New insights on the biology and host-pathogen interactions of the dollar spot pathogen,

Sclerotinia homoeocarpa

By

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ABSTRACT

Dollar spot, caused by the ascomycetous fungal pathogen *Sclerotinia homoeocarpa*, is one of the most economically important diseases of amenity turfgrass species worldwide. The research presented in this dissertation explores the basic biology of *S. homoeocarpa* with the aim of increasing understanding of pathogenesis to enable development of more sustainable, integrated disease management tactics for dollar spot control. Initial research focused on the sources of primary inoculum for dollar spot. A three-year field study assessing *in planta* survival of *S. homoeocarpa* revealed that this fungus overwinters predominantly in the shoots of symptomatic and asymptomatic turf and that environmental factors, particularly temperature and precipitation, may influence *S. homoeocarpa* overwintering. Commercial creeping bentgrass (CRB) seed contamination assays employing both culture-based and molecular detection techniques found that viable, pathogenic isolates of *S. homoeocarpa* could be recovered from seed and that *S. homoeocarpa* DNA was present in over 50% of seeds lots tested, suggesting a high potential for dispersal of this pathogen in seed. Additional research studies addressed development of a model host system for *S. homoeocarpa* and elucidation of the role of oxalic acid during host infection. Various model hosts and the natural host CRB responded similarly to *S. homoeocarpa* inoculation; however, symptoms developed more rapidly in plants with high

endogenous oxalate content. Investigation of this finding demonstrated that both host oxalate content and pathogen-produced oxalic acid may influence symptom development in response to *S. homoeocarpa* inoculation and that oxalic acid is likely associated with symptom development but not initial host colonization. Further, host tissue and the plant cell wall component xylan promoted production of oxalic acid by *S. homoeocarpa.* Additional studies with *Brachypodium distachyon* suggested potential for development of this host into a model system for elucidating host/pathogen interactions of *S. homoeocarpa* and two additional major fungal pathogens of turfgrass, *Microdochium nivale* and *Rhizoctonia solani*. Together, these studies provide new understanding of *S. homoeocarpa* epidemiology and biology and will facilitate development integrated disease management programs that minimize the use of fungicides for dollar spot suppression.

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CHAPTER 1: INTRODUCTION

Introduction

Turfgrasses have been cultivated for their aesthetic, recreational, and functional benefits for over ten centuries (5,6). Turf provides a relatively inexpensive means of erosion control, heat dissipation, and noise abatement; a safe and low-cost recreational surface; and numerous intangible benefits to mental and physical wellbeing (6). The turfgrass industry generates over $40 billion of revenue in the United States annually and makes significant contributions to the global economy (15). Though generally not thought of as an important 'crop' plant, turfgrass covers roughly 35 million acres of land in the United States (15) and was recently identified as the largest irrigated crop in the United States (42). Professional turfgrass sectors include golf courses, sod production, institutional facilities, and management services, such as lawn care and irrigation. Of these, the golf course industry is the single largest contributor, generating $33.2 billion in gross annual revenues and providing hundreds of thousands of jobs (15,29).

Dollar spot is one of the most serious and widespread diseases of turfgrass (1,61). Both warm and cool-season turfgrasses cultivated in temperate locations across the globe are susceptible to this disease (22,23,56). On individual blades of grass, the disease generally manifests as a bleached, hourglass-shaped lesion with reddish-brown borders (1,11,55,60). Dollar spot earns its name from the sunken, straw-colored, silver dollar-sized infection centers typically formed on closely mown turfgrass swards found on golf course putting greens and fairways. On less intensively maintained turfgrass, including home lawns and recreational athletic fields, stand symptoms are more diffuse with larger patches (1,61). Individual infection centers can coalesce when disease becomes severe, resulting in large blighted areas that detract from both aesthetic quality and playability of turfgrass swards (55,61).

Chemical control is the most widely used and effective method for management of dollar spot (55). A wide variety of fungicides are effective against this disease. These include multi-site chemistries, such as chlorothalonil, and site-specific chemistries, including those from the benzimidazole, demethylation inhibitor (DMIs), and dicarboximides classes (1). Fungicides are routinely applied to manage dollar spot on golf courses, resulting in upwards of ten fungicide applications or more in a single growing season at certain locations (25). This practice can inflict significant financial cost to the turf facility and can have negative consequences for the environment and human health (39,59).

Frequent fungicide applications also place strong selection pressure on *S. homoeocarpa* populations, which has resulted in frequent development of pathogen populations with reduced fungicide sensitivity (1,55,61). The presence of fungicide resistance in *S. homoeocarpa* was first detected in the late 1960s, when reduced sensitivity to the previously effective cadmium-based fungicides was reported (21,23,44). Since then, reduced sensitivity to the benzimidazole, DMI, and dicarboximide classes of fungicides have been reported for *S. homoeocarpa* (28,40,46,50,62,63). Resistant populations of *S. homoeocarpa* can develop quickly, in spite of the fact that this fungus is not known to undergo a sexual cycle, with the exception of isolated reports from the United Kingdom (4,35,36,48).

In response to the negative impacts of reliance on fungicides for dollar spot management, research in recent years has turned to integrated disease management (IDM) programs (27,61). These programs use simultaneous implementation of chemical, cultural, and biological controls, host resistance, and weather-based disease forecasting to significantly reduce the number of fungicide sprays required for adequate disease suppression. For IDM to succeed, it is important that all aspects of the phytopathological disease triangle—conducive environment, virulent

pathogen, and susceptible host—are considered. Most IDM research to date has focused on manipulation of environmental factors most conducive to dollar spot and on development of turfgrass cultivars resistant to dollar spot (38). However, more research is needed to understand the general pathogen biology, infection process, and host interactions of *S. homoeocarpa* for IDM on turfgrass to be successful.

It is generally assumed that the primary inoculum for dollar spot epidemics comes from *in planta* overwintering of *S. homoeocarpa* (1,61), but there is little data to back this claim. The only prior study of *S. homoecarpa* overwintering was performed on Kentucky bluegrass (*Poa pratensis* L.) in Rhode Island during the 1973-1974 winter season (23)(Fenstermacher 1980). This study, which destructively sampled symptomatic leaf blades by placing them inside a plastic straw and burying them in soil or placing them on the soil surface either in the field or inside a greenhouse, demonstrated that *S. homoeocarpa* survived the winter in less than 10% of samples. The pathogen was more frequently isolated from leaf blades with visible stroma but overall isolation rates remained under 10% and stroma did not give rise to apothecia or apothecial initials (23). However, conditions for overwintering were manipulated in this study and it was not replicated over multiple years. Further research on *S. homoeocarpa* overwintering is needed to better elucidate where in plants this pathogen overwinters, how much of the pathogen survives the winter, and whether or not winter survival of *S. homoeocarpa* is affected by environmental conditions.

Spatial analysis of dollar spot epidemics conducted by Horvath and colleagues (33) demonstrated that infection centers occur in small aggregates, indicative of a point source for *S. homoeocarpa* inoculum. These infection center clusters remain stable throughout the growing season, regardless of disease pressure and can have similar structures over multiple years. Based

on these results, it was concluded that spread of *S. homoeocarpa* on equipment or people is minimal (33). Similarly, Williams and colleagues (66) demonstrated that removal of grass clippings had no effect on dollar spot severity. The findings of these studies were in contrast to previous assumptions that dollar spot mycelia are dispersed through infected grass clippings or on people and mowers (61) and have generated new questions concerning where *S. homoeocarpa* comes from and how it is dispersed. Some have suggested that *S. homoeocarpa* is a soil-borne pathogen but recent research by Wilson (67) indicated that this fungus is a poor saprophyte and does not grow well on soil. Interestingly, Brede (13) and Brede and Dunfield (14) observed positive correlation between seeding rate and dollar spot severity in tall fescue and Kentucky blue grass, respectively. They assumed that this correlation was due to lower plant health resulting from increased stand density at higher seeding rates (13). More recently, however, it has been shown that turf with higher stand density is correlated with decreased dollar spot severity (11).

Surveys of *S. homoeocarpa* population biology in the United States and Canada determined that there is a small genetic base for this pathogen and that clonal reproduction of this pathogen is likely (34,52,58). Raina and colleagues (52) noted close genetic similarity between Oregon and Connecticut isolates of *S. homoeocarpa*, suggesting that the fungus may have been transported transcontinentally. In this same study, a *S. homoeocarpa* isolate from Belize was genetically distinct from all isolates collected in the United States. Though the genetic difference could be due to location, the Belize isolate used in this study was also the only isolate collected from a warm-season grass (52). Recently, multiple groups have found that *S. homoeocarpa* isolates collected from warm and cool-season grasses are phylogenetically distinct (43,51). Additionally, *S. homoeocarpa* isolates from warm-season grasses have much greater genetic

diversity than isolates from cool-season grasses (51). Interestingly, cool-season grasses are most often seeded and the majority of cool-season grass seed for North America is produced in a small region of the Pacific Northwest (8). Warm-season grasses, on the other hand, are generally vegetatively propagated and come from a much broader geographic range (38). Whether these differences in production contribute to the relative genetic diversity of *S. homoeocarpa* isolates collected from warm and cool season grasses is not known, but further research in this area is needed.

To date, much remains unknown about the infection process of *S. homoeocarpa.* It is generally accepted that this fungus infects its hosts through direct penetration, mowing-induced wounds, or stomata (1). More recently, formation of appresorium, infection vesicles, and primary hyphae inside infected cells have been documented on detached CRB leaves (47). While it has been generally assumed that *S. homoeocarpa* is a necrotrophic host specialist (3), various researchers have indicated a fairly broad host range for this pathogen, including members of the pink, morning glory, pea, sedge, and grass families (26,32,61). Orshinsky and colleagues (47) observed *S. homoeocarpa* infection on detached creeping bentgrass leaves and found that development of infection was more indicative of a hemibiotrophic than a necrotrophic pathogen. This was based on the observations that necrotic lesions did not develop immediately following host inoculations and fungal hyphae advanced well beyond the visibly infected areas of trypan blue-stained host tissue (47).

Though there is no cultivar with complete resistance to *S. homoeocarpa*, a number of creeping bentgrass cultivars with greatly enhanced resistance to infection and recovery following dollar spot outbreaks have been released (12,19,54,60). The exact mechanisms of resistance in these cultivars is unknown. Williams and Harrell (65) were unable to identify any correlation

between cultivar leaf surface morphology and dollar spot severity. A separate study, however, noted a correlation between cultivar resistance and trichome size as well as a tendency for resistant cultivars to maintain smaller lesion sizes (11). It is likely that the mechanisms of resistance to *S. homoeocarpa* are more apparent at the genetic level than at the physiological level. Various linkage maps and marker resources are available for CRB and have been used to understand the molecular mechanisms controlling dollar spot resistance (2,17,20,41,53). Bonos and colleagues (10) offered the initial insight that resistance is likely inherited quantitatively, the result of combined action of 2 to 5 (or more) genes, and that genetic improvement of dollar spot resistance in CRB should be possible. Subsequent to this, a major quantitative trait locus (QTL) for dollar spot resistance was identified on CRB linkage group 7.1, with additional smaller effect QTL found on linkage groups 2.1, 3.2, 4.1, 4.2, 6.2, and 7.2 (20). More recent publications have indicated that epistatic interactions may be involved in dollar spot resistance, which could decrease the efficacy of QTL analysis (9,53). Consequently, further research is required to pinpoint specific genes or traits important for resistance to dollar spot.

Various phytotoxic molecules have been identified in *S. homoeocarpa* culture filtrates, including norditerpenoid dilactones, oxalic acid, and an unidentified metabolite associated with root necrosis (23,31,57). Of these, oxalic acid is of particular interest because it is an important virulence factor for other *Sclerotinia* species, particularly *Sclerotinia sclerotiorum* (24,26). The role of oxalic acid in pathogenicity of *S. sclerotiorum* has been extensively studied (18,24,26,30,37,64) *S. homoeocarpa* is not, however, a true *Sclerotinia* species and is currently being reclassified into the *Rutstroemiaceae* family, a sister family to *Sclerotiniaceae* (7,16,45). Consequently, the role of oxalic acid in pathogenicity of *S. homoeocarpa* remains unclear.

Both oxalate oxidase activity (47) and expression of oxalate oxidase as well as related germin-like protein genes (49) have been found in *S. homoeocarpa*-infected creeping bentgrass tissue, suggesting that oxalic acid is important for infection of this turfgrass host. Conversely, Liberti and colleagues (43) found no correlation between oxalic acid production and pathogenicity or aggressiveness of 47 *S. homoeocarpa* isolates on detached leaf blades of St. Augustinegrass. The disparate results of these studies could be the result of a number of factors, ranging from the host species used to the method used for detection of oxalic acid production by *S. homoeocarpa* isolates. In any event, further research is necessary determine what, if any, role oxalic acid plays in host infection by *S. homoeocarpa*.

Rationale and Objectives for this Research

This work is in direct response to the desire by golf course superintendents, other turfgrass managers, and consumers for more sustainable, cost-effective, and environmentally conscious approaches to dollar spot management on high-input turfgrass. The response to development of integrated disease management approaches for dollar spot management has been positive and progressive; however, knowledge of pathogen biology and mechanisms of host defense response have lagged behind other areas. These knowledge gaps have slowed the development of highly effective integrated disease management programs for dollar spot can be developed. By contributing to the body of knowledge on *S. homoeocarpa* biology, I hope to address these shortcomings and facilitate the development of sustainable, integrated disease management practices for dollar spot suppression.

