Sclerotinia* must have rounded sclerotia with a hard outer rind and a soft inner medulla (Boudier, 1907, Whetzel, 1945). Sclerotial structures observed in the dollar spot fungus are described as isolated or expansive flakes with a parchment like thickness. This observation is a critical characteristic and illustrates the fungus's failure to satisfy the morphological requirements for taxonomic placement in *Sclerotinia*. Bennett discussed the difficulty in selecting the proper genus for the dollar spot fungus and even suggested possible placement in the *Stromatinia* (Boud.) Boud. rather than the *Sclerotinia*; however, during the characterizations of the strains, sporophores were observed originating from the edges of isolated sclerotial flakes which were thought to be aggregates of microsclerotia (Bennett, 1937). These observations of sporophore origination combined with the taxonomic placement of the *Stromatinia* in the family Sclerotiniaceae Whetzel led Bennett to conclude that it was acceptable to place the dollar spot fungus in the genus *Sclerotinia*. The specific name given to the dollar spot fungus was chosen for the similarity between the cupulate fruiting bodies (i.e. the apothecium and the conidiophores). The dollar spot fungus was referred to specifically as *homoeocarpa* (Bennett, 1937).

Colonies of *S. homoeocarpa* in culture are white with abundant aerial hyphae that can develop blue, yellow, green, or cinnamon colored appearance over time (Bennett, 1937). Wooly aerial mycelium eventually becomes compact and felt-like often turning a cinnamon color followed by the formation of a thin stroma layer that is olivaceous or black in color (Bennett, 1937). Ion-concentrations in the range of pH 4 to

7 were more favorable for growth. British isolates grew better at lower temperatures compared to the American isolates with optimal temperatures for both ranging from 20°C to 30°C (Bennett, 1937).

Sclerotinia homoeocarpa was believed to have been placed in an incorrect taxonomic group but was never renamed by mycologists (Whetzel, 1945b; Kohn and Greenville, 1989a, 1989b; Novak and Kohn, 1991; Carbone and Kohn, 1993; Holst-Jensen et al., 1997). A synopsis of the Sclerotiniaceae published shortly after Bennett's description of the dollar spot fungus did not include *S. homoeocarpa* (Whetzel, 1945). A monograph of *Sclerotinia* published the following year excluded *S. "heterocarpa*" from the genus, and it was noted that the dollar spot fungus was instead thought to be a species of *Rutstroemia* P. Karst. (Whetzel, 1945).

S. homoeocarpa rarely produces apothecia in nature and attempts to produce fruiting bodies in vitro have been unsuccessful until recently (Orshinsky and Boland, 2011; Fernstermacher, 1980). Since its description, there have been only three published reports of apothecial production in *S. homoeocarpa* two of which occurred at the Sports Turf Research Institute (STRI) in Great Britain (Jackson, 1973; Baldwin and Newell, 1992; Orshinsky and Boland, 2011). Apothecia very similar to those produced by *S. homoeocarpa* in the field were reproduced by Jackson in cultures of isolates obtained from fescue turf (*Festuca rubra* L. ssp. *Rubra*) with dollar spot symptoms. These observations, and examinations of the remnants of Bennett's original *S. homoeocarpa* specimens led Jackson to believe that dollar spot may be caused by more than one fungus, and that the dollar spot fungi were in fact species of *Rutstroemia* (Jackson, 1973); however, just prior to Jackson's findings, the genus *Rutstroemia* was

declared taxonomically invalid (Dumont and Korf, 1971; Dumont, 1972). Although a recircumscription of the Sclerotiniaceae resulted in the erection of Rutstroemiaceae Holst-Jensen, L.M. Kohn & T. Schumach., a new family to accommodate the substratal stromatal lineage of fungi previously placed in the Sclerotiniaceae, the dollar spot pathogen remains in the *Sclerotinia* genus.

Sclerotiniaceae and Rutstroemiaceae

Sclerotiniaceae

The Sclerotiniaceae was originally described by Whetzel (1945) to accommodate 15 genera of fungi (*Sclerotinia, Ciborinia, Monilinia, Stromatinia, Ciboria, Botryotinia, Septotinia, Streptotinia, Verpatinia, Ovulinia, Coprotinia, Martinia, Rutstroemia, Lambertella, and Seaverinia)* that produced primarily stipitate, cupulate, funnel-form, or saucer-shaped apothecia that arise from a sclerotium. Apothecia contain eight-spored, inoperculate asci. Ascospores are usually hyaline, unicellular, smooth, ellipsoidal, and flattened on one side. Spermatia are typically globose, and various conidia are present in certain genera. Sclerotia are comprised of a medulla that is typically covered by a rind and also serve a nutrient storage role. Sclerotia have a definite shape and have unique names to describe them (e.g. tuberoid, hollow-sphaeroid, mantleoid-sphaerulate, discoid, mummioid, and plano-convexoid).

Significant changes to the generic nomenclature of the Sclerotiniaceae were implemented by Dumont and Korf (1971). Although *Sclerotinia* was part of the original description of the Sclerotiniaceae when erected by Whetzel (1945), the generic name was incorrectly applied and was thus replaced by *Myriosclerotinia* N.F. Buchw. *Ciboria* Fuckel was changed to the then available and correctly applied *Sclerotinia*; *Stromatinia* was changed to *Tarzetta* (Cooke) Lambotte, and finally, the genus *Rutstroemia* was

restricted to one species (*Rutstroemia bulgarioides* (Rabenh.) P. Karst. 1871 = *Peziza bulgarioides* Rabenh.1867) that was not included in the original circumscription of the Sclerotiniacae (Whetzel, 1945). Retypifications and non-adherence to the International Code of Botanical Nomenclature led to *R. bulgarioides* exclusion from White's monograph of *Rutstroemia* (White, 1941; Dumont and Korf, 1971). The remaining species of *Rutstroemia* from Whetzel's original circumscription of the Sclerotiniaceae (Whetzel, 1945) (*R. americana, R. bolaris, R. calopus, R. echinophila, R. elatina, R. longiasca, R. longipes, R. luteo-virescens, R. macrospora, R. nerii, R. nervisequa, R. petiolorum, R. poluninii, R. pruni-serotinae, R. pruni-spinosae, R. renispora, R. setulata, R. sydowiana, and R. urceolus)* were excluded from *Rutstroemia* as a result of Dumont and Korf's (1971) review of the generic nomenclature of the Sclerotiniaceae. Dumont and Korf (1971) and Dumont (1972) were convinced, based on apothecial anatomy, that the remaining species of fungi brought together under the *Rutstroemia* were in fact members of three distinct genera (Dumont and Korf, 1972). *Poculum*, *Calycina* Nees ex Gray, and *Lanzia* Sacc. were proposed as appropriate generic names to accommodate the various species of *Rutstroemia* (Dumont, 1972). *Poculum* was proposed by Velonovsky (1934) to accommodate two species of fungi: *P. ruborum* and *P. juncorum*. Velonovsky's description of the two species is based on apothecial anatomy and Dumont's description of the stroma is limited in that it only describes it as substratal (Velonovsky, 1934; Dumont, 1972).

Rutstroemiaceae

A study of 34 taxa from 23 different genera highlighted the distinct differences between the sclerotial and substratal stromatal (stroma that are formed below the surface of the substrate) producing taxa, and the diphyletic nature of the Sclerotiniaceae

at that time (Holst-Jensen et al., 1997). As a result of that study and works leading up to it, the Sclerotiniaceae was recircumscribed to include only those taxa that produced sclerotial stroma as described by Whetzel (1945), while the remaining substratal stroma producing taxa (*Lambertella*, *Lanzia*, *Poculum*, *Rutstroemia*, and *Verpatinia*) would be collected in the newly formed Rutstroemiaceae (Holst-Jensen et al., 1997).

Two reports supporting the recircumscription of the Sclerotiniaceae examined details of stroma as a source of taxonomic characters similar to studies with *Rhizoctonia* spp. (Kohn and Greenville, 1989a; 1989b; Tu and Kimbrough, 1975). Detailed observations of the stroma with light microscopy, electron microscopy, and chemical staining enabled Kohn and Greenville (1989a, 1989b) to divide the taxa in their studies into three groups according to their specific stromatal structures which included determinate and indeterminate sclerotia, and substratal stromata. With so many genera present from the Sclerotiniaceae, Kohn and Greenville (1989a, 1989b) were able to compare isolates among different genera and either demonstrate similarities within the family, or illustrate differences between genera based on these stromatal characters (Kohn and Greenville 1989a, 1989b).

The observations on the anatomical and histochemical studies suggesting two distinct lineages were confirmed by Novak and Kohn (1991). Assays on developmental stromatal proteins found that the majority of proteins produced from stromatal extracts in the Sclerotiniaceae had a molecular weight between 31,000 and 39,500 compared to 44,000 for *Sclerotium rolfsii* Sacc. and 12,000 to 22,000 for *Aspergillus* P. Micheli. isolates in one dimensional electrophoretic analyses. Polypeptide differences were

observed within the Sclerotiniaceae between sclerotial and substratal stroma producing taxa as well (Novak and Kohn, 1991).

Finally, DNA sequence analysis of the internal transcribed spacer 1 (ITS1) within the Sclerotiniaceae further demonstrated the diphyletic nature of the family (Carbone and Kohn, 1993). Hierarchal clustering based on multiple sequence alignments of the ITS1 showed that sclerotial taxa were similar to *S. sclerotiorum* (Lib.) de Bary; however, the ITS1 locus alone was insufficient for concluding a separate lineage exists within the Sclerotiniaceae which led to comparing sequences of both internal transcribed spacers (ITS1 and ITS2), the 5.8s rRNA gene, and partial sequences of the small (SSU rDNA) and large (LSU rDNA) subunits (Holst-Jensen et al., 1997). Multiple gene genealogies using concatenated conserved sequences have been shown to differentiate fungal species and will be used to confirm to genus the identity of *Poculum*-clade isolates used in this study (Couch and Kohn, 2002; Slippers et al., 2004).

The objective of this research was to characterize putative *Poculum*-clade fungi isolated from turfgrass exhibiting symptoms of bleached white patches that rarely exceeded 9 cm in diameter that were similar to dollar spot disease. Characterization of the isolates included observations on the morphology of hyphae and stroma, nucleotide sequence analysis of phylogenetically informative loci, and pathogenicity on turfgrass hosts of isolation. These characters were compared to those known for *S. homoeocarpa* to determine if the novel isolates represent distinct sister species within Rutstroemiaceae.

CHAPTER 2
CHARACTERIZATION OF FUNGI ASSOCIATED WITH TURFGRASS IN FLORIDA

Introduction

Isolates of a sterile fungus were recovered from fine-textured turfgrass in Florida starting in 2004 with symptoms that included small patches of discolored turfgrass plants larger in diameter than symptoms typical of dollar spot but rarely exceeding 9 cm (Ruiz et al., 2006). The affected turfgrass plants turned bleached white over time compared to the tan to straw color associated with dollar spot symptoms as originally described (Monteith and Dahl, 1932; Bennett, 1937). The isolated fungus was initially identified as *Sclerotinia homoeocarpa* Bennett based on the growth habit of hyphae and general colony morphology on isolation media. After 2 to 3 weeks, discreet determinate stroma that were 0.2 mm in diameter were observed in cultures that were distinct compared to the indeterminate substratal stroma of *S. homoeocarpa* (Bennett, 1937; Whetzel, 1945; Holst-Jensen et al., 1997).

Sequences of the internal transcribed spacers (ITS) of the unknown isolates were found to be most similar to *Poculum henningsianum* (Plöttn.) T. Schumach. & L.M. Kohn than to *S. homoeocarpa* (Schumacher and Kohn, 1985; Ruiz et al., 2006; Cooper et al., 2011). *Poculum* Velen. species fall within the Rutstroemiaceae Holst-Jensen, L.M. Kohn & T. Schumach. With the exception of *S. homoeocarpa*, fungi in this family have been described as plant saprobes (Carbone and Kohn, 1993; Holst-Jensen et al., 1997). The Rutstroemiaceae family was proposed by Holst-Jensen et al. (1997) to accommodate the substratal stroma producing taxa in the Sclerotiniaceae Whetzel at that time. Fungi that remained in Sclerotiniaceae produced distinct, tuberoid, sclerotia with a differentiated rind and medulla as described by Whetzel (1945).

Fungi in the Rutstroemiaceae produce brown to green ascomata that are stipitate, and cupulate to verpoid and arise from indeterminate, brown to black, substratal stroma, included taxa belonging to the following genera: *Lanzia* Sacc., *Poculum*, *Rutstroemia* P. Karst., *Verpatinia* Whetzel & Drayton, and *Lambertella* Höhn. (Holst-Jensen et al., 1997). Relatively little information is available on these saprophytic fungi of little known economic importance.

The only pathogen belonging in the Rutstroemiaceae was described by Bennett (1937) as *S. homoeocarpa* (Carbone and Kohn, 1993; Holst-Jensen et al., 1997). This fungus is the causal agent of dollar spot disease which is one of the most common and costly fungal diseases of turfgrass throughout the world (Vargas, 1994; Couch, 1995; Smiley et al., 2005). Bennett described distinct strains of the dollar spot fungus while working to characterize isolates from Australia, Europe, and North America that included a “non-sporing” strain which formed no reproductive structures and produced sclerotial flakes in culture (Bennett, 1937). Since that time, questions concerning the taxonomy and phylogeny of the dollar spot fungus have remained. White (1941) published a monograph of the *Rutstroemia* P. Karst. (substratal stromatal producing fungi) but did not include *S. homoeocarpa* in his monograph. Whetzel (1945b) excluded a species he referred to as "*Sclerotinia heterocarpa*" that he stated was the cause of dollar spot on grasses of the cypericolous and juncicolous in his monograph of species of *Sclerotinia* Fuckel and was adamant about its placement within the *Rutstroemia*. Dumont (1971) challenged these monographs on the basis of non-adherence to the rules of the International Code of Botanical Nomenclature and proposed that *Rutstroemia* was comprised of at least three distinct genera for which he provided

proper generic names (Dumont, 1972). Jackson (1973) observed apothecia of *S. homoeocarpa* and suggested *Rutstroemia* as the correct generic classification; however, discussion on the taxonomy of the *Rutstroemia* by Dumont (1971, 1972) prevented conclusive placement of *S. homoeocarpa* at that time. Carbone and Kohn (1993) added support for renaming *S. homoeocarpa* with phylogenetic reconstructions from internal transcribed spacer 1 (ITS1) sequences, but a single locus comparison of the fungi included was insufficient evidence for where *S. homoeocarpa* should be moved. Later, Holst-Jensen et al. (1997) recommended a recircumscription of the Sclerotiniaceae based on biological and molecular studies that resulted in the erection of the Rutstroemiaceae. This new family accommodated the substratal stromatal lineage of fungi that used to be part of the Sclerotiniaceae, but with a general lack of sexual fruiting structures and very few sister species with which to define genera that make up the Rutstroemiaceae, no reclassification of *S. homoeocarpa* has occurred.

The objectives of this study were to genetically and morphologically characterize isolates of the unknown fungus associated with atypical dollar spot symptoms on turfgrass; develop a method to distinguish the unknown fungus from *S. homoeocarpa;* and evaluate the pathogenicity of the isolates on various turfgrass species.

Materials and Methods

Fungal Isolate Collection

Fungi collected in or referenced by this study are included in Table 2-1. The first 13 fungi listed were isolated from various species of warm-season and cool-season turfgrass including common bermudagrass (*Cynodon dactylon* (L.) Pers.), hybrid bermudagrass (*Cynodon dactylon* (L.) Pers*. x C. transvaalensis* Burtt Davy), St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze), seashore paspalum

(*Paspalum vaginatum* Swartz), zoysiagrass (*Zoysia japonica* Steud.), roughstock bluegrass (*Poa trivialis* L.) and perennial ryegrass (*Lolium perenne* L.) according to the methods described in Liberti et al. (2012) from locations throughout Florida between 2004 and 2009. Fungi were isolated from turfgrass leaves with disease symptoms by first surface-sterilizing plant tissue for 60 to 90 s in 50 ml of 0.65% sodium hypochlorite solution with a drop of Tween 20 (Sigma-Aldrich, St. Louis, MO), and then triple rinsing in 50 ml of sterile dH2O. Surface sterilized plant tissue was then plated on 20% strength potato dextrose agar (PDA) media prepared by adding 12.0 g/L Agar (Difco™ Laboratories, Sparks, MD) to 7.8 g/L PDA (Difco™ laboratories, Sparks, MD) amended with 150 µg/L rifampicin (Fisher Scientific, Fair Lawn, NJ) and 0.5 g/L ampicillin (ACROS Organics, Geel, Belguim) antibiotics. Isolation plates were incubated at room temperature (RT) approximately 22°C to 24°C for 18 to 24 h. Pure cultures were obtained by transferring a subculture of each fungal colony to a fresh 20% PDA plate. Isolated fungi were maintained on 20% strength PDA and were stored on sterile filter paper (Whatman International Ltd, Maidstone, England) that had been placed on the media in a culture plate. The isolate was transferred onto the filter paper in a colonized agar plug. Plates were incubated until cultures produced stroma. Stromatized paper was then collected, dried in sterile petri dishes for 48 to 96 h, and stored in sterile glass vials at 4°C for long term storage. When needed, fungi were revived from storage by plating on 20% PDA and were transferred after 48 to 72 h at 24°C.

Morphology

Hyphae and stroma dimensions were measured, mycelial compatibility between isolates was determined, and growth rates at different temperatures were measured for all isolates. All assays were performed with three replicates and repeated twice. All

data subjected to analysis of variance were analyzed with proc anova (SAS v9.2), and means were separated with Fisher's least significant difference test at $\alpha < 0.05$.

Cover slips were placed directly on mycelium and stroma that formed on plates of 20% PDA at 24°C, and images of hyphae and stroma were captured with a compound microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 250X magnification. Diameters of hyphae and stroma were measured with Q Capture Pro software version 6.0.0.412 (Q Imaging Inc., Surrey BC, Canada). Calibration of measuring software was confirmed with an improved Neubauer hemocytometer. Five large-diameter hyphae and five small-diameter hyphae were measured after 7 days. Two perpendicular diameters of five stroma were recorded after 2 to 3 weeks.