In this research my specific objectives were to:

1) Develop a semi-selective medium for improved isolation of *S. homoeocarpa* from host tissues
2) Quantify overwintering of *S. homoeocarpa* in the field and assess the potential of commercial creeping bentgrass seed as a source of primary inoculum for dollar spot epidemics
3) Characterize the infection process and study the role of oxalic acid in pathogenesis of *S. homoeocarpa* on the natural and model hosts and
4) Evaluate the potential of *Brachypodium distachyon* as a model host for fungal pathogens of turfgrass

In previous discussions of the proposed research for this dissertation, I considered using *Arabidopsis thaliana* mutants and virus-induced gene silencing in *B. distachyon* to study molecular aspects of host resistance to *S. homoeocarpa*. Subsequent research findings indicated that these methods were not the best suited for study of host-pathogen interactions relevant to the *S. homoeocarpa*/turfgrass pathosystem. Consequently, these lines of investigation were abandoned and preliminary research findings from these experiments have not been included in this dissertation.

Each of the four research chapters in this dissertation has been submitted or prepared for submission to a peer-reviewed journal. Chapter 2 is currently under review at the Canadian Journal of Plant Pathology. Chapters 3, 4, and 5 have been prepared for submission to Phytopathology, PLOS One, and BMC Plant Biology, respectively. For consistency, all references are in the format requested by Phytopathology.

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CHAPTER 2: DEVELOPMENT OF A SEMI-SELECTIVE MEDIUM FOR ISOLATION OF THE DOLLAR SPOT PATHOGEN, *SCLEROTINIA HOMOEOCARPA*, FROM PLANT TISSUES

This chapter is a slightly modified version of the following manuscript submission:

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Contribution: R.R. was the primary author. She designed and performed experiments, analyzed data, and wrote the manuscript. B.V.R assisted with experimental set-up and data collection. J.P.K helped design experiments and write the manuscript.

Abstract

Dollar spot, caused by *Sclerotinia homoeocarpa*, is one of the most economically devastating diseases of amenity turfgrasses worldwide. *S. homoeocarpa* is easily isolated from active lesions, but detection from seed, dormant host tissue, and other plant debris that may serve as source of primary inoculum is difficult. In this study, a semi-selective medium was developed to enhance isolation of *S. homoeocarpa*. Various turfgrass fungicides, the pH indicator dye bromophenol blue, and two pH levels were assessed for their effects on, and, in the case of bromophenol blue, response to, growth of *S. homoeocarpa* and contaminant fungi frequently isolated from field and seed samples. Amendment of medium pH to 4 promoted growth of *S. homoeocarpa* in the absence of fungicides and had a positive effect on growth of *S. homoeocarpa* relative to contaminant isolates on media amended with 0.1ppm triticonazole or 5ppm azoxystrobin. *S. homoeocarpa* growth rate on these three media, as determined by *in vitro* radial growth assays, was consistent across many isolates representing the majority of *S. homoeocarpa* vegetative compatibility groups. Isolation efficiency of the three pH-amended media was tested against antibiotic-amended PDA on field samples and artificially inoculated turfgrass seed. In both cases, the medium amended with 5ppm azoxystrobin significantly decreased contaminant isolation with no adverse effects on recovery of *S. homoeocarpa* in comparison with antibiotic-amended PDA. The enhanced specificity of this medium will be a useful tool for selective detection of *S. homoeocarpa* and identification of sources of initial inoculum for dollar spot.

Keywords: Dollar spot, semi-selective medium, *Sclerotinia homoeocarpa*, pathogen detection, turfgrass pathology

Introduction

Dollar spot, caused by the ascomycetous fungus *Sclerotinia homoeocarpa* (F.T. Bennett), is one of the most serious and widespread diseases of turfgrasses (2,6,36), as well as other economically important plants including switchgrass (35) and perennial peanut (15). On individual blades of grass, the disease manifests as bleached, hourglass-shaped to oblong lesions with reddish-brown borders (2,26). These symptoms vary between host species as well as between cultivars with varying susceptibility to *S. homoeocarpa* (4,8,25,34). Dollar spot earns its name from the sunken, straw-colored, roughly silver dollar-sized infection centers that are typically present when the disease occurs on closely mown turfgrass swards, such as golf course putting greens or fairways (11,31). Severe disease outbreaks on such surfaces result in the coalescence of individual foci into large blighted areas, which detract from both aesthetic and playing quality of the affected turf (11,26). On less rigorously maintained turfgrass, including homelawns and playing fields, dollar spot symptoms typically include larger, more diffuse patches of blighted grass.

Chemical control is the most common and effective method for managing dollar spot (26). However, increasingly stringent regulatory measures and concerns about economic and environmental costs have necessitated decreased dependence on fungicides for dollar spot management (22). Chemical control of dollar spot has also been complicated by development of *S. homoeocarpa* populations with resistance to benzimidazole (9,37,38), dicarboximide (12), and sterol demethylation-inhibitor (DMI) fungicide classes(14,16,21,23,38).

Identifying the source of initial inoculum for dollar spot is critical for development of integrated, pathogen biology-based control methods that limit dependence on fungicides (36). Research demonstrates that *S. homoeocarpa* does not thrive in soil without a source of organic

matter; thus, it is unlikely that this fungus exists as a soil-inhabiting saprophyte (40). Infected seed, dormant overwintering mycelia, and symptomless infections are all possible sources of *S. homoeocarpa* inoculum (13,40). Attempts to isolate *S. homoeocarpa* from such tissues on antibiotic amended potato dextrose agar (PDA^{+++}) are largely unsuccessful due to lack of medium specificity and the large number of contaminant fungi present on such samples. Consequently, the objectives of this study were (i) to develop a semi-selective medium that inhibits the growth of common contaminant fungi while minimizing inhibitory effects on *S. homoeocarpa* and (ii) to compare the effectiveness of such media against PDA^{+++} for isolation of *S. homoeocarpa* and contaminant fungi from field and seed samples.

Materials and methods

Fungal strains

Four isolates of *S. homoeocarpa* and eight contaminant fungi obtained from field and seed samples were used for preliminary growth assays on trial media (Table 1). All isolates were obtained by plating turfgrass leaves or seed samples on petri plates containing potato dextrose agar (PDA) amended with 50 mg/L of the antibiotics chloramphenicol, streptomycin sulfate, and tetracycline (PDA^{+++}). Hyphal tips of putative *S. homoeocarpa* and contaminant colonies were excised and transferred to fresh PDA^{+++} to obtain pure cultures. When possible, isolates were identified to genus by sequencing of the ITS region with primers ITS1 and ITS4 (39); however, not all contaminant isolates were amenable to common DNA extraction methods and were left as 'unidentified.' Additionally, not all contaminant fungi could be identified to genus by BLASTN searches of resulting sequences and were only identified to the lowest definite taxonomic group (3). *S. homoeocarpa* vegetative compatibility grouping (VCG) tester isolates for validation studies were obtained from Dr. Lane Tredway, formerly at North Carolina State University, and

were initially described by Viji and colleagues (33) (Table 2). All isolates were maintained by weekly transfer to fresh PDA and stock cultures were stored on filter paper disks at -80°C.

Medium additives and preparation

Three antibiotics, five fungicides at varying concentrations, the pH indicator dye bromophenol blue, and 12.1N hydrochloric acid (HCl) were used as medium additives in this research (Table 3). The base medium PDA was prepared according to the manufacturer's instructions and cooled to 50°C prior to addition of amendments. All amendments were added to media on a 50°C stir plate to ensure homogeneity and media was poured immediately after amendment addition to avoid settling. To decrease variability inherent in manual pouring, a 10mL pipettor (Eppendorf, New York, USA) was used to pipet 6.5mL medium into each 60mm x 15mm petri plate. Plates were allowed to solidify within a biological safety cabinet and then stored in the dark at 4°C until use. To avoid breakdown of media additives, all plates were used within 5 days of pouring.

Mycelial growth assays on trial media

Agar plugs (6mm diameter) were excised from the advancing edge of one-week-old fungal colonies of *S. homoeocara* and contaminant fungi with a sterile cork borer. Plugs were placed in the center of 60mm x 15mm petri plates containing semi-selective media. Plates were inverted, arranged randomly, and incubated at room temperature and ambient lighting for 48 h. To prevent edge or other position effects, plates were arbitrarily rearranged 24 h after transfer. At 48 h post-transfer, radial growth was measured and percent growth versus the standard medium PDA^{+++} was calculated using the formula:

1 [[(Average colony diameter on trial media) / (Average colony diameter on PDA+++)] * 100% **]**

Each fungal isolate x medium combination was performed in triplicate and the experiment was repeated three times.

Acid production on bromophenol blue amended media

To test acid production as a positive indicator for *S. homoeocarpa* 6mm diameter agar plugs were excised from the advancing edge of one-week old fungal colonies with a sterile cork borer and placed in the center of 60mm x 15mm agar plates amended with 50mg/L bromophenol blue, as previously described (29). Plates were sealed with parafilm and incubated upside down at room temperature and ambient lighting for 48 h. Yellow color production was then rated as (0) for no coloration, (1) for weak coloration, and (2) for strong coloration. Due to the subjective nature of this rating scale, all ratings were performed by the same individual and all plates in a repetition were rated at the same time. Each fungal isolate x medium combination was performed in triplicate and the experiment was repeated three times.

Effect of decreased pH on fungal growth

HCl (12.1N) was added to cooled medium with a pipet to achieve a desired medium pH of 4. For fungicide-amended media, fungicides were added prior to HCl to prevent interference of pH with solubility. Mycelial growth of *S. homoeocarpa* and contaminant isolates was determined as described above. Each isolate x pH x fungicide interaction was performed in triplicate and the experiment was repeated three times.

Evaluation of semi-selective media for detection of S. homoeocarpa *in field samples*

In the spring of 2012, a total of 50 soil cores were collected for use in validating semi-selective medium efficiency: Ten from active dollar spot infection centers, 20 from asymptomatic turf, and 20 from dollar spot infection centers that had formed the previous fall (overwintering samples). Fewer soil cores were selected from symptomatic infection centers

because *S. homoeocarpa* is easily isolated from active lesions on PDA^{+++}. For each sample, *S. homoeocarpa* isolation efficiency with and without surface disinfestation was determined.

To determine isolation efficiency, 12 grass blades were extracted from a single core using sterile forceps. Three blades were then plated on each of the four previously mentioned media in a circular pattern with at least 10 mm between each blade to ensure that none overlapped. This process was repeated for each soil core collected. Plates were sealed with parafilm, inverted and incubated at room temperature for 48 h. At 48 h, total fungal colony forming units (CFUs) per plate and number of putative *S. homoeocarpa* colonies per plate were determined. Putative *S. homoeocarpa* colonies were subcultured to obtain pure cultures and to confirm identity.

After the initial plating of grass blades, the same cores were immersed in a 0.06% sodium hypochlorite (10% Clorox) solution for 15 s and the shoots were gently rubbed, then rinsed in distilled water twice for 30 s each time. Samples were then allowed to dry on sterile paper towels and the entire leaf blade plating process was repeated as described above.

Evaluation of potential semi-selective media for detection of S. homoeocarpa *in seed*

Five grams of commercially available creeping bentgrass seed (cv. 'Penncross') was mixed in a 50 mL beaker with 2.5 mL of deionized water. The beaker was sealed with a double layer of aluminum foil and sterilized on a dry cycle at 121°C and 15 psi for 30 min. The sterilized seed was allowed to cool overnight and infested the following day with two agar plugs taken from the advancing edge of a 5-day-old colony of *S. homoeocarpa* isolate 2F92-1. The beaker was sealed with Parafilm and allowed to incubate for 3 weeks at room temperature and ambient lighting with daily shaking to disperse inoculum and prevent clumping. After 3 weeks, an arbitrary sample of approximately 100 seeds was plated on PDA^{+++} to determine colonization. The remaining infested seeds were placed on sterile paper towels and allowed to dry overnight in

a biological safety hood. One gram of infested seed was then mixed with 9g of untreated commercially available CRB seed (cv. ‘Penncross) for a 1:9 ratio of infested to non-infested seed and this was repeated three times. Seed mixtures were stored in 50mL conical tubes (BD Falcon, San Jose, CA, USA) at room temperature with ambient lighting.

Two days after mixing infested and non-infested seed, 400 seeds were collected from the mixtures and distributed evenly among the four media (100 seeds plated per medium, 10 petri plates of each medium with 10 seeds on each). Seeds were plated individually and spaced so that none touched. Plates were sealed with parafilm and incubated upside down at room temperature and ambient lighting for 5 days. The proportion of seed *S. homoeocarpa* was isolated from was recorded after 24 h, and after 5 days the number of colonies of contaminant fungi per plate was determined. This entire experiment was repeated 9, 13, and 14 days after the initial seed mixtures were prepared, for a total of four experimental repetitions.

Statistical analysis

All experimental data were analyzed using the generalized linear mixed model (GLIMMIX) procedure in SAS Version 9.3 (17). Model fitting criteria and studentized residual plots were assessed to determine the best model for each data set. Generally, isolate and media were treated as fixed effects and experimental replicate (block) was set as a random effect. Replicate was not included as a random effect when model fitting criteria indicated a negligible effect on variance components.

Mycelial growth assays were best fit by a lognormal distribution. Pre-planned single degree of freedom contrast statements were used to make meaningful comparisons between groups of interest. These included: *S. homoeocarpa* versus contaminant isolates on trial semi-selective media, growth on media at pH6 versus pH4, and growth of Wisconsin *S. homoeocarpa*

isolates versus VCG tester isolates on pH-amended media. Yellow coloration on bromophenol blue amended media was best fit by a normal distribution and differences between *S. homeoocarpa* and contaminant isolates were again evaluated with pre-planned contrast statements.