Mycelial compatibility was tested on 20% PDA amended with red food coloring (McCormick and Company Inc., Sparks, MD) (Kohn et al., 1990; Mitkowski and Colucci, 2006). Compatibility was tested among all unknown isolates and two *S. homoeocarpa* isolates, one isolate each from the common and Floridian types designated by Liberti et al. (2012). Five-millimeter agar plugs from cultures of different isolates were arranged radially on plates so that each isolate was paired with itself and all others at least three times. Where colonies grew together, compatibility was assessed after at least 7 days. Deng et al. (2002) described four possible reactions at the zone of interaction. Compatible reactions occurred if the two isolates merged seamlessly with no observable zone of interaction. Gap reactions occurred if the zone had two dark lines on the bottom of the plate with a 3 to 10 mm space at the zone of interaction. Line gap reactions occurred if there was a small gap (approximately 1 mm) with 1 or 2 dark lines

on the bottom of the plate. Barrage reactions occurred if no gap was present but a thick mycelial zone occurred with a dark area on the bottom of the plate (Deng et al., 2002).

The effect of temperature on the rate of growth for the selected isolates: UF0421, UF0404, UF0476, UF0523, and UF0525 was compared on plates of 20% PDA. A 5 mm plug from a 3-day-old culture was transferred and incubated at 5°C increments between and including 15°C and 40°C in a dark incubator. Growth was measured in mm on 2 perpendicular axes at 24 h intervals for four consecutive days. Calculated means and standard error were plotted to compare growth rate differences within each temperature tested.

Fungal DNA Extraction

DNA was extracted according to the methods described in Liberti et al. (2012). Fungi were grown on Petri plates containing 20% PDA covered with sterile cellophane (Gel Drying Film, Promega Corporation, Madison, WI) for 3 days at 24°C. Collected mycelium lifted from the plate on cellophane was ground in liquid nitrogen with a pestle in a 1.5 ml microcentrifuge tube. Total DNA was extracted using the Qiagen Dneasy Plant Mini Kit following the manufacturer's instructions in 100 µl elution buffer and stored at -80°C until needed for PCR amplification with no further preparation (Qiagen Inc, Valencia, CA).

PCR and Cloning of Amplicons

Reaction volumes for amplification of specific loci totaled 25 µl. Reactions contained 5 to 10 ng of template DNA, 10 to 20 pmol of each primer, 0.2 mM dNTPs, 2.5 µl of 10x reaction buffer, 1.5 mM $MgCl_2$, and 0.5 U of Platinum® Taq (Invitrogen, Carlsbad, CA). Selected loci and primers included in this study are listed in Table 2-2 (White et al., 1990; Glass and Donaldson, 1995; Rehner, 2001). DNA fragments were

amplified under the following conditions for the rDNA internal transcribed spacer (ITS) and beta-tubulin loci: initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 1 min; 55°C for 1 min; and 72°C for 1 min; final extension at 72°C for 10 min; reaction products were held at 4°C. DNA fragments were amplified under the following conditions for the elongation factor 1-alpha locus: initial denaturation at 95°C for 5 min; 9 cycles at 95°C for 25 s; 66°C -1°C per cycle for 30 s; and 72°C for 90 s followed by 36 cycles at 95°C for 30 s; 56°C for 30 s; and 72°C for 90 + 0:01 s per cycle; final extension at 72°C for 15 min; reaction products were held at 4°C. Amplifications of loci were confirmed by UV visualization of DNA bands following electrophoresis in 0.8% agarose gels amended with 1 µl/100 ml of 1% ethidium bromide (Fisher Scientific, Fair Lawn, NJ) at 75V for 45 to 60 min.

PCR products were purified with the MinElute PCR Purification Kit (Qiagen Inc., Valencia, CA) and cloned into *Escherichia coli* JM109® high efficiency competent cells (Promega Corporation, Madison, WI) using the pGEM®-T Easy vector system II Kit (Promega, Corporation, Madison, WI) following each manufacturer's instructions. Plasmids were purified using QIAprep® MiniPrep Kit (Qiagen Inc., Valencia, CA). Not1 and EcoRI restriction enzymes were added to purified plasmids for EF1-alpha and beta-tubulin, and ITS loci respectively for digestion from plasmid to verify clone. Purified plamids were sent to the International Center for Biotechnological Research (ICBR, University of Florida, Gainesville, FL) for bidirectional sequencing with T7 (TAATACGACTCACTATAGGG) and SP6 (TATTTAGGTGACACTATAG) primers.

Sequence Analysis

Consensus sequences were assembled and edited with Sequencher software v4.8 (GeneCodes Corporation, Ann Arbor, MI) and compared to homologous

sequences published in GenBank (Table 2-1). Multiple sequence alignments (MSA) were executed for individual and combined loci with Molecular Evolutionary Genetics Analysis version 5.0 (MEGA v5.0) or 6.06 (MEGA v6.06) software program. MSA reliability was confirmed by DNA sequence identity using the non-coding sequence criterion (average distance < 0.33) (Hall, 2008). Loci were subjected to a partition homogeneity test using PAUP* v4.0 software with 1,000 replicates (heuristic search; stepwise addition; TBR; max-trees = 5000) (Swafford, D. 2002) and determined not to be significantly different (*P*=0.25). The individual data sets were concatenated with SeaView software v4.3.5 (Gouy et al., 2010). Neighbor joining (NJ) and maximum parsimony (MP) analyses were conducted for each locus, and the combined data set, using default settings for MEGA v6.06 software (Tamura et al., 2011). Average nucleotide sequence divergence between groups was calculated with MEGA v6.06 software (Tamura et al., 2011). Tree topologies were evaluated by bootstrap (BS) analysis 50% majority-rule consensus for 1,000 replicates.

Restriction fragment length polymorphism

Primers ITS1 and ITS4 (White et al., 1990) were used to amplify rDNA including the internal transcribed spacers as described above. PCR products were purified with MinElute® PCR Purification Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Restriction enzyme candidates were selected by comparing recognition sites within sequences of the internal transcribed spacer amplicon with NEBcutter (New England Biolabs Inc., Beverly, MA). A CviQi restriction enzyme recognition site (New England Biolabs Inc., Beverly, MA) was determined to be polymorphic and was added to 10 µl of reaction volume for digestion of purified PCR products and incubated for 30 min according to manufacturer's recommendations. Digestions were separated

electrophoretically and visualized with UV light on 2% agarose gels amended with 1 μl/100 ml of 1% ethidium bromide (Fisher Scientific, Fair Lawn, NJ).

Detached leaf assay

A 3 mm colonized agar plug from the expanding edge of a colony of *S. homoeocarpa* (UF0606, UF0420, or UF0421), *P. henningsianum* (LMK734), and all isolates of *Poculum*-clade fungi was placed directly onto six St. Augustinegrass leaf blades that were wounded by cutting a 2 to 3 mm section from the edge of the leaf and three non-wounded leaf blades per isolate. Inoculated leaf blades were incubated at 24°C for 6 to 7 days. Disease incidence was recorded once at 6 or 7 days post inoculation as a (+) or (-) for chlorosis and necrosis.

Maintenance of turfgrass hosts

Pots of 'Penncross' creeping bentgrass, 'Sea Isle Supreme' seashore paspalum, 'Champion' bermudagrass and 'Sonesta' bermudagrass were prepared for pathogenicity assays as follows. Pots containing calcine clay (Turface® MVP; Profile Products LLC, Buffalo Grove, IL) were seeded with either creeping bentgrass or 'Sonesta' bermudagrass, or established from asexual stolon fragments of either 'Sea Isle Supreme' seashore paspalum, or 'Champion' bermudagrass. Turfgrass was established in 3 to 4 weeks and maintained in a greenhouse at 24°C to 30°C until use in assays. Pots were irrigated as needed, trimmed on 3 to 4 day intervals, and fertilized as needed (6-12-18 starter fertilizer; Harrell's LLC, Lakeland, FL).

Inoculum Preparation and Inoculation

Inoculum was prepared by autoclaving 25 g of hard red winter wheat seed (*Triticum aestivum* L.) with 15 ml dH_2O for 30 min daily over three consecutive days. Sterilized wheat seed was then infested with fungus by inoculating seed with 3 to 5

colonized agar plugs from the outer edge of a 2- to 3-day-old culture. Inoculated seed was incubated at 22°C to 24°C for 3 days, and infested seed was prepared for use by loosening with a spatula immediately prior to inoculation. Four unknown isolates, one *S. homoeocarpa* isolate, and an untreated check were included in each assay.

Turfgrass was trimmed to 1/2 inch with scissors prior to inoculation. Pots of turf were treated with 10 to 20 infested seed, misted with dH_2O, enclosed in a 30-by-50 cm polyethylene bag, and incubated at 24°C for 24 to 48 h. Leaf tissue was collected after 48 h incubation, surface sterilized, and plated onto 20% PDA. Identity of the isolated fungi was determined by stroma morphology. Disease incidence for each replicate was recorded based on the presence or absence of a necrotic spot in the turf. Reisolation of the inoculated fungus was attempted for three replicates of each isolate and recovery was recorded as a (+) or (-) for each replicate. A total of six pathogenicity trials were conducted that included two on 'Sea Isle Supreme' seashore paspalum, two on 'Champion' bermudagrass, and a single test each on 'Sonesta' bermudagrass and 'Pencross' creeping bentgrass.

Results

Molecular Characterization

A partition homogeneity test of the three loci indicated that the sequences could be combined (P=0.25). The combined sequence included 2551 aligned characters with 87.3% DNA sequence identity indicating > 66% multiple sequence alignment accuracy among nucleotide residues (Thompson et al., 1999; Hall 2008). Tree topologies for the combined data set were the same for NJ and MP analyses (Figures 2-1 and 2-2). Strongly supported lineages (Bootstrap value > 70%) in the phylogenies were consistent with groups of isolates based on morphological features. The unknown isolates formed

three distinct groups that were most closely related to *P. henningsianum* and *S. homoeocarpa*. The first group included isolates UF0518, UF0523, UF0816, UF0824, and UF0912. The second group included isolates UF0404 and UF0525. These seven isolates had higher average sequence similarity to *P. henningsianum* (93.1%) compared to *S. homoeocarpa* (84.7%). The third group was formed by the single *Poculum*-clade isolate UF0476 and had a higher average sequence similarity to *S. homoeocarpa* (93.8%) than *P. henningsianum* (86.7%). The *S. homoeocarpa* isolates included common (UF0420, UF0606) and Floridian (UF0421) as described by Liberti et al. (2012). Tree topologies for the combined data sets were identical but had slight differences in branch length for NJ analyses, and MP analyses for three equally parsimonious trees (Figures 2-1 and 2-2).

Morphology

Compatibility was observed for all isolates in self pairings. Aside from self-compatibility, no compatibility between isolates was observed. All incompatible reactions were characterized by barrage reactions with no gap in the mycelium. A thick layer of mycelium 3 mm wide formed at the zone of interaction and a dark zone appeared on the bottom of the plate when scored.

Hyphae of the Poculum-clade isolates were septate and hyaline. Hyphae of two distinct widths were observed for each of the *Poculum*-clade and *S. homoeocarpa* isolates. Width of the large hyphae for group 2 *Poculum*-clade isolates and a representative from each of the two *S. homoeocarpa* groups were smaller than the large hyphae of *Poculum*-clade groups 1 and 3 ($P<0.05$). Group 2 *Poculum*-clade isolates and the common *S. homoeocarpa* isolate were smaller than the smaller diameter hyphae of the Floridian *S. homoeocarpa* isolate. The width of the smaller hyphae for

the Group 3 *Poculum*-clade isolates and the Floridian *S. homoeocarpa* isolate were larger than the common *S. homoeocarpa*. Group 1 *Poculum*-clade isolates varied significantly in the diameter of the smaller hyphae within the group (Table 2-3).

Poculum-clade isolates formed discreet, uniform, black to dark brown substratal stroma resembling sclerotial flecks after 14 to 21 days incubation at 24°C on 20% PDA. Stroma observed in cultures of the *Poculum*-clade isolates varied in size between 0.3 and 1.3 mm in diameter. Stroma of group 2 were smaller in size than the other two groups of *Poculum*-clade isolates. Stroma of group 2 and 3 were black in color while stroma from group 1 were light brown to reddish brown. The isolates of dollar spot fungus produced plate-like stroma on the surface and within the agar as previously described (Liberti et al., 2012). A summary of the hyphal and stromatal characteristics are presented in Table 2-3.

Growth rates for UF0421, UF0404, UF0476, UF0523, and UF0525 were not significantly different at the tested temperatures: 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C. None of the selected isolates produced abundant hyphae at 35°C or 40°C. Growth rates at the optimal temperature (25°C) were higher for the *Poculum*-clade isolates compared to the Floridian *S. homoeocarpa* isolate (UF0421); however, the difference was not significant (Figure 2-3). Growth rates for the group 1 *Poculum*-clade isolates were moderate compared to the other *Poculum*-clade groups and *S. homoeocarpa* (UF0421). Growth rates were highest for group 2 at lower temperatures compared to other isolates. Growth rates for group 3 were similar to Floridian *S. homoeocarpa* at lower temperatures, and slightly higher at higher temperatures up to 30°C.

Pathogenicity

No disease symptoms were observed in control pots inoculated with sterilized seed. Isolates were recovered from surface sterilized plant tissue inoculated with *S. homoeocarpa* and *Poculum*-clade isolates (Table 2-4). Disease symptoms were not observed in pots treated with any *Poculum*-clade isolate for any turfgrass species in this study for at least 7 days post-inoculation. A virulent isolate of *S. homoeocarpa* (UF0421) served as a positive control, produced symptoms, and was recovered from 3 of 3 replicates in all pathogenicity tests except Sea Isle Supreme Trial 1 where it produced symptoms in 3 of 3 replicates, but was recovered from only 2 of 3 replicates. *Poculum*-clade isolates, UF0476 and UF0523, were recovered from 3 replicates in all six pathogenicity trials. UF0525 was recovered from 3 replicates in five tests. UF0525 was recovered from 2 out of 3 replicates in the 'Pencross' creeping bentgrass trial. UF0404 was recovered from 3 replicates in two seashore paspalum trials and two 'Champion' bermudagrass trials, but only 2 replicates in the 'Sonesta' bermudagrass trial. UF0404 was not recovered in the 'Pencross' creeping bentgrass trial.

Detached leaf bio-assay

No symptoms were observed on detached leaf blades of St. Augustinegrass inoculated with non-colonized agar plugs in either trial. UF0606 developed chlorosis and necrosis 2 to 3 days after inoculation in wounded and non-wounded assays similar to symptoms reported by Liberti et al. (2012). The common (UF0420) and Floridian (UF0421) *S. homoeocarpa* developed chlorosis and necrosis 2 to 3 days after inoculation on St. Augustinegrass leaf blades in wounded leaf blade trials (not tested on non-wounded leaves). Inoculations with *P. henningsianum* (LMK734) did not produce

any necrosis or chlorosis on non-wounded leaves, or one wounded leaf trial (LMK734 was not included on the second wounded leaf trial).

Leaves inoculated with *Poculum*-clade isolates UF0404, UF0476, UF0518, UF0523, UF0525, UF0816, UF0824, and UF0912 did not develop any chlorosis or necrosis on non-wounded detached leaf assays (Figure 2-4). In the first wounded leaf trial UF0476 produced a relatively small (3 to 4 mm^2) lesion on one leaf replicate directly under a wounded inoculation site; however, no chlorosis was observed. A dark area appeared at the wound site for the remaining *Poculum*-clade isolates but did not expand like the one leaf replicate for UF0476. In the second wounded leaf assay UF0476 produced necrosis on 30 to 40% of one leaf replicate and 2% on two leaf replicates, and chlorosis throughout three leaf replicates. No necrosis developed in the remaining *Poculum*-clade isolates, but chlorosis was observed in three leaf replicates for UF0523, UF0525, UF0816, UF0824, and UF0912. Chlorosis was observed in 2 out of 3 leaf replicates for UF0404. UF0518 did not develop in culture and was not included in the second wounded leaf assay.

RFLP

The CviQi restriction enzyme cut the 554 bp ITS1 and ITS4 amplicon of all *Poculum*-clade isolates and none of the *S. homoeocarpa* isolates (Figure 2-5). The amplicons were cut at position 161 to 163 which produced restriction fragments of 163 bp and 391 bp. The *Poculum*-clade isolate UF0518 had fragments of all three lengths (Figure 2-5).

Discussion

The novel fungi included in this study were isolated from samples of turfgrass sent to the University of Florida Rapid Turfgrass Diagnostic Service by golf course

superintendents. The samples had symptoms similar to dollar spot disease, but distinct in that the patches were more white in color and slightly larger than typical dollar spot symptoms. Additionally, the fungi isolated from these distinct symptoms were similar to the dollar spot pathogen, *S. homoeocarpa*, and some were initially misidentified as such in our culture collection. While characterizing the dollar spot pathogen in Florida, morphological and genetic evidence mounted to suggest the isolates represented one or more novel species which became the focus of the research presented here.

Similarities between the dollar spot pathogen and the novel misidentified fungi include similar and overlapping growth rates, diameters of primary and secondary hyphae, and general colony growth habit among isolates of dollar spot pathogen and the *Poculum*-clade isolates on the standard isolation medium 20% PDA. Primary among the morphological differences between the well-studied dollar spot pathogen and the novel fungi were the discreet determinate substratal stroma that formed in culture plates after extended incubation as compared to the indeterminate stroma of *S. homoeocarpa*. In some cases, the small sclerotia-like stroma were observed at the time of isolation to be embedded in necrotic tissue (Ruiz et al., 2006)

Stroma of the *Poculum*-clade isolates were characteristic of fungi in the Rutstroemiaceae as described by Whetzel (1945) and Kohn and Greenville (1989a, 1989b). Two groups based on morphology of stroma were distinguished and included five isolates in group 1 that had light to reddish brown stroma that were on average roughly three times larger than the diameter of black stroma from the two isolates in group 2. The differences, though obvious, offered limited practical usefulness as a diagnostic character since the stroma formed after 2 to 3 weeks of incubation, and

isolation plates from disease samples are not typically kept more than a few days to a week.