The seed and field sample validation experiments both consisted of count data and were best fit by a Poisson and negative binomial distribution, respectively. Dunnett's test was used to compare each candidate semi-selective medium against the control PDA^{+++} for isolation frequency of both *S. homoeocarpa* and contaminants. For field samples, the analysis was sliced by sample type to allow for comparison of candidate semi-selective media against PDA^{+++} only within the same sample type.

Results

Effect of fungicide amendments

Mycelial growth assays were conducted on media containing five fungicides at varying concentrations, for a total of 13 fungicide-amended media tested in the absence of other additives. The average colony diameter and growth relative to the standard isolation medium PDA^{+++} were compared for four isolates of *S. homoeocarpa* and eight contaminant isolates (Table 4). In general, *S. homoeocarpa* and contaminant isolates had similar colony diameter and growth relative to PDA^{+++} on fungicide-amended media (Table S1). Greater average colony diameter and relative growth of *S. homoeocarpa* when compared to contaminant fungi were the criteria used to select potential semi-selective media. None of the initial media tested met these criteria.

Even at the lowest concentrations of PCNB and thiophanate methyl, large differences were noted in growth of the four *S. homoeocarpa* isolates. Isolates S10 and OJN9 grew nearly as well on media amended with 10 ppm thiophanate methyl as on the standard isolation medium,

PDA^{+++}. Conversely, growth of isolates 2F92-1 and Heath1A was roughly 10% of growth on PDA^{+++}. Medium containing 5ppm PCNB severely limited or prevent growth of all *S. homoeocarpa* isolates except Heath1A. Due to the variability in *S. homoeocarpa* growth responses to these media, they were not considered for selective isolation of *S. homoeocarpa* and were not included in pH or BB trials.

Specficity of bromophenol blue for detection of S. homoeocarpa

The pH indicator dye BB was added to media containing 5, 10, and 20 ppm azoxystrobin. The purpose of this additive was to visualize a reduction of medium pH in response to oxalic acid production by *S. homoeocarpa* (31). All isolates were rated at 48 h for their production of yellow coloration in the medium and average pigmentation was compared between *S. homoeocarpa* and contaminant isolates (Table 5). On bromophenol blue amended medium with 5 or 20 ppm azoxystrobin, contaminant isolates produced more yellow pigmentation than *S. homoeocarpa* isolates. In medium containing 10ppm azoxystrobin, an orthogonal contrast comparing *S. homoeocarpa* isolates to contaminants indicated that *S. homoeocarpa* produced significantly more pigmentation than contaminants, however, this was the result of very strong medium acidification by some *S. homoeocarpa* isolates and negligible color change by others (Table S2).

Effect of medium pH

PDA^{+++}pH4 had a strong positive effect on *S. homoeocarpa* growth relative to PDA^{+++} (Fig. 1A). Growth of all contaminant isolates, with the exception of 24FR, was suppressed on PDA^{+++}pH4 and was significantly less than that of the four *S. homoeocarpa* isolates. Growth of 24FR on PDA^{+++}pH4 was similar to that on PDA^{+++} (Table S1). Both the average colony diameter (41.14 mm) and growth relative to PDA^{+++} (138.30%) of *S. homoeocarpa* isolates were

greater than those of contaminants (17.82 mm and 70.92%, respectively) on PDA^{+++}pH4 (Table 6).

Growth of *S. homoeocarpa* isolates on A5pH4 was greater than that on A5 (two-way independent t-test, $P<0.0001$; Fig. 1F). Contaminant isolates grew equally well on both A5 and A5pH4 media. Direct comparison between *S. homoeocarpa* and contaminant isolate growth on A5pH4 revealed significantly greater colony diameter and growth relative to PDA^{+++} for *S. homoeocarpa* than for contaminant isolates (Table 6). *S. homoeocarpa* colony diameter and relative growth remained higher than that of contaminants on pH4 azoxystrobin-amended media with 10 and 20ppm azoxystrobin, though both were decreased by the additional active ingredient (Table 6). Additionally, contaminant isolates also showed increased growth on A10pH4 compared with A10 (Fig. 1G). This pH-mediated growth benefit was not observed for contaminant isolates at A20pH4 (Fig. 1H).

The DMI-fungicides triticonazole and propiconazole suppressed the growth of both *S. homoeocarpa* and contaminant isolates in acidified media relative to media containing the same concentration of active ingredient at neutral pH (Fig. 1B-E). There was no growth penalty on *S. homoeocarpa* isolates on T01pH4 medium relative to T01 medium. Contaminant growth was strongly suppressed on T01pH4 relative to T01 (Fig. 1B). Consequently, both *S. homoeocarpa* growth and growth relative PDA^{+++} were greater than that of contaminants on T01pH4 (Table 6). *S. homoeocarpa* colony diameter was greater than that of contaminants on T1pH4 and P01pH4 but growth relative to PDA^{+++} was not different or only slightly better on these two media, respectively.

Selection of candidate semi-selective media

Candidate semi-selective media were chosen from those media for which colony diameter and growth relative to PDA^{+++} were both significantly greater for *S. homoeocarpa* than for contaminant isolates. Media meeting these criteria included PDA^{+++}pH4, T01pH4 and azoxystrobin-amended media at pH4 for all concentrations evaluated. Of the three azoxystrobin-amended media, A5pH4 was selected as a candidate semi-selective medium because the largest differences between *S. homoeocarpa* and contaminant isolate colony diameter and relative growth were observed on this medium.

Growth of S. homoeocarpa VCG tester isolates on candidate semi-selective media

A total of 12 VCG tester isolates from groupings A through F, as well as three isolates of undefined VCG, were included in growth assays on candidate semi-selective media (Table S3). Pre-planned single degree of freedom contrasts were used to compare colony diameter between Wisconsin *S.homoeocarpa* isolates used for initial growth assays and VCG tester isolates. No differences were found between colony diameter of Wisconsin *S.homoeocarpa* isolates and VCG tester isolates on PDA^{+++} as well as the three candidate semi-selective media (Table 7). Growth relative to PDA^{+++} did not differ between Wisconsin *S. homoeocarpa* and VCG isolates on A5pH4 or T01pH4 media, but was higher for Wisconsin *S. homoeocarpa* isolates on PDA^{+++}pH4. Growth of all *S. homoeocarpa* isolates was greater on PDA^{+++}pH4 than on PDA^{+++}.

Isolation from field samples

The three candidate semi-selective media were compared to PDA^{+++} for their isolation efficiency from field samples both with and without surface disinfestation. In general, isolation frequency was very low, with the exception of symptomatic samples (Table 8). Dunnett's test with PDA^{+++} as the control group revealed no differences in *S. homoeocarpa* isolation efficiency, regardless of sample type or treatment. Overall, the most *S.homoeocarpa* isolations were made

on the A5pH4 medium and the fewest were made on PDA^{+++}. Additionally, *S. homoeocarpa* was not recovered from surface disinfested samples on PDA^{+++} (Table 8).

The number of contaminant fungi isolated per sample on candidate semi-selective media was also compared to the standard PDA^{+++} with Dunnett's test. Fewer contaminants colonies were isolated on A5pH4 than on PDA^{+++} for all samples types, regardless of surface disinfestation (Fig. 2A-C). PDA^{+++}pH4 and T01pH4 only performed better than PDA^{+++} on the prior-year symptomatic samples that had been surface disinfested (Fig. 2A).

Isolation from infested seed

Candidate semi-selective media did not significantly increase isolation of *S. homoeocarpa* from artificially infected seed, though isolation of *S.homoeocarpa* was slightly greater on all semi-selective media than on PDA^{+++} (Fig. 3A). Isolation of *S. homoeocarpa* from infested seed was closer to the starting ratio of 1:9 (infected:untreated) seeds on candidate semi-selective media than on PDA^{+++}. Of the three candidate semi-selective media, only A5pH4 significantly decreased isolation of contaminant fungi when compared with PDA^{+++} (Fig. 3B). The average number of contaminants per plate was reduced 2.65 on PDA^{+++} to 1.25 on A5pH4.

Discussion

Both *in planta* overwintering (13) and introduction of *S. homoeocarpa* on contaminated seed have been hypothesized as possible sources of initial inoculum for epidemics of dollar spot. Isolation of the *S. homoeocarpa* from such samples, however, is difficult due to the large number of contaminant fungi living on or in the plant material, as well as decreased activity of *S. homoeocarpa* in asymptomatic plant tissues. Molecular detection methods, such as *S. homoeocarpa*-specific PCR primers have recently become available, but do not to confirm viability of the pathogen and may not be sensitive enough to detect minute amounts of *S.*

homoeocarpa DNA (1). Consequently, a medium that can select for *S. homoeocarpa* is essential for determining the contribution of infected seed, pathogen overwintering, and latent periods for dollar spot epidemics. The A5pH4 medium developed in this study, which significantly decreases contaminant isolation without effecting detection of *S. homoeocarpa* will with isolations from these difficult samples and contribute to understanding of dollar spot epidemiology.

Many semi-selective media for phytopathogenic fungi rely on specific morphological characteristics for aid in identification of the target organism. These include such traits as profuse sporulation (10,28), microsclerotia production (20), or pigmentation (29,30). North American isolates of *S. homoeocarpa*, as well as most isolates worldwide, do not produce spores, fruiting bodies, or true survival structures (24) and the white, aerial mycelia initially produced by *S. homoeocarpa* in culture are indistinguishable from those of many common saprophytic fungi, as well as other plant pathogens. The formation of substratal stroma in culture medium is fairly exclusive to *S. homoeocarpa* but may not form for weeks and can be difficult to detect in the presence of many competing fungi. Consequently, the primary goal of a semi-selective medium for *S. homoeocarpa* was to significantly reduce growth of contaminant fungi.

All fungicides tested in this study had a negative effect on the growth of *S. homoeocarpa*, despite the fact that resistance or insensitivity has been reported for many classes of fungicides (9,14,18,19,21,23,37,38). The exception was PCNB, which was included in this study as a result of its efficacy in semi-selective medium for isolation of *S. sclerotiorum* (29). *S. homoeocarpa* isolates differed significantly in their responses to PCNB and thiophanate methyl; thus, these fungicides would not be useful in a semi-selective medium for *S.homoeocarpa* because only resistant populations would be selected. Conversely, all *S. homoeocarpa* isolates grew similarly

on media amended with various concentrations of azoxystrobin, triticonazole, and propiconazole. This indicated that any of these could be a potential additive in a semi-selective medium for *S. homoeocarpa* because they would not preferentially isolate specific populations. Fungicide amendments alone, however, did not produce significant differences in growth between *S. homoeocarpa* and common contaminant fungi. Additionally, bromophenol blue was determined to be an unnecessary media additive due to variability in acid production between *S. homoeocarpa* isolates and media acidification by some of the contaminant fungi tested.

Decreasing media pH with the addition of HCl was the most important amendment for selection of *S. homoeocarpa* and inhibition of contaminant fungi. Increased growth of *S. homoeocarpa* on PDA at pH4 has been demonstrated previously (32) and is in agreement with our results for growth of *S. homoeocarpa* isolates on PDA^{+++}pH4 relative to PDA^{+++}. *S. homoeocarpa* produces oxalic acid (32), and may acidify its own environment to create conditions amenable for growth. Various acids, including tannic, gallic, lactic and polygalacturonic, have been used in semi-selective for other phytopathogenic fungi (7,20,28,30). In addition to promoting growth of specific target species over competitors, acids are frequently selected for addition to selective media as a result of the specific morphological changes they produce in the target organism or promotion of sporulation (28,30).

This study reveals that there is a complex interplay between medium pH, fungicide amendment, and growth of *S. homoeocarpa*. Of particular interest, is the increase in *S. homoeocarpa* growth on acidified azoxystrobin-amended media relative to azoxystrobin-amended media at neutral pH (Fig. 1). This could be a result of decreased fungicide solubility, however, fungicide amendments were added to media first to avoid this possibility and prior research indicates that solubility of azoxystrobin is not pH dependent (27). Research shows that,

in certain cases, azoxystrobin application can exacerbate epidemics of dollar spot in the field (5). In general, this disease enhancement has been attributed to effects on competing microbes or the phyllosphere. The present data indicate a possible role of environmental pH as well. Further studies are needed to determine to the mechanism by which decreased pH allows *S. homoeocarpa* to grow better in the presence of azoxystrobin.

In validation experiments with both naturally infected field samples and artificially infected seed, semi-selective media did not perform significantly better than the current standard isolation medium PDA^{+++}. This result was not unexpected. It is well-known that *S. homoeocarpa* is readily isolated from symptomatic tissues without selective agents, as demonstrated by our high isolation rates on symptomatic field samples and artificially infected seed for all media tested. Semi-selective medium for *S. homoeocarpa* is needed for isolation from more difficult sample types, such as seed and asymptomatic tissues, where the pathogen may linger but is not easily isolated from due to the presence of saprophytic fungi. These events are so rare that it is difficult to generate a sample size large enough to detect significant differences between media types. Indeed, a power analysis from the field data set indicated that roughly 200 samples of each type would be needed to detect a difference between *S. homoeocarpa* isolation rates on A5pH4 and PDA^{+++}, although more of both the surface disinfested and non-surface disinfested samples were positive on A5pH4 in validation studies. Similarly, natural infection of turfgrass seed with *S. homoeocarpa* is so low (<1:1,000; Rioux *et al*., unpublished) that an impractical number of naturally-infected would need to be plated to detect a statistically significant difference between media.

A5pH4 performed better than PDA^{+++} at decreasing isolation of contaminants in all samples tested. The other candidate semi-selective media were not as successful at reducing

contaminant isolation and would not lead to significant gains in *S. homoeocarpa* isolation efficiency. In contrast, the suppression of contaminant fungi achieved by A5pH4 decreases competition with and potential outgrowth of *S. homoeocarpa* by saprophytic fungi and contributes to improved isolation, as well as decreased identification of false positives in difficult samples. A5pH4 suppressed contaminant isolations in both field and seed samples, which may have very different saprophyte populations, indicating that it will be a useful medium for selective isolation of *S. homoeocarpa* from a broad range of sample types.

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Table 1. *S. homoeocarpa* and common contaminant fungi used in preliminary media assessments

Isolate	Isolate Identity[w]	Collection Site
2F92-1	*S. homoeocarpa*	The Oaks Golf Course; Cottage Grove, WI
S10	*S. homoeocarpa*	O.J. Noer Turfgrass Research Center; Verona, WI
Heath1A	*S. homoeocarpa*	Heath Sod Farms; Coloma, WI
OJN9	*S. homoeocarpa*	O.J. Noer Turfgrass Research Center; Verona, WI
13NSF	*Nigrospora* sp.	O.J. Noer Turfgrass Research Center; Verona, WI
PCU-3A	Unidentified	Penncross CRB seed
24FR	*Neofusicoccum* sp.	O.J. Noer Turfgrass Research Center; Verona, WI
24FS	Unidentified Hypocrea	O.J. Noer Turfgrass Research Center; Verona, WI
4FS	*Fusarium* sp.	O.J. Noer Turfgrass Research Center; Verona, WI
5FSA	*Epicoccum nigrum*	O.J. Noer Turfgrass Research Center; Verona, WI
R8-1	Unidentified Sordariomycete	O.J. Noer Turfgrass Research Center; Verona, WI
A404	Unidentified	Penncross CRB Seed

Notes: [w] The ITS region was sequenced with primers ITS1 and ITS4 to confirm isolate identity (White *et al*., 1990). In cases where DNA extraction was not possible or BLASTN searches received low hits, isolate identity is left as 'unidentified' or as the lowest taxonomic level that can be identified with certainty.

Table 2. *S. homoeocarpa* VCG tester isolates used for candidate semi-selective medium validation

Isolate	VCG[w]	Host	Collection Site
A1421	A	*Agrostis palustris*	Michigan
A1426	A	*Agrostis palustris*	Michigan
A1442	B	*Agrostis palustris*	Michigan
A1612	C	*Agrostis palustris*	Michigan
A1619	D	*Agrostis palustris*	Michigan
A1623	F	*Agrostis palustris*	Michigan
A167	C	*Agrostis palustris*	Michigan
HE-10G-14	Unknown	Unknown	North Carolina
LWC40	Unknown	Unknown	North Carolina
ML715	E	*Agrostis palustris*	Michigan
ML729	D	*Agrostis palustris*	Michigan
RE-126-38	Unknown	Unknown	North Carolina
ML75	E	*Agrostis palustris*	Michigan

Notes: [w]VCG of Michigan isolates were determined by Viji and colleagues (2004). Three North Carolina isolates of *S. homoeocarpa* that did not fit into these VCGs were included to increase representation of *S. homoeocarpa* genetic diversity.

Table 3. Amendments added to experimental semi-selective media

Additive	Trade Name	Concentration	Media Abbreviations[w, x]
Streptomycin	N/A	50mg/L	N/A
Chloramphenicol	N/A	50mg/L	N/A
Tetracycline	N/A	50mg/L	N/A
Azoxystrobin	Heritage TL	5, 10, 20 ppm	A5, A10, A20
Pentachloronitrobenzene (pure AI)	N/A	5, 10, 20 ppm	PCNB5, PCNB10, PCNB20
Propiconazole	Banner Maxx	0.1, 1, 10 ppm	P01, P1, P10
Thiophanate methyl	Cleary's 3336	10, 100, 1000 ppm	TM10, TM100, TM1000
Triticonazole	Trinity	0.1, 1, 10 ppm	T01, T1, T10
Bromophenol blue	N/A	25mg/L	BB
Hydrochloric acid	N/A	to pH4 (~600µL/L)	pH4

Notes: [w] The standard medium PDA^{+++} was used as a base for all trial semi-selective media
[x] Media abbreviations given in this table and combinations thereof (for example, A5ph4) are consistent throughout the remainder of this text

Table 4. Relative growth of *S. homoeocarpa* and contaminant isolates on fungicide-amended media

Medium	Colony diameter (mm)				Growth relative to PDA^{+++} (%)			
	S. homoeocarpa mean (se)	Contaminant mean (se)	T[y]	P>T	*S. homoeocarpa* mean (se)	Contaminant mean (se)	T	P>T
A5	6.13 (1.09)[w]	7.12 (1.93)	0.43	0.67	17.92 (2.84)[x]	31.98 (9.71)	3.64	0.0005
A10	5.70 (1.17)	5.58 (1.44)	0.80	0.43	16.67 (3.15)	24.71 (6.73)	3.33	0.0012
A20	10.67 (1.29)	6.02 (1.40)	9.18	<0.0001	31.63 (4.25)	26.04 (7.82)	1.77	0.08
T0.1	20.48 (1.17)	19.72 (2.37)	0.41	0.69	60.42 (2.87)	71.15 (10.19)	1.94	0.06
T1	17.64 (0.94)	12.22 (3.30)	0.59	0.55	52.18 (3.26)	45.39 (9.95)	3.57	0.0006
T10	2.18 (0.87)	5.67 (1.67)	Non-est[z]	Non-est	6.49 (2.56)	20.76 (5.83)	Non-est	Non-est
P0.1	19.82 (1.14)	17.35 (2.09)	2.14	0.03	58.60 (3.83)	69.29 (11.14)	5.74	<0.0001
P1	13.34 (0.99)	7.61 (1.01)	9.76	0.21	43.00 (2.64)	34.56 (6.72)	2.06	0.12
P10	1.28 (1.02)	2.9 (1.08)	Non-est	Non-est	3.66 (2.89)	12.12 (4.67)	Non-est	Non-est
TM10	17.46 (7.97)	3.6 (1.60)	Non-est	Non-est	53.02 (24.59)	17.28 (8.14)	Non-est	Non-est

Notes: [w]Colony diameter is reported as the mean colony diameter (mm) of nine replicates for each isolate; (se) represents one standard error of the mean.
[x]Growth relative to PDA^{+++} is reported as % of growth on PDA^{+++} of nine replicates for each isolate; (se) represents one standard error of the mean.
[y]|T| and P values were obtained from orthogonal contrast statements comparing *S. homoeocarpa* isolate means to contaminant isolate means
[z]A number of tests were non-estimable due to large frequency of zero values

Table 5. Yellow color change of *S. homoeocarpa* and contaminant isolates on fungicide-amended media

	Color Change Rating			
Medium	*S. homoeocarpa* mean (se)	Contaminant mean (se)	T[x]	P>T
A5BB	0.50 (0.29)	0.04 (0.04)	15.51	<.0001
A10BB	0.14 (0.14)	0.43 (0.24)	4.10	<.0001
A20BB	0.19 (0.20)	0.54 (0.22)	3.73	0.0004

Notes: [w]Color change rating is represented as the mean rating for yellow pigmentation (scale of 0-4) of nine replicates for each isolate; (se) represents one standard error of the mean.
[x]T and P values were obtained from orthogonal contrast statements comparing *S. homoeocarpa* isolate means to contaminant isolate means

Table 6. Growth of *S. homoeocarpa* and contaminant isolates on pH-amended media

Medium	Colony diameter				Growth relative to PDA^{+++}			
	S. homoeocarpa mean (se)	Contaminant mean (se)	T[y]	P>T	*S. homoeocarpa* mean (se)	Contaminant mean (se)	T	P>T
PDA^{+++}pH4	41.14 (1.26)	17.82 (2.88)	33.38	<.0001	138.30 (7.74)	70.92 (11.81)	27.25	<.0001
A5pH4	16.61 (1.36)	6.55 (0.89)	21.66	<.0001	48.92(9.27)	26.08 (3.62)	16.84	<.0001
A10pH4	14.38 (1.32)	7.46 (1.52)	24.01	<.0001	42.26 (3.08)	25.09 (4.33)	18.33	<.0001
A20pH4	13.29 (1.52)	4.23 (0.64)	12.78	<.0001	39.09 (3.86)	16.74 (2.58)	9.52	<.0001
T0.1pH4	19.49 (0.82)	7.25 (1.03)	13.90	<.0001	57.53 (2.29)	29.08 (5.40)	7.33	<.0001
T1pH4	12.57 (1.97)	6.87 (0.84)	9.32	<.0001	35.84 (4.52)	27.51 (4.72)	1.79	0.08
P0.1pH4	19.82 (1.14)	17.35 (2.09)	9.76	<.0001	39.31 (2.55)	30.76 (5.64)	2.06	0.04
P1pH4	13.34 (0.99)	7.61 (1.01)	1.95	0.05	34.87 (4.47)	20.16 (4.53)	1.69	0.09

Notes: [w]Colony diameter is reported as the mean colony diameter (mm) of nine replicates for each isolate; (se) represents one standard error of the mean.

[x]Growth relative to PDA^{+++} is reported as % of growth on PDA^{+++} of nine replicates for each isolate; (se) represents one standard error of the mean.

[y]|T| and P values were obtained from orthogonal contrast statements comparing *S. homoeocarpa* isolate means to contaminant isolate means

Table 7. Growth of Wisconsin *S. homoeocarpa* and VCG tester isolates on candidate semi-selective media

	Colony diameter			
Medium	*S. homoeocarpa* mean (se)	VCG Tester mean (se)	$\|T\|^x$	P>|T|
PDA^{+++}	29.93 (1.36)	33.83 (1.75)	1.66	0.10
PDA^{+++}pH4	41.14 (1.26)	42.83 (0.72)	1.54	0.13
A5pH4	16.61 (1.36)	18.57 (0.80)	1.40	0.17
T0.1pH4	19.49 (0.82)	17.47 (1.01)	1.59	0.11

Notes: [w]Colony diameter is reported as the mean colony diameter (mm) of nine replicates for each isolate; (se) represents one standard error of the mean.

[x]|T| and P values were obtained from orthogonal contrast statements comparing *S. homoeocarpa* isolate means to contaminant isolate means

Table 8. Proportion of field samples positive for *S. homoeocarpa* on standard and candidate semi-selective media

	Sample Type					
	Symptomatic		Prior year symptomatic		Asymptomatic	
Medium	NSD	SD	NSD	SD	NSD	SD
PDA^{+++}	0.70[x]	0.00	0.05	0.00	0.05	0.00
PDA^{+++}pH4	0.90	0.10	0.00	0.10	0.05	0.00
A5pH4	0.80	0.20	0.05	0.00	0.15	0.00
T0.1pH4	0.70	0.20	0.00	0.00	0.05	0.00

Notes: [x]The proportion of field samples positive for *S. homoeocarpa* was calculated by dividing the number of positive cores over the total number of cores for that sample type. N=10, 20, and 20 for symptomatic, prior year symptomatic, and asymptomatic, respectively.

Table S1. *Sclerotinia homoeocarpa* and contaminant isolate growth relative to PDA^{+++} on all trial media