Although vegetative compatibility is common among isolates of *S. homoeocarpa*, VCG pairings between the novel fungi and two dollar spot pathogen isolates produced no compatible reactions. A small number of isolates were included in the pairings, but these results provide further evidence that the novel fungi are not likely clonal representatives of a single species. In general, VCG pairings can provide useful information where large populations of clonal isolates have been identified but are of limited usefulness in this study since no compatibility was identified within the *Poculum*-clade isolates (Sonoda, 1989; Powell and Vargas, 2001; Ruiz et al., 2006)

Single gene phylogenies reconstructed with internal transcribed spacer sequences have provided evidence that the dollar spot pathogen belongs in the Rutstroemiaceae (Carbone and Kohn, 1993; Holst-Jensen et al., 1997). In general phenotypic characters along with multi-gene genealogies have been used to differentiate between fungal species (Slippers et al., 2004). We conducted a multi-gene genealogy, similar to that recommended by Kohn (2004) and used by Slippers et al. (2004), that included concatenated sequences of the internal transcribed spacer, beta-tubulin, and elongation factor 1-alpha loci (Figures 2-2 and 2-3) to characterize the novel *Poculum*-clade isolates.

Our novel fungi were associated with isolates of *P. henningsianum* and *S. homoeocarpa* in a well-supported clade distinct from sister genera also in the Rustroemiaceae and from fungi in the Sclerotiniaceae (*Botrytis cinerea* Pers. and *Sclerotinia sclerotiorum* (Lib.) de Bary. The phylogenies from NJ and MP analyses

presented are in agreement with those reported by Carbone and Kohn (1993) and Holst-Jensen et al. (1997) and with biological studies (Kohn and Greenville, 1989a, 1989b) identifying the diphyletic nature of the Sclerotiniaceae that ultimately resulted in the erection of the Rutstroemiaceae.

Symptoms that included leaf blade necrosis, white discoloration, and blighting were among the symptoms expected from pathogenicity tests with the *Poculum*-clade isolates. Although the isolates were recovered from inoculated, incubated, and then surface sterilized plant tissue, symptoms associated with the *Poculum*-clade isolates were not observed from any inoculation of turfgrass pots. Even when detached leaf blades were wounded and inoculated with the *Poculum*-clade isolates, only UF0476 produced inconsistent symptoms that were not repeatable when pots of grass were inoculated. The specific environmental and host factors required for pathogenesis (if possible) were not elucidated through this research. We do not know if the *Poculum*-clade isolates are only capable of endophytic and subsequent saprophytic colonization of turfgrass plants, but the isolates tested were not pathogenic under conditions favorable for dollar spot disease development.

One goal of this research was to develop a tool to distinguish between *Poculum*-clade isolates and the dollar spot pathogen. A restriction enzyme digestion of the internal transcribed spacer of all of the *Poculum*-clade isolates tested, including *P. henningsianum* produced a distinct polymorphism following CviQi restriction digestion. Dollar spot pathogen ITS amplicon was uncut and the full length was 554 base pairs. Although the digestion of the amplicon from isolate UF0518 was incomplete, the results of the test are still valid in that each of the *Poculum*-clade isolates was at least partially

digested at the expected nucleotide position. Moving forward this assay could be useful for screening collections to identify other *Poculum*-clade isolates that may have been collected and stored as *S. homoeocarpa* based on colony morphology and can serve as a rapid diagnostic technique for distinguishing samples in the disease clinic.

In conclusion, there is strong morphological and molecular data to support that the novel fungi characterized here are species from the Rutstroemiaceae family, and likely in the *Poculum* genus. Additional isolates and research are needed to conclusively identify and adequately describe these potentially novel *Poculum* species. The *Poculum* species represented are likely sister species whose description and characterization may eventually provide the context needed to reclassify *Sclerotinia homoeocarpa* within the Rutstroemiaceae.

Table 2-1. Source, origin, and GenBank accession numbers for isolates and respective sequences used in this study

			GenBank accession numbers		
Isolate Code[a]	Host[b]	Origin[c]	ITS	Beta-tubulin	EF1-α
UF0401	BG	Collier Co.	KF725724	KF725737	KF725754
UF0404	BG	Marion Co.	KF725729	KF725742	KF725759
UF0420	BG	Alachua Co.	KF725725	KF725738	KF725755
UF0421	SP	Santa Rosa Co.	KF725726	KF725739	KF725756
UF0476B	BG	Alachua Co.	KF725730	KF725743	KF725760
UF0480	RG	Alachua Co.	KF725727	KF725740	KF725757
UF0518	BG	Alachua Co.	KF725731	KF725744	KF725761
UF0523	BG	Santa Rosa Co.	KF725732	KF725745	KF725762
UF0525A	SP	Santa Rosa Co.	KF725733	KF725746	KF725763
UF0606	RB/BG	Lady Lake	KF725728	KF725741	KF725758
UF0816B	SP	Marion Co.	KF725734	KF725747	KF725764
UF0824	SP	Palm Beach Co.	KF725735	KF725748	KF725765
UF0912	SP	Marion Co.	KF725736	KF725749	KF725766
LMK5	*Aster ageratoides*	Japan	Z81438	KF725751	KF725768
LMK734	*Carex rostrata*	Norway	Z81442.1	KF725750	KF725767
LMK758	*Filipendula ulmaria*	Norway	Z81448	KF725752	KF725769
S. homoeocarpa TN	unknown	USA	DQ448302.1	DQ448299.1	DQ448301.1
S. sclerotiorum 1980	*Phaseolus vulgaris*	New York	XM_001585239	XM_001594794	XM_001594041
B. cinerea BC1	*Phaseolus vulgaris*	Netherlands	XM_001547652	XM_001560987	XM_001551736
F. graminiearum PH1	*Zeae mays*	Kansas	XM_384429	XM_389706	XM_388987
M. grisea 7015	*Oryza sativa*	USA	XM_361658	XM_003718381	XM_003716200

[a] UF = Philip Harmon's collection at the University of Florida; LMK = Linda Kohn's collection at the University of Toronto.

[b] BG is *Cynodon dactylon* (L.) Pers.; SP is *Paspalum vaginatum* Sw.; RG is *Lolium perenne* L.; RB is *Poa trivialis* L.

[c] Counties located in Florida unless otherwise specified.

Table 2-2. Primer sequences and conditions for amplification of selected loci

Primer Name	Sequence	Temp (°C) and Extension Time	Primer pair
ITS1[b]	5' - TCCGTAGGTGAACCTGCGG - 3'	55, 2 min	White et al. (1990)
ITS4[b]	5' - TCCTCCGCTTATTGATATGC - 3'		
Bt2a[c]	5' - GGTAACCAAATCGGTGCTGCTTTC - 3'	55, 2 min	Glass and Donaldson (1995)
Bt2b[c]	5' - ACCCTCAGTGTAGTGACCCTTGGC - 3'		
EF1-526F[d]	5' - GTCGTYGTYATYGGHCAYGT - 3'	66-56, Touchdown[a]	Rehner (2001)
EF1-1567R[d]	5' - ACHGTRCCRATACCACCRATCTT - 3'		

[a] Touchdown PCR (Rhener, 2001) consisted of 9 cycles with decreasing annealing temperatures beginning at 66°C (66 to 56 °C) for 1 min 30 seconds followed by 36 cycles at 56°C with an extension time of 1 min 30 seconds plus 1 second for each additional cycle.

Table 2-3. Comparison of hyphae and stroma, ancestral group identification, and compatibility reactions for *Poculum*-clade isolates and *S. homoeocarpa* isolates.

ISOLATE	Species	Large Hyphae		Small Hyhpae		Stroma		Phylogeny Group
		Width (µm)[b]						
UF0404	*Poculum sp.*	8.6 ± 0.9	d	2.7 ± 0.2	bc	283.1 ± 28.6	e	2
UF0476	*Poculum sp.*	10.5 ± 1.0	bc	3.1 ± 0.4	a	824.4 ± 87.8	b	3
UF0518	*Poculum sp.*	10.8 ± 0.6	ab	2.8 ± 0.2	ab	537.2 ± 142.4	d	1
UF0523	*Poculum sp.*	11.5 ± 0.5	ab	2.4 ± 0.1	bcd	593.0 ± 114.2	cd	1
UF0525	*Poculum sp.*	9.2 ± 0.9	d	2.5 ± 0.1	bcd	303.6 ± 23.0	e	2
UF0816	*Poculum sp.*	9.5 ± 0.9	cd	2.5 ± 0.3	bcd	691.9 ± 112.2	bc	1
UF0824	*Poculum sp.*	11.1 ± 0.9	ab	2.4 ± 0.0	cd	1286.6 ± 173.7	a	1
UF0912	*Poculum sp.*	11.1 ± 1.0	ab	2.2 ± 0.2	d	1323.8 ± 189.5	a	1
UF0420	*S. homoeocarpa*	8.6 ± 0.6	d	2.5 ± 0.1	bcd	n/a		n/a
UF0421	*S. homoeocarpa*	9.1 ± 1.1	d	3.1 ± 0.2	a	n/a		n/a

[a] Vegetative compatibility group reactions from Deng et al. (2002): a=compatible, b=gap, c=line gap, d=barrage.
[b] Means with the same letter are not significantly different (Fisher's LSD, α = 0.05).