	Growth relative to PDA^{+++} (%)^{w} (se)											
	Isolate											
Medium	2F92-1	Heath1A	S10	OJN9	13NSF	24FR	24FS	4FS	5FSA	A404	PCU3A	R8-1
pH4	118 (0.9)^{x}	120 (1.7)	112 (1.3)	137 (0.9)	77 (5.6)	104 (2.7)	58 (1.2)	31 (1.2)	65 (4.3)	81 (2.8)	89 (1.1)	27 (2.5)
A5	26 (1.5)	23 (1.1)	18 (1.2)	20 (0.8)	10 (2.3)	20 (1.3)	36 (3.8)	64 (2.8)	40 (2.0)	4 (2.4)	10 (1.0)	3 (0.0)
A10	26 (1.7)	17 (1.0)	20 (1.1)	17 (0.8)	4 (1.0)	19 (1.2)	30 (2.2)	28 (1.2)	24 (1.7)	1 (0.7)	9 (0.8)	2 (0.4)
A20	22 (1.4)	6 (0.9)	17 (0.8)	18 (1.1)	10 (1.6)	19 (0.9)	25 (1.1)	25 (1.4)	31 (1.6)	5 (1.9)	8 (0 .9)	1 (0.3)
A5pH4	59 (1.2)	44 (1.3)	46 (1.9)	47 (1.7)	12 (2.0)	45 (1.4)	29 (0.7)	20 (3.0)	21 (3.4)	30 (1.4)	25 (0.6)	8 (0.3)
A5BB	48 (1.1)	32 (1.2)	32 (0.9)	47 (1.7)	12 (2.6)	40 (4.0)	14 (3.4)	34 (2.1)	59 (2.8)	35 (5.5)	3.5 (1.8)	11.8 (2.6)
A10pH4	51 (1.0)	41 (1.6)	39 (2.5)	38 (2.1)	10 (1.0)	46 (2.3)	23 (0.6)	23 (0.7)	19 (3.4)	37 (0.7)	18 (0.9)	6 (0.4)
A10BB	44 (6.8)	31 (6.0)	40 (5.1)	27 (5.3)	15 (1.5)	18 (4.5)	47 (11.9)	35 (2.9)	95 (10.8)	47 (6.5)	22 (4.0)	9 (2.3)
A20pH4	35 (2.0)	22 (0.5)	25 (1.7)	21 (1.2)	7 (0.8)	21 (0.9)	20 (0.8)	22 (1.0)	21 (1.7)	6 (1.6)	13 (0.8)	1 (0.3)
A20BB	54 (7.6)	31 (6.8)	33 (4.9)	27 (5.1)	10 (1.6)	19 (0.9)	52 (5.1)	50 (5.5)	85 (9.6)	37 (6.3)	25 (5.0)	6 (1.3)
T0.1	67 (2.6)	58 (4.5)	54 (2.9)	62 (4.8)	69 (20.2)	126 (18.8)	62 (5.4)	58 (13.0)	87 (2.6)	29 (4.6)	86 (18.5)	53 (12.4)
T1	53 (3.2)	43 (3.7)	56 (6.2)	54 (7.2)	76 (18.3)	50 (8.7)	54 (5.3)	28 (9.1)	57 (1.7)	7 (3.9)	69 (12.6)	5 (1.4)
T10	11 (5.6)	0 (0.0)	5 (2.5)	10 (3.3)	48 (18.7)	24 (7.0)	30 (8.3)	27 (16.0)	10 (4.7)	0 (0.0)	26 (9.6)	0 (0.0)
T0.1pH4	62 (3.0)	53 (3.9)	54 (1.3)	61 (3.6)	10 (1.2)	51 (2.2)	45 (1.6)	22 (1.1)	43 (2.0)	23 (2.7)	20 (1.3)	18 (1.3)
T1pH4	45 (1.9)	24 (1.6)	39 (1.4)	35 (2.8)	17 (1.0)	49 (2.7)	42 (1.5)	26 (2.1)	37 (1.0)	17 (1.9)	15 (1.4)	18 (2.3)
P0.1	62 (3.1)	54 (4.2)	51 (3.4)	68 (2.6)	66 (10.7)	123 (17.8)	71 (4.2)	79 (17.7)	88 (1.5)	28 (4.0)	58 (25.9)	28 (5.6)
P1	4.3 (2.7)	37 (5.8)	45 (3.1)	48 (3.6)	36 (12.6)	57 (7.1)	33 (3.9)	55 (11.7)	33 (8.2)	8 (2.5)	35 (5.2)	4 (1.9)
P10	1 (0.9)	0 (0.0)	2 (1.7)	0 (0.0)	0 (0.0)	4 (2.2)	19 (4.2)	36 (10.2)	10 (4.7)	0 (0.0)	9 (4.1)	0 (0.0)
P0.1pH4	45 (2.1)	33 (2.9)	41 (2.3)	38 (2.2)	13 (1.5)	57 (1.9)	46 (1.1)	35 (2.2)	39 (1.1)	18 (2.1)	19 (0.7)	18 (1.6)
P1pH4	40 (1.4)	21 (2.4)	39 (1.5)	29 (1.4)	7 (2.4)	35 (1.5)	32 (1.1)	30 (2.6)	28 (1.5)	17 (4.1)	12 (2.2)	1 (0.6)
PCNB5	0 (0.0)	54 (4.8)	0 (0.0)	4 (1.3)	---	---	---	---	---	---	---	---

Notes: ^{w}Growth relative to PDA^{+++} calculated by the equation: **[**[(Average colony diameter on trial media) / (Average colony diameter on PDA+++)] * 100% **].**

^{x}Growth relative to PDA^{+++} was calculated from nine replicates for each isolate; (se) is one standard error of the mean

Table S2. Yellow color production on bromophenol blue-amended media by *S. homoeocarpa* and contaminant isolates

	Color change rating (se)											
	Isolate											
Medium	2F92-1	Heath1A	OJN9	S10	13NSF	24FR	24SF	4FS	5FSA	A404	PCU3A	R8-1
A5BB	1.0 (0.0)	1.0 (0.0)	0.0 (0.0)	0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.33 (0.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
A10BB	0.0 (0.0)	0.6 (0.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.83 (0.4)	0.0 (0.0)	0.67 (0.5)	1.0 (0.9)	0.0 (0.0)	0.0 (0.0)
A20BB	0.0 (0.0)	0.78 (0.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.00 (0.0)	0.67 (0.5)	1.33 (1.0)	1.33 (1.0)	0.0 (0.0)	0.0 (0.0)

Notes: [w]Color change rating represents the average of nine replicates per isolate (0-3 rating scale); (se) is one standard error of the mean

Table S3. Colony diameter of Wisconsin and VCG tester isolates *S. homoeocarpa*

	Colony diameter (mm) (se)[w]		
	Medium		
Isolate	PDA^{+++}pH4	A5pH4	T01pH4
2F92-1	41.4 (0.3)	20.7 (0.4)	21.8 (1.0)
Heath1A	41.8 (0.6)	15.4 (0.4)	18.4 (1.4)
OJN9	43.7 (0.3)	15.0 (0.6)	19.4 (1.1)
S10	37.7 (0.4)	15.3 (0.6)	18.3 (0.4)
A1421	44.8 (0.4)	17.3 (1.2)	13.9 (1.2)
A1426	44.8 (0.4)	18.9 (1.4)	18.8 (1.6)
A1442	44.5 (0.4)	23.3 (2.4)	15.6 (1.0)
A1612	43.8 (0.9)	17.8 (2.3)	17.9 (1.5)
A1619	43.8 (0.8)	15.6 (1.3)	15.9 (1.5)
A1623	42.9 (1.2)	20.3 (1.5)	16.2 (1.2)
A167	44.3 (0.3)	21.9 (0.9)	16.2 (1.0)
HE10619	44.6 (0.5)	21.4 (1.4)	17.9 (1.2)
LWC46	40.6 (3.5)	19.8 (1.6)	15.9 (2.9)
ML715	37.4 (1.5)	17.6 (1.2)	15.5 (1.1)
ML729	45.2 (0.2)	16.4 (1.6)	27.7 (1.3)
ML75	41.2 (1.7)	13.2 (2.3)	15.7 (3.4)
RE12638	38.9 (2.0)	17.9 (0.4)	22.3 (0.4)

Notes: [w]Colony diameter represents the mean of nine replicates per isolate; (se) is one standard error of the mean

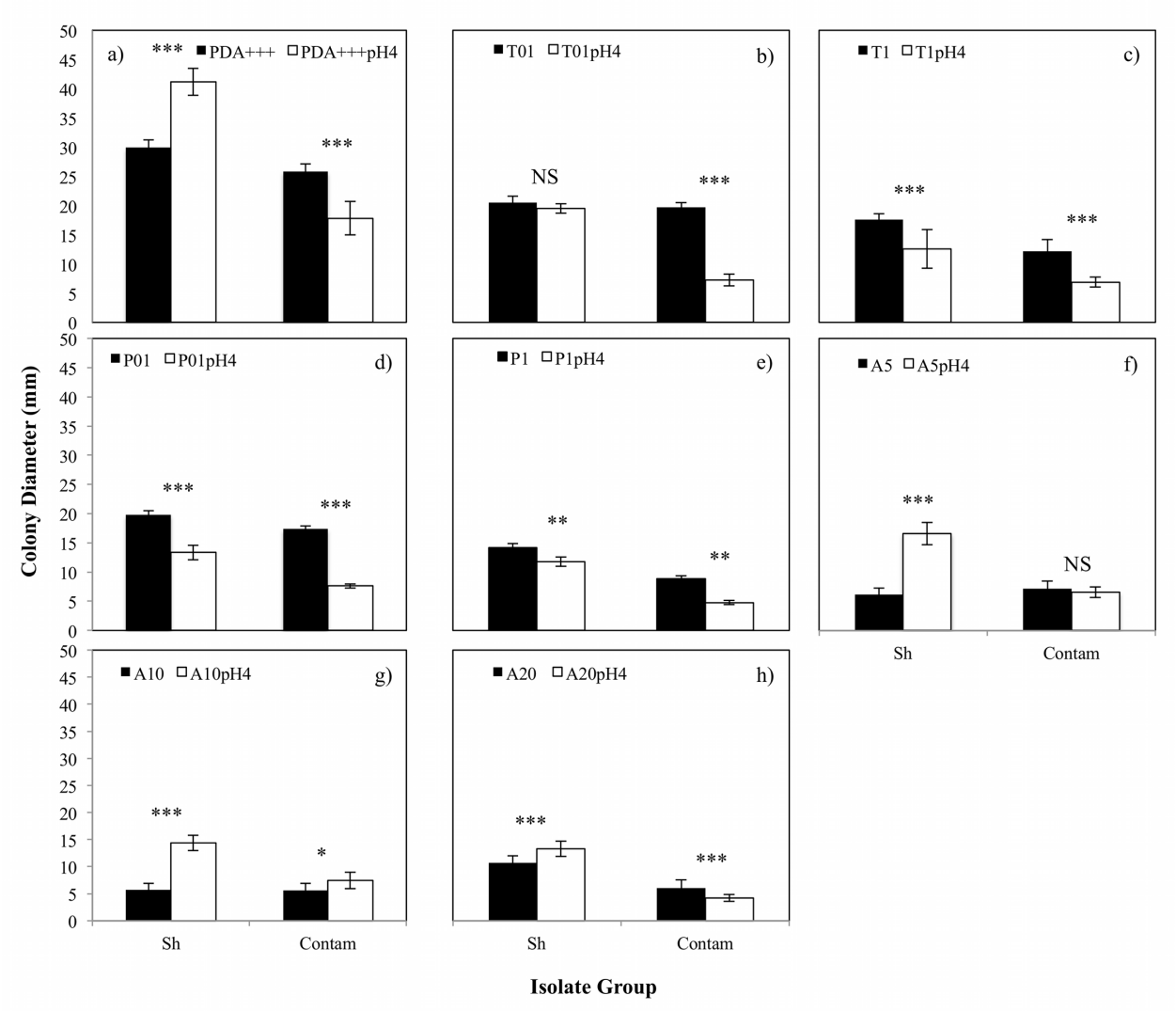

Figure. 1. Effect of medium pH-amendment on growth of *S. homoeocarpa* and contaminant isolates. Columns represent mean colony diameter (mm) of *S. homoeocarpa* and contaminant isolates with (black) and without (white) addition of HCl to medium for (a) PDA+++, (b) T01, (c) T1, (d) P01, (e) P1, (f) A5, (g) A10, and (h) A20, respectively. Errors bars represent one standard error of the mean. Two-sided independent t-tests were used to compare colony diameter between the two media and significance is indicated as follows: * *P<0.05;* ** *P<0.01;* and *** *P<0.001*.

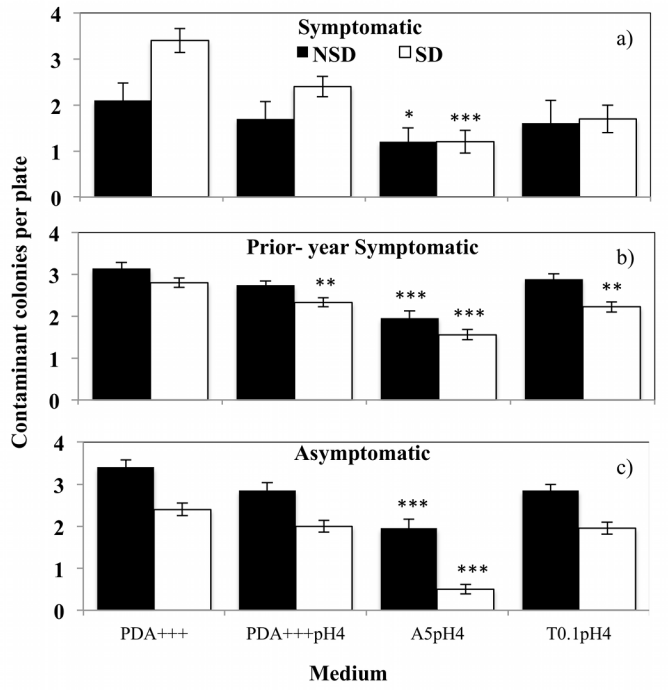

Figure. 2. Isolation of contaminant fungi from field samples on candidate semi-selective media. Each column represents the mean number of contaminant fungal colonies isolated per plate from (a) symptomatic (N=10), (b) prior-year symptomatic (N=20), and (c) asymptomatic field samples (N=20) either before (black) or after (white) surface disinfestation. Errors bars represent one standard error of the mean. Dunnett's test used to compare contaminant isolations between the control medium, PDA^{+++} the two media and each candidate semi-selective medium. Significance is indicated as follows: * *P<0.05;* ** *P<0.01;* and *** *P<0.001*.

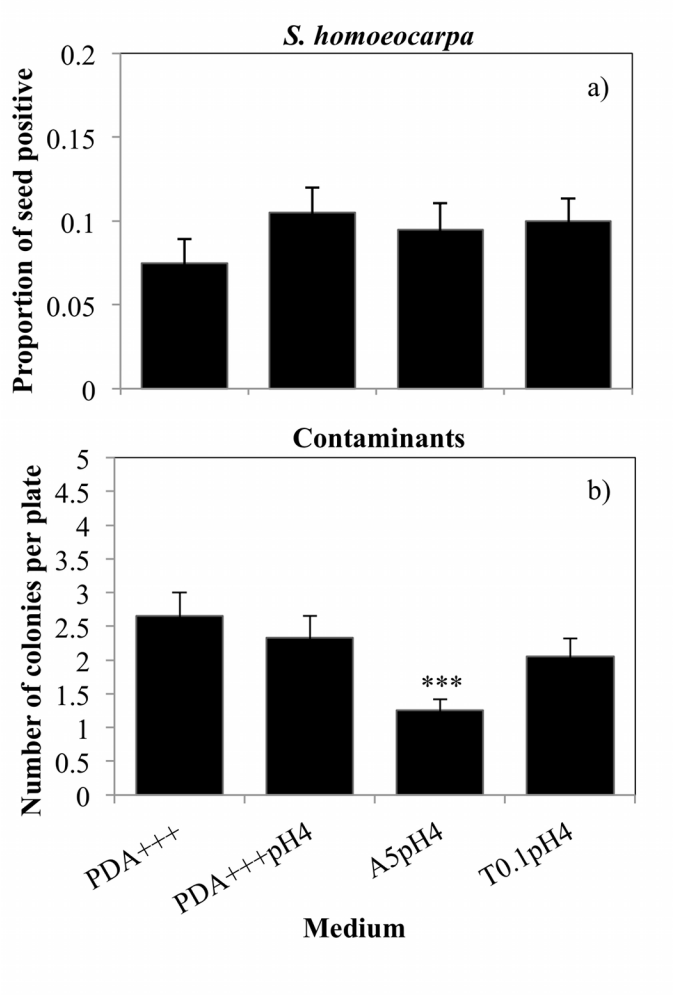

Figure. 3. Isolation of *S. homoeocarpa* and contaminant fungi from inoculated seed samples. (a), The mean proportion of seeds out of 100 positive for *S. homoeocarpa*. (b) The mean number of contaminant fungal colonies isolated per plate (N=10). Columns represent the mean of four replicated experiments treated as blocks in statistical analyses. Dunnett's test used to compare contaminant isolations between the control medium, PDA^{+++} the two media and each candidate semi-selective medium. Significance is indicated as follows: * *P<0.05;* ** *P<0.01;* and *** *P<0.001*.

CHAPTER 3: QUANTIFICATION OF *SCLEROTINIA HOMOEOCARPA* OVERWINTERING *IN PLANTA* AND DETECTION IN COMMERCIAL SEED

This chapter is a preliminary version of the following manuscript:

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Contribution: R.R. was the primary author. She designed, performed, and analyzed all experiments in conjunction with other authors and wrote the manuscript. M.G. and J. S. assisted with seed plating and molecular detection, respectively. D.K.W. assisted with design and implementation of Q-PCR experiments. S.B. contributed to experimental design and acquisition of materials. D.S. contributed to nested PCR experimental design. M.D.C. assisted with experimental design and data analysis. J.P.K. helped to design experiments and write the manuscript.

Abstract

Dollar spot is the most economically important disease of amenity turf grasses in the United States, yet little is known about the source of primary inoculum for this disease. With the exception of a few isolates from the United Kingdom, *Sclerotinia homoeocarpa*, the causal agent of dollar spot, does not produce spores. Consequently, it is assumed that overwintering of this organism in soil, thatch, and plant debris provides primary inoculum for dollar spot epidemics. Overwintering of *S. homoeocarpa* in roots and shoots of symptomatic and asymptomatic creeping bentgrass turfgrass was quantified over the course of a three-year field experiment. Roots did not consistently harbor *S. homoeocarpa*, whereas *S. homoeocarpa* was isolated from 30% of symptomatic shoots and 10% of asymptomatic shoots in the spring of two out of three years. The presence of stroma-like pathogen material on leaf blades was associated with an increase in *S. homoeocarpa* isolation and colony diameter 48 hpi . Commercial seed has also been hypothesized as a potential source of initial inoculum for *S. homoeocarpa*. In this research, two or more commercial seed lots of six creeping bentgrass cultivars were tested for contamination with *S. homoeocarpa* using culture-based and molecular detection methods. A viable, pathogenic isolate of *S. homoeocarpa* was isolated from one commercial seed lot and contamination of this lot was confirmed with nested PCR using *S. homoeocarpa* specific primers. Nested PCR detected the presence of *S. homoeocarpa* contamination in five additional seed lots. Seed source, but not cultivar or resistance to dollar spot, influenced contamination by *S. homoeocarpa*. Overall, this research reveals potential sources of initial inoculum for dollar spot epidemics and may lead to novel options for managing pathogen introduction.

Keywords: Dollar spot, *Sclerotinia homoeocarpa*, overwintering, primary inoculum

Introduction

Dollar spot, caused by the fungus *Sclerotinia homoeocarpa*, is the most important disease of turfgrass in North America and is the primary expenditure for disease management in turf throughout the world (2,56,58). Under intense disease pressure, fungicide application costs for dollar spot management may exceed $170/ha per year on high-value golf course greens and fairways (22). These high costs, coupled with concerns over the environmental impacts of fungicide use, and prevalence of fungicide resistance in *S. homoeocarpa* populations have necessitated the development of alternative methods for dollar spot management (58,59).

Various alternatives to chemical management for dollar spot have been attempted in golf course settings yet none have achieved acceptable control in the absence of chemical applications (8,34,37,58,59). Biological control with microorganisms possessing antimicrobial activity, including *Pseudmonas aureofaciens* and *Fusarium heterosporum*, or with hypovirulent, mitovirus-infected isolates of *S. homoeocarpa* showed moderate efficacy in the field (47,58,64). The performance of such products is too variable for adoption when there is little tolerance for disease in golf course settings. Nitrogen sources and application rates have also been evaluated for dollar spot management, yet inconsistent results and interference with increased ball roll distances demanded in golf course settings limit the practicality of nitrogen for cultural management of dollar spot (34,37,59). Mowing strategies, dew removal, and lightweight rolling, all of which can decrease leaf wetness duration, reduce dollar spot severity, but still must be used in combination with fungicide programs to achieve consistent disease suppression (14,19,61). Similarly, turfgrass cultivars with relative resistance to dollar spot are available but this resistance is not complete and must be used in conjunction with repeated fungicide applications (33).

None of the alternative dollar spot management methods described above target the source of initial inoculum for *S. homoeocarpa*. The initial descriptions of *S. homoeocarpa* by Bennett (5) reported three strain types for this fungus: Perfect, ascigerous, and nonsporiferous. Since that time, researchers have shown that only *S. homoeocarpa* isolates collected from fescues (*Festuca* sp.) in the United Kingdom are capable of producing conidia or fertile apothecia (4,5,31). Original strains of *S. homoeocarpa* from Australia and the United States examined by Bennett (5) were categorized as nonsporiferous and subsequent studies reported *S. homoeocarpa* isolates collected from outside of the United Kingdom produce apothecial initials but fail to mature and produce sexual ascospores (16,44). Consequently, vegetative mycelia and substratal stroma, which is formed by *S. homoeocarpa* under adverse conditions, are believed to be the primary means of survival for this pathogen (2,52).

The stroma formed by *S. homoeocarpa* consists of a thin, plate-like mat of melanized hyphal cells and lacks the structural features of true sclerotia (43,44). With the exception of *S. homoeocarpa*, this type of indeterminate stroma is formed by saprophytic fungi and has been termed a survival structure (43). There is only a single report associating the presence of stroma on leaf blades with overwintering of *S. homoeocarpa* (16). In this study, the pathogen was isolated from less than 10% of symptomatic leaf blades after a three-month winter incubation period, though a considerable number of these isolations were made from stromatized leaf blades (16). The limited frequency of *S. homoeocarpa* isolation in this study was surprising, given the polyetic nature of dollar spot epidemics and the aggregated distribution of dollar spot outbreaks each year (27). Additionally, the study was conducted in artificially constructed environments that may not reflect true overwintering capacity for *S. homoeocarpa* and was restricted to a single year (16). Since *in planta* overwintering is considered an important source of initial

inoculum for this pathogen, additional years of data and more in-depth investigation of *S. homoeocarpa* overwintering is warranted.

Dollar spot is ubiquitous in golf course settings and appears within a year of seeding, even following methyl bromide fumigation (9,21). The presence of dollar spot after methyl bromide application coupled with evidence that *S. homoeocarpa* is a poor soil saprophyte (Wilson, unpublished), suggests that this is not a soil-borne pathogen. Further, lack of spore production by *S. homoeocarpa* isolates originating outside of the United Kingdom indicates that long-distance dispersal via wind or rain is unlikely (16,44). Geostatistical analysis of dollar spot epidemics demonstrated limited spread of this pathogen within a season, indicating equipment does not contribute to spread of inoculum. Consequently, the authors concluded that epidemics initiated from individual clusters of infection centers (27).

A possible source of long-distance dissemination for *S. homoeocarpa* that corresponds with initiation of epidemics from aggregated areas in a turfgrass sward is introduction of the pathogen with turfgrass seed. In the United States, the vast majority of cool-season turfgrass seed production is in the Pacific Northwest (6,11). The isolated area of cool-season turfgrass seed production corresponds with reports of very limited genetic diversity in *S. homoeocarpa* isolates collected from cool-season grasses in the United States and Canada (28,46,50). This is in contrast to the greater genetic diversity of *S. homoeocarpa* isolates collected from warm-season grasses, which are vegetatively propagated and cultivated in many regions (6,38,49). Additionally, the finding that seeding rate of both Kentucky bluegrass (10) and fine fescue (9) is positively correlated with dollar spot severity supports the hypothesis that *S. homoeocarpa* can be dispersed by seed. Although evidence supports *S. homoeocarpa* dispersal on seed,

commercial seed has not previously been evaluated as a possible source of inoculum for *S. homoeocarpa*.

In the present study, both *in planta* pathogen overwintering and prevalence of *S. homoeocarpa* in commercial creeping bentgrass seed were evaluated. The goal of the study was to further elucidate the sources of primary inoculum for the dollar spot pathogen as a means to advance the understanding of dollar spot epidemiology. The specific objectives of this research were to determine: (1) the rate of pathogen overwintering in roots and shoots of symptomatic and asymptomatic creeping bentgrass turf; (2) the importance of stroma in *S. homoeocarpa* overwintering; and (3) the viability, pathogenicity, and prevalence of *S. homoeocarpa* on commercial creeping bentgrass seed.

Materials and methods

Study site and sample collection. The overwintering study was conducted on a United States Golf Association sand-based creeping bentgrass (*Agrostis stolonifera* L.; cv. 'Penncross') putting green maintained at a height of 0.40 cm and located at the O.J. Noer Turfgrass Research and Education Facility in Verona, Wisconsin. The same site was resampled in all three years of the study. In each year, late season dollar spot development permitted sampling from turf within and outside dollar spot infection centers. Fall sampling was conducted in December of all three years, after the ground had frozen but prior to lasting snow cover. Spring sampling was conducted in late-March to mid-April of each year, after the ground had thawed and snow cover had melted (Table 1). Samples were collected using a standard 2 cm diameter soil probe to a depth of approximately 8 cm to allow for collection of root tissue. In the first winter of the study, 20 samples were collected from both symptomatic and asymptomatic turf. Twenty-five samples were collected in the second and third years of the study. Symptomatic samples were collected

from the perimeter of the arbitrarily selected dollar spot infection center. Asymptomatic samples were collected from symptomless turf approximately 15 cm from the respective infection center; thus, here was a symptomatic and an asymptomatic sample collected for each infection center. Infection centers were marked with golf tees and were resampled in the spring to allow for direct comparisons of fall and spring isolation rates. All samples were collected in individual zip-lock plastic bags and stored at 4°C until plating.

Sample plating and *S. homoeocarpa* isolation. Intact soil cores were surface disinfested prior to plating by submerging in 10% Clorox (0.06% sodium hypochlorite) for 15 s and gently massaging the root area to remove attached sand. Disinfestation was immediately followed by two 30 s rinses in deionized water and samples were then dried on fresh paper towels. Sterile forceps were used to select and plate individual leaf blades and roots onto antibiotic-amended PDA (BD Difco, Sparks, MD; amended with 100mg/L each tetracycline, chloramphenicol, and streptomycin sulfate). For each sample, four leaf blades and four root pieces were placed on two replicate 50 mm diameter petri dishes (Fisher Scientific, Hanover Park, IL), resulting in a total of eight organ subsamples per sample. All petri dishes were inverted and incubated at ambient temperature (22±2°C) for 48 h.

From 48-96 h after plating, fungal colonies resembling *S. homoeocarpa* were marked and sub-cultured onto fresh antibiotic-amended PDA to obtain pure cultures. Colony morphology and sequencing of the ITS region were used to positively confirm *S. homoeocarpa* isolations. Fungal DNA was extracted from freshly harvested mycelia of *S. homoeocarpa* using a modified CTAB extraction protocol (51) and fungal ITS region-specific primers ITS1 and ITS2 (60) were used for amplification and sequencing of fungal DNA. DNA sequencing was performed at the University of Wisconsin-Madison DNA Sequencing Facility (Madison, WI).

Stroma plating experiments used a procedure similar to that described above. A dissecting scope was used to visually identify leaf blades with and without stroma-like material from samples previously identified as positive for *S. homoeocarpa* isolation in fall 2012 and spring 2013. Leaf blades were plated individually in 50 mm diameter petri dishes and *S. homoeocarpa* isolation and identification were repeated as above. Colony diameter measurements were taken at 48 h after plating and were calculated by averaging the lengths of two perpendicular transects across the colony. In total, 20 leaf blades and 20 leaf blades without stroma-like material were used in these studies. Sample size was constrained by the limited number of leaf blades on which stroma-like material was observed.