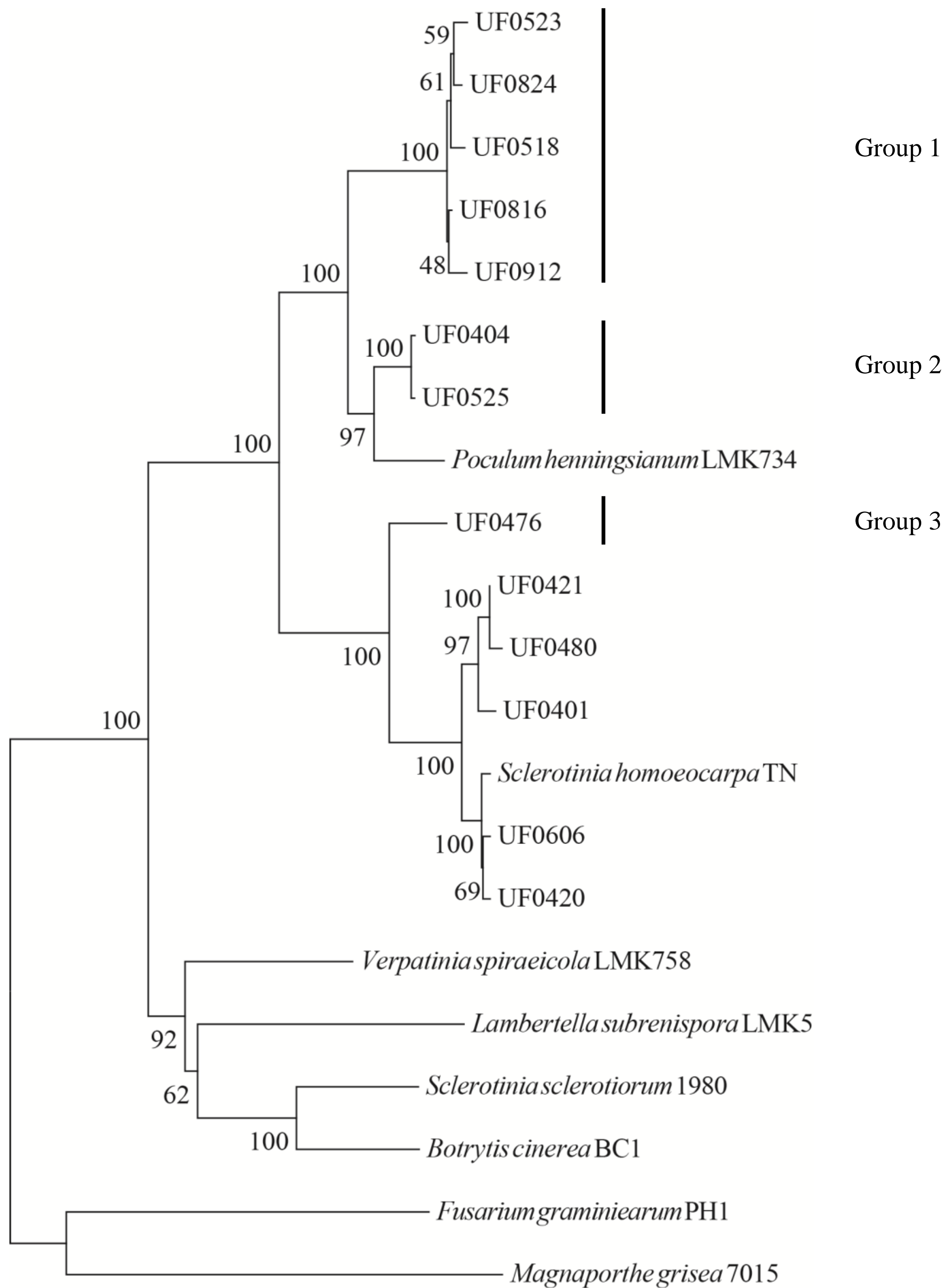

Figure 2-1. Neighbor joining tree showing the relatedness of *Poculum* isolates to *P. henningsianum* and *S. homoeocarpa* relative to other related fungi in the Rutstroemiaceae and Sclerotiniaceae based on combined sequences of ITS, beta-tubulin, and elongation factor 1-alpha loci.

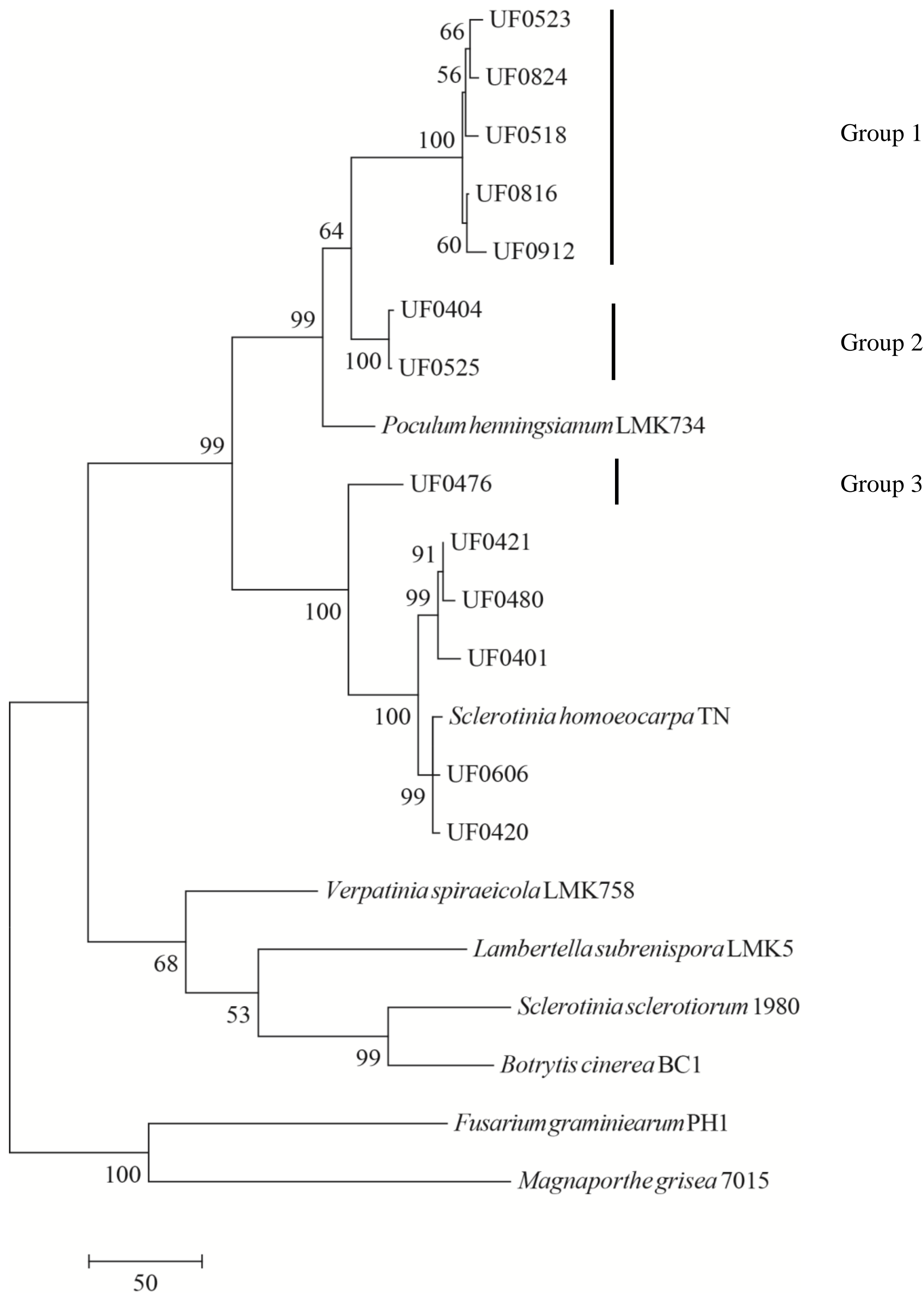

Figure 2-2. Maximum parsimony tree showing the relatedness of *Poculum* isolates to *P. henningsianum* and *S. homoeocarpa* relative to other related fungi in the Rutstroemiaceae and Sclerotiniaceae based on combined sequences of ITS, beta-tubulin, and elongation factor 1-alpha loci.

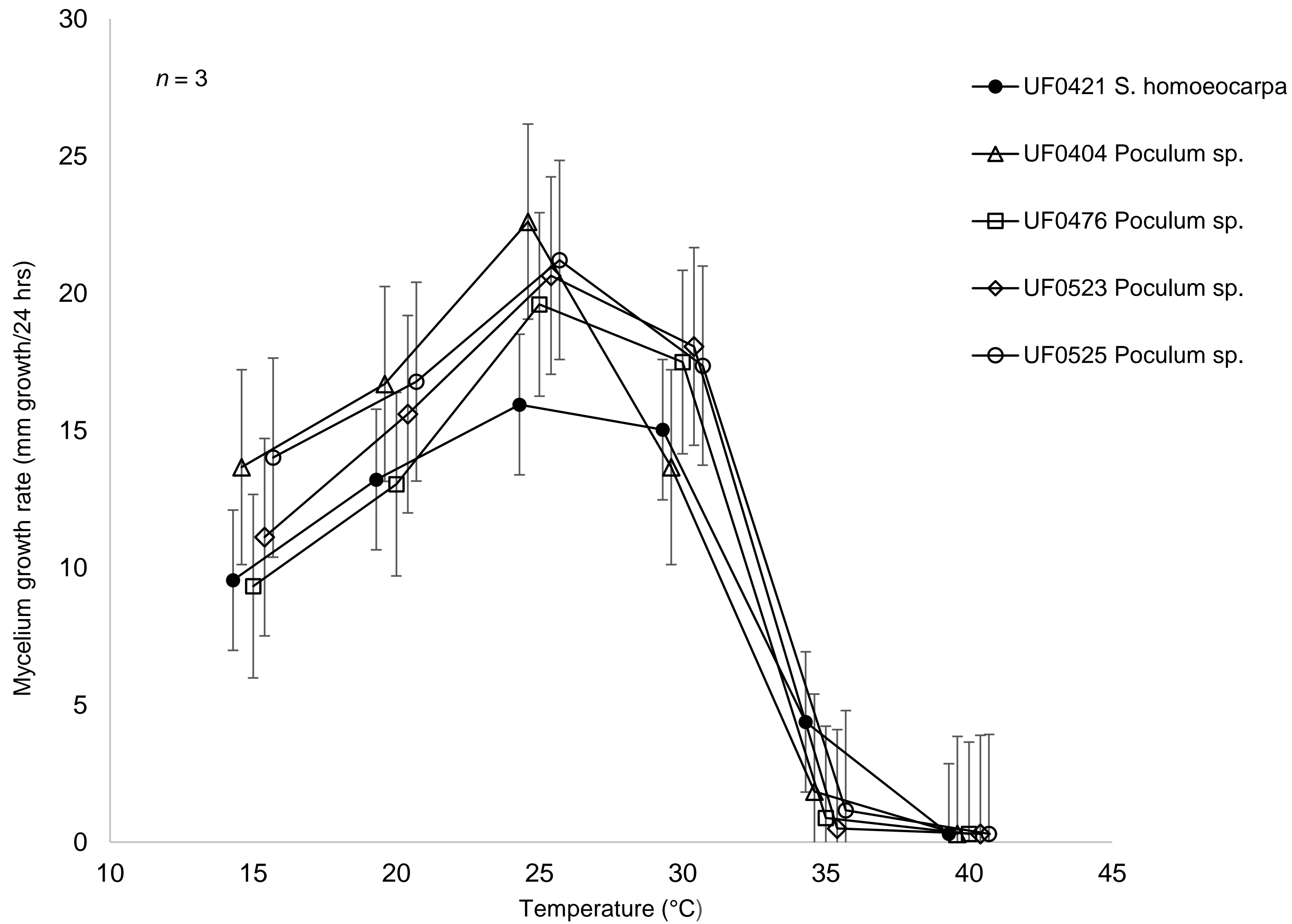

Figure 2-3. Mean mycelium growth rate and SE bars of selected *Poculum* isolates and *S. homoeocarpa* isolates at 6 temperatures in mm/day.

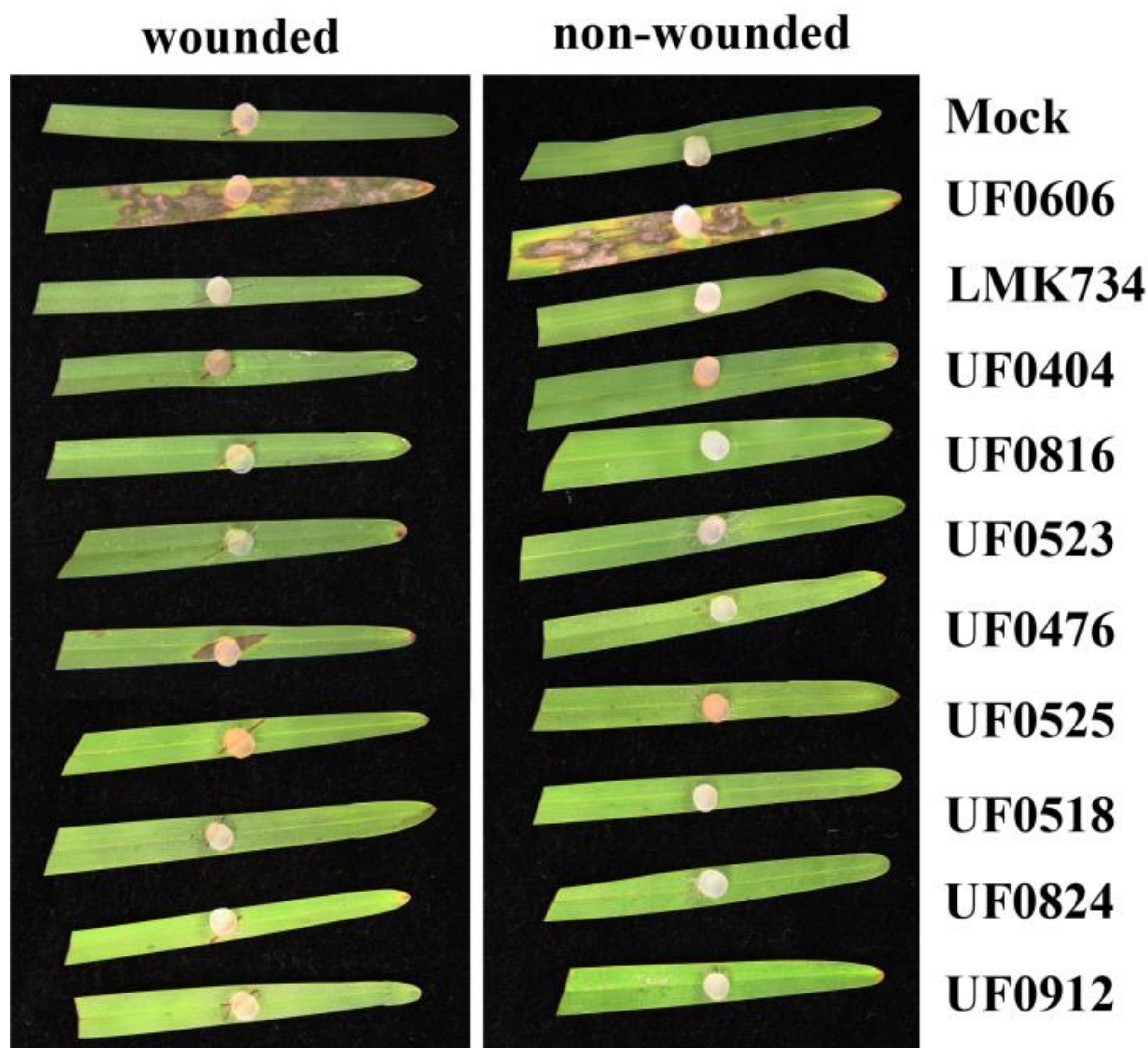

Figure 2-4. Detached leaves of St. Augustinegrass 2 to 3 days post-inoculation with colonized agar plugs of selected *Poculum* (LMK734, UF0404, UF0816, UF0523, UF0476, UF0525, UF0518, UF0824, UF0912) and *S. homoeocarpa* (UF0606) isolates.

Table 2-4. Recovery of selected *Poculum*-clade and *S. homoeocarpa* isolates from surface sterilized plant tissue collected from pots of healthy turfgrass 24 to 48 h post inoculation from three replicates for each isolate (+) recovered, (-) not recovered for individual replicates.

Isolate	Sea Isle Supreme	Sea Isle Supreme	Champion Bermuda	Champion Bermuda	Sonesta Bermuda	Pencross Bentgrass
Check	- - -	- - -	- - -	- - -	- - -	- - -
UF0421	+ + -	+ + +	+ + +	+ + +	+ + +	+ + +
UF0404	+ + +	+ + +	+ + +	+ + +	+ + -	- - -
UF0476	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
UF0523	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
UF0525	+ + +	+ + +	+ + +	+ + +	+ + +	+ + -

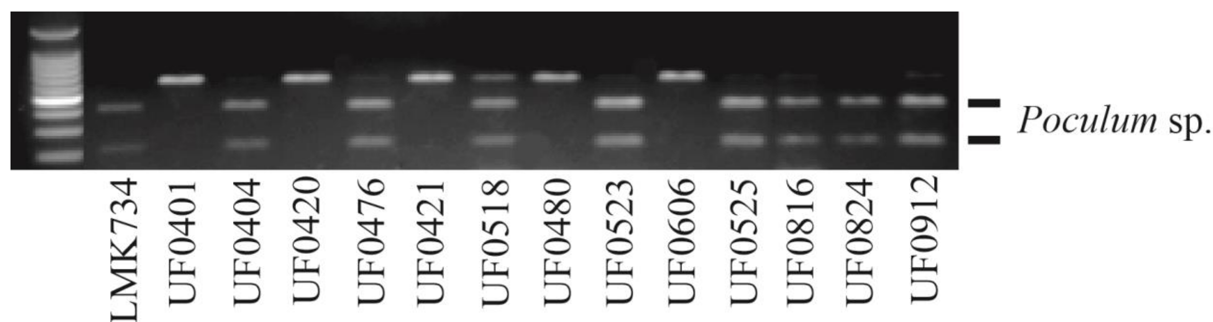

Figure 2-5. CviQi digestion of ITS1 and ITS4 amplicons on 2% agarose gel with DNA fragments for selected *Poculum*-clade isolates and *P. henningsianum* (LMK 734) compared to intact ITS1 and ITS4 amplicons for *S. homoeocarpa*.

APPENDIX
ADDITIONAL ASSAYS

Introduction

Additional assays were completed during the course of this dissertation. These experiments included fungicide sensitivity assays and inoculum source evaluations. Fungicide sensitivity can impact disease management success. Fungicide resistance in populations of the dollar spot pathogen has been documented for numerous groups of fungicides. The goal of this research was to determine the sensitivity of *Poculum*-clade isolates to fungicide products available to turfgrass managers.