Seed sources. Samples from commercial CRB seed lots were obtained directly from seed production companies. CRB cultivars for seed assays were selected based on their resistance to dollar spot in National Turfgrass Evaluation Program trials from 2009-2011 (http://www.ntep.org; Table 2). A minimum of two seed lots per cultivar were tested for *S. homoeocarpa* contamination by both culture-based and molecular detection methods.

Culture-based detection. *Individual seed plating.* In an experimental replicate, a total of 1000 seeds from each seed lot were plated on two semi-selective media for *S. homoeocarpa* (antibiotic-amended PDA at pH4 with either 0.1ppm triticonazole or 25ppm azoxystrobin; Rioux et al., this thesis) in 100 mm diameter petri plates (Fisher Scientific, Hanover Park, IL). Twenty-five seeds were placed on each petri dish, resulting in a total of 20 plates of each medium and 40 total plates total per seed lot in each experimental replicate. A dissecting needle was used to select and plate individual seeds. The entire experiment was repeated three times; thus, an overall total of 3,000 seeds per lot were plated. For each experimental replicate, plates were sealed, inverted, and incubated at 22±2°C for 72-120 h. Fungal colonies resembling *S. homoeocarpa*

were marked and positively identified as described above for overwintering samples. To allow for identification of other fungal species commonly present in commercial CRB seed, the ten most common fungal colony morphotypes were noted and isolated in pure culture. DNA was extracted, using methods described above (67), from fresh mycelia of these cultures and the ITS region was sequenced with the fungal ITS-region primers ITS1/ITS2 (60).

Enrichment and dilution plating. Potato dextrose broth was prepared according to the manufacturer's instructions and amended with 100mg/L of the antibiotics streptomycin sulfate, chloramphenicol, and tetracycline and 25 ppm azoxystrobin (Heritage TL; Syngenta, Greensboro, NC). HCl was added to the medium to decrease the pH to four. Twenty mL of antibiotic and fungicide-amended PDB at pH4 and 0.25g of seed from each commercial seed lot were added to 12 sterile flasks. Flasks were sealed with a double layer of parafilm and incubated with constant shaking for 72 h at ambient temperature (22±2°C). After 72 h, the suspension was homogenized in a commercial blender and four 10-fold serial dilutions were made for each sample. Three 0.5mL aliquots of each dilution were plated onto separate azoxystrobin-amended PDA plates prepared as described above, except that PDA was substituted for PDB. All plates were incubated for 24-48 h. Potential *S. homoeocarpa* colonies were then marked and sub-cultured to obtain pure cultures, as previously described.

Pathogenicity assays with *S. homoeocarpa* isolates collected from seed were conducted as follows. Four-week-old CRB (cv. 'Penncross') plants were inoculated by placing two agar plugs, collected from the edge of four day-old fungal cultures, deep within the center of the turfgrass canopy. Mock-inoculated controls were included for comparison. All pots in the experiment were randomized in a plastic flat and covered with a humidity to dome to promote infection. Four-week-old barley (cv. 'Parkland') plants were inoculated using the parafilm sachet

method (Jia et al., 2013) and control plants were mock-inoculated with PDA plugs. Disease symptoms on both CRB and barley were photographed at 120 hpi. Both CRB and barley plants were cultivated in a growth room at 24±2°C with a 14 h day-length and inoculations were performed under these conditions.

Molecular detection. For nested and quantitative PCR, primer sets were designed to the outside and inside, respectively, of a previously described primer set specific to the *S. homoeocarpa* EF1- α gene (1) (Table 3; Fig. 4A). The NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/ tools/primer-blast/) was used to design primers specific to the *S. homoeocarpa* EF1-α DNA sequence (GenBank Accession DQ448301).

Seed DNA was extracted from ten 50 mg subsamples of each seed lot using the Qiagen DNeasy Plant Mini-Kit (Qiagen, Carlsbad, CA) according to the manufacturer's instructions except with slight modifications in sample preparation. Specifically, seeds were ground in Lysing Matrix A tubes (MP Biomedicals, Irvine, CA) containing two 0.64 cm diameter ceramic beads with a FastPrep-24 (MP Biomedicals, Irvine, CA). Samples were homogenized twice for 40 s at 6 m s^{-1} in Qiagen DNeasy kit buffer AP1. RNAse A was added to samples after homogenization and no further adjustments to the manufacturers protocol were made. DNA quantity and quality were assessed with an ND-1000 Spectrophotomer (NanoDrop Technologies, Wilmington, DE).

Both primary and nested PCR reactions were performed in 25 µL reaction volumes with GoTaq Colorless PCR Master Mix (Promega, Madison, WI), following the manufacturers protocol. Primary reactions contained: 12.5 µL GoTaq Colorless PCR Master Mix, 2 µL seed DNA, 0.2 µM of primers EF1α_outerF and EF1α_outerR, and nuclease free water (NFW).

Nested reaction mixtures were identical, with the exceptions that 2 µL of primary PCR products diluted 1:30 with NFW were used as template and the EF1α primer set was used. Cycling conditions for primary and nested PCR included an initial two minute denaturation period at 95°C; 30 cycles of 30 s denaturation at 95°C, 30 s annealing at 56°C and 52°C for primary and nested PCR, respectively, and 30 s elongation at 72°C; and a final five minute elongation period. A positive control of seed DNA spiked with *S. homoeocarpa* genomic DNA and a no-template negative control were included in all runs and PCR was performed in a MasterCycle Pro S (Eppendorf, Hamburg, Germany). Five µL of all primary and nested PCR products were mixed with 1 µL of 6X DNA loading dye and subject to electrophoresis in a 1% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA). Gels were visualized with UV light in a Universal Hood II Gel Doc System (BioRad, Hercules, CA) and analyzed with Quantity One imaging software (BioRad, Hercules, CA). Positive bands were excised from the gel with a sterile razor blade and cleaned up with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) then sequenced at the University of Wisconsin-Madison DNA Sequencing facility (Madison, WI). NCBI BLASTn was used to confirm sequences were specific to the *S. homoeocarpa* EF1- α gene sequence (3).

Quantitative PCR (qPCR) reactions were performed in hard-shell 96-well skirted PCR plates and sealed with Microseal 'B' adhesive seals (BioRad, Hercules, CA). qPCR reactions contained 1 X SsoFast EvaGreen Supermix, 8 µL template (seed DNA or EF1- α plasmid DNA standard), 0.2 µM EF1α_nestF and EF1α_nestR, and NFW for a final reaction volume of 20 µL. A standard curve with concentrations of EF1- α plasmid DNA ranging from 10^6 to one copy/ µL was included on each plate and all DNA samples were assayed in triplicate. Cycling conditions included an initial 2 minute denaturation at 98°C, followed by 40 cycles of 98°C for 2 s and

annealing at 55°C for 2 s. A melt curve was created after the final cycle by increasing the temperature from 70-90°C in 0.2°C intervals and holding at each temperature for 10 s. qPCR reactions were performed in a CFX-96 Real-Time System (Bio-Rad, Hercules, CA).

Statistical analysis. Statistical analyses were performed using the generalized linear mixed model procedure (PROC GLIMMIX) in SAS version 9.3 (29). Studentized residuals and tests in PROC UNIVARIATE were used to assess normality and determine the best fitting distribution for each data set. Full models with all random effects and their interactions with fixed effects were then fit to the appropriate distribution and model fitting criteria (AIC of -2RLL, depending on distribution) were used to select the best fitting model.

Overwintering data was coded as a binary variable (0=no *S. homoeocarpa* detected; 1=*S. homoeocarpa* detected) and subject to analysis of variance using the binary distribution. Sample (block) was considered a random effect and all other factors (treatment, organ, and season) were fixed. The full data set was analyzed to test for a season effect. Individual years were then analyzed independently and factors (season or treatment) were sliced to compare specific levels of each factor. Stroma isolation data were also subject to analysis of variance under the binary distribution. Stroma colony diameter data were analyzed using the normal distribution. In both cases, a simple one-way ANOVA was used to test for the main effect of the presence or absence of stroma.

Nested PCR seed detection data were subject to analysis of variance under a normal distribution. Because lots were exclusive to the cultivar from which they came and cultivars were exclusive to the source from which they came, both of these factors were treated as nested in statistical analyses. Source and cultivar(source) were considered fixed effects while lot(cultivar)

was treated as a random effect. Fixed treatment effects were assessed with a simple two-way ANOVA and a single degree of freedom orthogonal contrast was used to test for a difference between resistant and susceptible cultivars.

Results

Pathogen overwintering. In all three years of this study, fall isolation from symptomatic shoots was approximately 70%. (Fig. 1). *S. homoeocarpa* was isolated from asymptomatic shoots in the fall of all years, but the percentage of positive samples varied, with 15%, 32%, and 12% of samples positive for *S. homoeocarpa* in 2010, 2011, and 2012, respectively (Fig. 1). The percentage of root samples positive for *S. homoeocarpa* was 15% in fall 2010 but negligible in all other seasons. Consequently, roots were not included in statistical analyses of *S. homoeocarpa* overwintering.

The majority of spring isolations of *S. homoeocarpa* were made from samples taken from areas with dollar spot symptoms the previous fall (Fig. 1). The rate of *S. homoeocarpa* spring isolation from symptomatic tissue ranged from 0.05% in spring of 2010 to 40% in spring of 2011. In two of three years of this study, spring isolations of *S. homoeocarpa* were also made from tissue collected from asymptomatic areas in the previous fall (Fig. 1). *S. homoeocarpa* was isolated from 8 and 12% of asymptomatic samples in the spring of 2012 and 2013, respectively. In winter 2012-2013, there was no difference in the percentage of samples positive for *S. homoeocarpa* in the fall and spring. A single spring isolation of *S. homoeocarpa* was made from roots in spring 2011. *S. homoeocarpa* was not recovered from roots at any other spring sampling date.

Across all years, season had an effect on *S. homoeocarpa* isolation from both symptomatic (*P<0.001*) and asymptomatic (*P=0.03*) samples. In individual years, a significant effect of season across sample types (symptomatic or asymptomatic) was detected only in Year 2 (Fig. 1). By separating sample types, an effect of season was detected in symptomatic samples for all three years (*P<0.05*) and for asymptomatic samples in Year 2 (*P=0.03*). Sample type had an effect on *S. homoeocarpa* isolation in the fall of each year (*P<0.05*), but had no effect on spring isolations (*P>0.1*) in any year. No season by sample type interaction was detected in any year of the study (*P>0.1*).

Stroma. Leaf blades with black to brown stroma-like material on or within epidermal tissue were readily identified with a dissecting scope and were selected as potentially harboring *S. homoeocarpa* stroma (Fig. 2A). Leaf blades serving as non-stroma controls were green to yellow in color and did not appear to contain fungal material. *S. homoeocarpa* was isolated from leaf blades with and without stroma,yet the presence of stroma-like material affected the rate of isolation (*P=0.03*; Fig. 2B) and growth rate (*P=0.0002*; Fig. 2C) of *S. homoeocarpa*.

Culture-based seed detection. *Individual seed plating*. A single isolate of *S. homoeocarpa* was collected from commercial seed with the culture-based detection method (Table 4). The isolate was collected from Lot 1 of cv. 'Shark.' using triticonazole–amended medium. Colony morphology (Fig. 3A) and amplification with *S. homoeocarpa*-specific primers confirmed the identification of this isolate. Tests on CRB and barley demonstrated pathogenicity and resulting symptoms were similar to those observed with a virulent isolate of *S. homoeocarpa* (Fig. 3B-C). Specifically, symptoms on CRB included water-soaking around the site of inoculation, accompanied with reddish brown lesions on individual leaf blades. Light-tan lesions with reddish brown borders, characteristic of *S. homoeocarpa* infection, developed on barley.

Overall, 16 different fungal colony morphotypes were frequently identified during seed plating. The most commonly isolated seed contaminant was a pink yeast in the genus *Rhodotorula*. The fungus *Epicoccum nigrum* was the second most commonly isolated seed contaminant. Other frequently isolated fungi included *Trichoderma* sp., *Penicillium polonicum*, *Mucor fragilis*, and *Aureobasidium proteae*. Lack of morphological characteristics needed for identification, inability to extract quality DNA, or poor sequencing results made it difficult to identify many of the fungi isolated.

Enrichment and dilution plating. After 24 h of incubation, plates from the enrichment experiment were overrun with Zygomycete species; consequently, no *S. homoeocarpa* colonies were identified by this method.

Molecular seed detection. *Nested PCR*. Nested PCR using the primary primer set EF1α_Outer and nested primer set EF1α was able to detect a single *S. homoeocarpa*-infested seed in 50 mg of seed (Fig. 4). A minimum of two seed lots per CRB cultivar were tested for *S. homoeocarpa* contamination with nested PCR. Eight of the twelve seed lots tested were positive for *S. homoeocarpa* DNA (Table 4), including both seed lots from cultivars 'Penncross' and 'Shark'. The highest contamination was present in 'Shark' Lot 1, from which *S. homoeocarpa* was isolated using the culture-based detection method. Three additional 'Shark' seed lots were tested for *S. homoeocarpa* contamination and two were positive (Table 4).

Seed source affected detection of *S. homoeocarpa* (*P=0.01*) based on ANOVA of nested PCR data. CRB cultivar did not affect detection (*P>0.05*). Cultivars were nested within seed source; thus, no source by cultivar interaction was tested. An orthogonal contrast between cultivars with high and low dollar spot resistance, according to NTEP creeping bentgrass variety

trials, indicated that dollar spot resistance did not influence detection levels for *S. homoeocarpa* (*P>0.05*; Fig. 5).