In attempts to satisfy Koch's postulates, the *Poculum*-clade isolates were grown on various media prior to inoculation of turfgrass and incubation in a moist environment for 24 to 48 h at 24°C. The various inoculum sources that were tested are described. The goal of this research was to develop a reliable method to inoculate *Poculum*-clade isolates onto turfgrass in pathogenicity assays.

Materials and Methods

Fungicide Sensitivity

Fungicide sensitivity of *Poculum*-clade isolates including UF0404, UF0476, UF0523, UF0525, UF0816, UF0824, and UF0912 as well as the dollar spot pathogen isolate UF0420 were determined. Isolates were maintained on 20% strength potato dextrose agar (PDA) media prepared by adding 12.0 g/L Agar (Difco™ Laboratories, Sparks, MD) to 7.8 g/L PDA (Difco™ laboratories, Sparks, MD). Sensitivity to fungicides was tested on 20% PDA compared to 20% PDA amended with EC_{50} concentrations taken from the literature for the following formulated fungicides: Heritage 50WG (azoxystrobin) at 30 mg a.i. + SHAM (salicylhydroxamic acid) 10 µg/ml/L (Vincelli

and Dixon, 2002); Emerald (boscalid) at 2 mg a.i./L (Zhang et al., 2009); 3336 (thiophanate-methyl) at 60 mg a.i./L (Burpee, 1997); 26GT (iprodione) at 530 mg a.i./L (Burpee, 1997); BannerMaxx (propiconazole) at 30 mg a.i./L (Burpee, 1997); SHAM at 10 µg/ml (Vincelli and Dixon, 2002). Salicylhydroxamic acid (SHAM) at 0.1% was added to media containing azoxystrobin to prevent alternative oxidase (Vincelli and Dixon, 2002). A five mm plug was transferred from 3-day-old colonies to prepared plates and incubated at 24°C. Colony growth was measured in mm on 2 perpendicular axes at 24 h intervals for three consecutive days. Sensitivity assays were performed with three replicates and repeated twice. Data subjected to analysis of variance were analyzed with proc anova (SAS v9.2), and means were separated by treatment for each isolate with Fisher's least significant difference test at $\alpha < 0.05$ (Tables A-1 and A-2).

Inoculum Source Evaluation

Turfgrass colonization by different inoculum preparations was tested by inoculating turfgrass with colonized PDA, 20% PDA, V8 juice agar, and water agar (WA) media, sterile colonized wheat seed, and stromatized filter paper. Four *Poculum*-clade isolates were included for each inoculum preparation. Isolates from each ancestral group identified in Chapter 2 (UF0404, UF0476, UF0523, and UF0525) were included in the inoculum preparation evaluation. Sterile colonized wheat seed and pots of 'Sonesta' bermudagrass (*Cynodon dactylon* (L.) Pers.), 'Aloha' seashore paspalum (*Paspalum vaginatum* Swartz) and 'SeaDwarf' seashore paspalum (*Paspalum vaginatum* Swartz) were prepared with the same methods as described in Chapter 2.

Turfgrass was trimmed to 1/2 inch with scissors prior to inoculation. Pots of turf were treated with three colonized 7 mm agar plugs, 10 to 20 infested seed, or three pieces of 4 mm^2 stromatized paper. Turfgrass was misted with dH_2O, enclosed in a 30-

by-50 cm polyethylene bag with a moist paper towel, and incubated at 24°C for 24 to 48 h. Inoculations were evaluated by the presence (+) or absence (-) of dense mycelium production on turf (Table 3-3).

Results

Growth rates for individual isolates was inhibited by the same products in both trials, but data could not be combined (P<0.0001). Differences in mean growth rate were observed for each isolate when grown on plates containing Emerald 70WG, Chipco 26GT, Banner Maxx, and Heritage 50WG + 10% SHAM compared to untreated plates and plates treated with 10% SHAM alone or Cleary's 3336 (Tables A-1, A-2.). Cleary's 3336 did not inhibit growth as expected.

Colonization of turfgrass with infested wheat seed inoculum produced abundant aerial hyphae and was deemed successful. Inoculations with all other inoculum preparations failed to produce visible hyphae in an acceptable timeframe needed for pathogenicity assays (Table A-3). Agar plugs had no production of hyphae after placement on turf plots. Inoculations with stromatized paper were ineffective and did not appear to result in actively growing fungal tissue. No production of hyphae was observed in pots inoculated with stromatized paper.

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Table A-1. Average growth per day for selected isolates on fungicide amended media for each product.

Treatment	Active Ingredient	Rate	*Poculum* clade isolates UF0404	UF0476	UF0523	UF0525	UF0816	UF0824	UF0912	*S. homoeocarpa* UF0420
Contol	n/a	n/a	23.8 a	23.1 b	24.9 a	26.6 b	24.5 b	22.2 b	25.1 b	26.7 b
Emerald 70WG	boscalid	2 mg/l	8.3 d	13.8 e	13.0 d	9.2 f	9.7 e	10.5 d	11.9 f	15.7 d
Cleary's 3336	thiophanate-methyl	60 mg/l	22.2 b	23.9 ab	25.1 a	28.2 a	25.7 a	24.3 a	25.8 a	29.3 a
Chipco 26GT	iprodione	530 mg/l	6.6 e	13.8 e	11.0 e	10.6 e	11.7 d	14.6 c	12.5 e	13.5 d
Banner Maxx	propiconazole	30 mg/l	16.2 c	16.1 d	14.2 c	17.1 d	12.5 d	15.3 c	13.9 d	8.4 e
SHAM	salicylhydroxamic acid	10 mg/l	22.6 b	24.1 a	25.4 a	26.9 b	24.3 b	22.7 b	25.6 a	26.4 b
Heritage 50WG + SHAM	azoxystrobin + salicylhydroxamic acid	30mg/l + 10 mg/l	15.7 c	20.8 c	20.2 b	22.6 c	20.4 c	22.3 b	23.3 c	21.7 c

Table A-2. Average growth per day for selected isolates on fungicide amended media for each product.

			Poculum clade isolates							*S. homoeocarpa*
Treatment	Active Ingredient	Rate	UF0404	UF0476	UF0523	UF0525	UF0816	UF0824	UF0912	UF0420
Contol	n/a	n/a	24.6 a	25.4 a	26.3 b	23.9 a	22.7 b	26.1 a	27.3 b	28.2 a
Emerald 70WG	boscalid	2 mg/l	8.7 e	13.5 g	10.0 e	11.6 e	10.7 d	14.2 d	16.2 e	18.7 d
Cleary's 3336	thiophanate-methyl	60 mg/l	24.2 a	23.7 c	25.8 b	22.7 b	24.6 a	24.1 bc	25.6 c	27.5 ab
Chipco 26GT	iprodione	530 mg/l	10.1 d	16.8 e	9.4 f	11.7 de	14.9 c	14.1 d	15.1 f	13.1 e
Banner Maxx	propiconazole	30 mg/l	16.0 c	15.6 f	16.6 d	12.5 d	15.4 e	14.1 d	8.4 g	11.8 e
SHAM	salicylhydroxamic acid	10 mg/l	24.4 a	24.5 b	27.5 a	22.9 b	22.9 b	24.5 b	28.4 a	26.5 b
Heritage 50WG + SHAM	azoxystrobin + salicylhydroxamic acid	30mg/l + 10 mg/l	18.6 b	20.2 d	22.3 c	19.2 c	22.3 c	23.6 c	21.8 d	23.2 c

Table A-3. Comparison of the effect of medium source on production of hyphae during inoculation on turfgrass.

		Growth Medium Source						
		Agar Plugs				Seed		
Turf Species	Variety	1/5th PDA	PDA	V8	WA	Wheat	Oat	Paper
Bernmudagrass	‘Sonesta'	- - -	- - -	- - -	- - -	+ + +	n/a	- - -
Paspalum	‘Aloha'	- - -	- - -	- - -	- - -	+ + +	n/a	- - -
Paspalum	‘SeaDwarf'	- - -	- - -	- - -	- - -	+ + +	n/a	- - -

(+) - turf colonization, (-) – no colonization

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BIOGRAPHICAL SKETCH

Gary "Todd" Cooper received his bachelor's degree from Otterbein College in 2005. His major was Life Science with a concentration in Plant Science. During his undergraduate years Todd was employed as an automobile technician repairing automatic transmissions for Ford, Lincoln, and Mercury dealers in the Columbus area. While finishing his degree he accepted an offer to work at Battelle Memorial Institute as a Research Technician for the Aerosol Process Technologies group in Columbus, Ohio. Upon graduation Todd accepted an offer to work on a master's degree at the University of Florida studying angular leaf spot on strawberries. He completed this program in August of 2007 and immediately began work to characterize an unknown fungus with Dr. Philip Harmon for his doctoral degree which he completed in 2014. Todd was offered a position as a Research Scientist with Syngenta Inc. and accepted the offer to begin work in July 2011. Todd coordinates field research with several faculty members from the University of Florida but his current and primary responsibilities include evaluating various fungicides and herbicides for disease control, weed control, and product safety on various turfgrasses at Syngenta's Vero Beach Research Center located in Vero Beach, Florida.