Quantitative PCR. The primer set EF1α_NestF/ EF1α_NestR had >99% efficiency in standard curves constructed from plasmid DNA and a detection limit as low as 10 target copies/μL template (Fig. 6A). Six seed lots positive for *S. homoeocarpa* by nested PCR were also tested by qPCR. Five of the six lots, including Shark Lot 1, tested negative for *S. homoeocarpa* contamination by qPCR (Table 4). The low success rate of qPCR indicated the presence of PCR inhibitors in seed DNA samples. To test this, seed DNA samples that had tested positive by nested PCR were diluted with NFW and used as template in qPCR or spiked with known amounts of plasmid DNA. The detection threshold value decreased with sample dilution factor and spiked samples had much higher threshold values than for the same target concentration in standards, confirming the presence of PCR inhibitors in seed DNA samples (Fig. 6B).

Discussion

This is the first multi-year study on *in planta* overwintering of *S. homoeocarpa* and the first evidence that commercial seed may serve as an important source of initial inoculum for dollar spot epidemics. Previously, the only study on *S. homoeocarpa* overwintering had been conducted on Kentucky bluegrass (*Poa pratensis*) at the University of Rhode Island in the winter of 1973-1974 (16). In that study, *S. homoeocarpa* was recovered from less than 10% of symptomatic leaf blades and never from asymptomatic leaf blades (16). In the present research, spring recovery of *S. homoeocarpa* ranged from 5-40% and 0-12% in symptomatic and asymptomatic samples, respectively. The authors of that previous study (16) noted the presence of numerous saprophytic fungi in their samples and degradation of leaf blades by fungal contaminants, which may help to explain the higher rates of *S. homoeocarpa* isolate in our study.

While saprophytes were frequently recovered in our study they did not appear to significantly impair *S. homoeocarpa* isolation or contribute to host tissue degradation.

The rate of fall isolation from symptomatic shoots was similar in all three years of our study, but spring isolation rates varied, indicating that environmental factors may influence winter survival of *S. homoeocarpa*. Consequently, average daily temperature and precipitation data were collected and compared for the three years of our study (Fig. 1B,D,F). *S. homoeocarpa* survival rates were highest in the second year of the study, during which average winter temperatures were approximately 4°C higher than in years one and three. The warmer winter temperatures in 2011-2012 may have promoted overwintering of *S. homoeocarpa* when compared to the other two years of this study. Warmer winter temperatures are also associated with increased survival of the turfgrass stem rust pathogen, *Puccinia graminis* subsp. *graminis* on tall fescue and perennial ryegrass (45). Spring isolations of *S. homoeocarpa* were higher in the third year of our study than in the first year, despite similar average winter temperatures in both years. A snowfall event in late January of 2013 may have insulated *S. homoeocarpa* from fluctuating air temperatures, resulting in a greater number of spring isolations in year three. Direct comparisons of these studies to prior research are difficult because most research on fungal pathogen overwintering focuses on survival of spores and often involves artificial manipulations of the pathogen itself or of infected host material (24,26,62). However, a few studies have demonstrated the ability of fungal pathogens to survive harsh winter conditions in host debris that serves as primary inoculum in the following spring (18,23,63).

Increased isolation of *S. homoeocarpa* from leaf blades with stroma in our study agrees with the findings of Fenstermacher (16) on Kentucky bluegrass and supports the general conclusion that stroma is a survival structure for this pathogen (5,52,58). Stroma was not

observed on creeping bentgrass roots and isolations of *S. homoeocarpa* from roots were limited, indicating that overwintering in roots does not contribute to primary inoculum for dollar spot epidemics. To our knowledge, this is the first report that stroma influences overwintering of *S. homoeocarpa* in creeping bentgrass. The abnormal nature of *S. homoeocarpa* stroma and the fact that this survival structure is generally restricted to saprophytic fungi warrant further investigation of the role of this structure in *S. homoeocarpa* biology and epidemiology (43). Potentially, control methods aimed at the decreasing the formation of stroma in the fall could decrease overwintering of *S. homoeocarpa* and reduce initial inoculum for dollar spot epidemics.

The isolation of *S. homoeocarpa* from asymptomatic samples in the spring of 2011-2012 and 2012-2013 was unexpected. Since diseased tissue from the previous year's epidemic was considered the source of inoculum for subsequent years (12,52,58), detection of this pathogen in asymptomatic material indicates that latent infections may be an important source of overwintering and inoculum. Isolation of *S. homoeocarpa* from asymptomatic turfgrass also relates directly to the detection of *S. homoeocarpa* in commercial creeping bentgrass seed. In the Pacific Northwest, where the majority of cool-season turfgrass seed is produced, environmental conditions are generally not suitable for dollar spot epidemics (53). Asymptomatic infection with *S. homoeocarpa* may allow contamination of seed fields to go unnoticed; thus, allowing for harvesting and distribution of seed that appears to be pathogen free yet is contaminated with *S. homoeocarpa* through latent or asymptomatic infections. Symptomless infection arising from seeds has previously been described for *Botrytis cinerea* in lettuce (54). The role of asymptomatic colonization of turfgrasses by *S. homoeocarpa* should be investigated further.

To our knowledge, this is the first report of a viable, pathogenic isolate of *S. homoeocarpa* obtained from commercial seed of any turfgrass species. A single isolation from

the 36,000 total seeds plated in this study may seem trivial, yet a kilogram of CRB seed contains two to four million individual seeds (15). This translates to approximately 70 *S. homoeocarpa*-infected seeds per kilogram of creeping bentgrass seed or a minimum of thousands of infected seeds on a freshly seeded golf course putting green at a standard seeding rates (15). In the present study, we only detected the presence of *S. homoeocarpa* in commercial seed and did not relate this to establishment of dollar spot epidemics in the field. Consequently, the influence of seed contamination on dollar spot development in the field is needed.

The detection of *S. homoeocarpa* in seed corresponds well to numerous observations on dollar spot epidemiology and population structure. For example, it is common for natural inoculum to be used for initiation of dollar spot epidemics in the season following methyl bromide fumigation and reseeding of turf (9,21). Since movement of *S. homoeocarpa* inoculum by humans or equipment is negligible (27), introduction on seed provides a practical explanation for both the aggregated fashion of dollar spot epidemics and rapid establishment of this disease in newly seeded areas. Introduction of *S. homoeocarpa* on seed also agrees with the observation that seeding rate is positively correlated with dollar spot severity in the field over multiple years (9,10). The authors of these studies hypothesized that this correlation may have been due to higher plant density resulting in lower plant health. However, Bonos (7) demonstrated that higher plant density is actually associated with increased dollar spot resistance . It is plausible that increased seeding rate also increases the number of *S. homoeocarpa*-infected seeds introduced to a newly seeded area, resulting in higher inoculum levels and disease severity.

The limited genetic diversity of *S. homoeocarpa* isolates collected from cool-season, as opposed to warm-season grasses, lends further support to the possibility that dispersal on seed is an important aspect of dollar spot epidemiology (28,49,50,57). In particular, Raina and

colleagues (50) were unable to detect any genetic difference between Oregon and Rhode Island isolates of *S. homoeocarpa* by RAPD analysis. Similarly, isolates from the same *S. homoeocarpa* genotype appeared to cause dollar spot epidemics in multiple regions of the United States and Canada (57). Both of these findings indicate that long-distance dispersal of *S. homoeocarpa* occurs from a limited number of point sources, which corresponds well with spread of this pathogen through seed.

In our study, eight of twelve (75%) of commercial seed lots tested positive for *S. homoeocarpa* with nested PCR. In these lots, the number of positive subsamples ranged from 1-5. Nested PCR gene was more effective than qPCR at detecting seed lot contamination and could detect a single *S. homoeocarpa* infested seed in a 50 mg sample, approximately 1 in 450 creeping seeds (0.002% infestation) (1) . This detection rate is higher than the 0.01-0.1% detection rates reported for other molecular detection methods (13,39,48), though not as high as that of some qPCR detection methods (30,40). Though qPCR detection methods are popular at present, regular and nested PCR-based detection methods are still highly effective and provide an efficient means of qualitative pathogen detection (17,20,39,55). In the future, the nested PCR method could lead to development of a loop-mediated isothermal amplification (LAMP) assay for detection of *S. homoeocarpa* in seed. LAMP assays are less sensitive to PCR inhibitors than conventional and real-time PCR methods and can be performed in the field with minimal equipment or training (25,32,41,42).

Analysis of data from nested PCR indicated that seed source but not cultivar resistance to dollar spot had an effect on contamination with *S. homoeocarpa*. This was somewhat surprising as an influence of host cultivar resistance on seed contamination has previously been demonstrated for other cereal pathogens (35,36), however, the sample size of two lots from each

of six cultivars limited our power to detect significant differences between cultivars. In the future, it will be necessary to screen more seed lots for *S. homoeocarpa* contamination. The effect of source on seed lot contamination may be due to a number of factors. It is possible that previous field history or environmental conditions differ between production fields used by individual seed companies. Climatic conditions in the Pacific northwest, where the majority of creeping bentgrass seed is grown, are fairly stable and unlikely to vary drastically from one company's fields to the next (6). It is possible, however, that certain fields or areas within a field have microclimates conducive to plant infection by *S. homoeocarpa*. Alternatively, management or harvest practices may vary between companies and contribute to the differences observed. It is unclear whether *S. homoeocarpa* is harbored on or in the seed itself or associated with debris mixed in with the seed. If the latter is the case, seed with minimal debris may also have lower rates of *S. homoeocarpa* contamination. Future experiments to determine the source of *S. homoeocarpa* in commercial seed will help in identifying which practices, if any, promote or limit contamination of seed with this pathogen.

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Table 1. Sampling dates for *S. homoeocarpa* overwintering sample collections.

Year	Season	Sampling Date	No. of Samples
2010-2011	Fall	12/1/2010	20
2010-2011	Spring	3/24/2011	20
2011-2012	Fall	12/7/2011	25
2011-2012	Spring	3/29/2012	25
2012-2013	Fall	12/17/2012	25
2012-2013	Spring	4/16/2013	25

Table 2. Seed sources, cultivars, and National Turf Evaluation Program performance results for creeping bentgrass commercial seed lots used in *S. homoeocarpa* culture-based and molecular seed detection studies

Source	Cultivar	NTEP Rating[2]	Resistance Designation
Seed Research of Oregon	96-2	4.6[3]	VS
Mountain View Seed	Shark	5.3	MS
Tee-2-Green	Penncross	5.5	MS
Seed Research of Oregon	SR1150	6.3	R
Lebanon Turf	Declaration	7.4	VR
Seed Research of Oregon	Focus[1]	7.9	VR

[1]The experimental name for cv. 'Focus' in NTEP trials was 'GMC comp'
[2]NTEP ratings provided are the average from dollar spot trials in 2009, 2010, and 2010 that included all six cultivars
[3]Ratings are based on turfgrass quality and are given on a 0-9 scale; 9= highest turf quality/resistance to dollar spot and 0=lowest turf quality/resistance to dollar spot

Table 3. Primers used for molecular detection of *S. homoeocarpa* in creeping bentgrass commercial seed lots

Primer Name	Sequence	Prod. Length (bp)	Use	Reference
EF1α_F	CGGTATGACTTCTCCACCTTTC	219	Nested PCR	Al-Elmagid et al. 2013
EF1α_R	GAACCCTTTCCCATCTCCTT	219	Nested PCR	Al-Elmagid et al. 2013
EF1α_Outer_F	CGGTAAGCAGAACCCTCGAC	554	Primary PCR	This paper[1]
EF1α_Outer_R	CAGCTTGGGAGGTACCAGTG	554	Primary PCR	This paper
EF1α_Nest_F	TTATCGGGTTGCGTTTTCTC	102	Q-PCR	This paper
EF1α_Nest_R	AACGGGTTAGCAAAGGGATT	102	Q-PCR	This paper

[1]Primers developed in the present research were designed using the NCBI Primer-BLAST tool with the DNA sequence for the *S. homoeocarpa* EF1- α input as the sole target sequence. Primer specificity was checked by sequencing of primary and nested PCR products and qPCR melt curve analysis

Table 4. Results for *S. homoeocarpa* contamination of creeping bentgrass commercial seed lots by culture-based and molecular detection methods

Cultivar	Lot[1]	Culture-based Detection[2]	Nested PCR[3] (Subsamples +)	Q-PCR (Subsamples +)
96-2	1	-	+ (1)	n/a
	2	-	- (0)	n/a
Shark	1	+	+ (5)	- (0)
	2	-	+ (3)	- (0)
Penncross	1	-	+ (1)	- (0)
	2	-	+ (2)	n/a
SR1150	1	-	- (0)	- (0)
	2	-	- (0)	n/a
Declaration	1	-	+ (2)	n/a
	2	-	- (0)	n/a
Focus	1	-	+ (1)	- (0)
	2	-	+ (1)	n/a

[1]Two representative lots of each cultivar were selected by for detection of *S. homoeocarpa* contamination by each method; additional lots of 'Shark' and 'Penncross' seed were tested by the nested PCR detection method only

[2]Three 1,000 seed replicates for all lots were performed by plating 500 seeds from each lot onto two different media semi-selective for *S. homoecarpa* (1,000 seeds total) and repeating three times (3,000 seeds/lot total)

[3]Nested PCR was repeated twice for each seed lot; numbers shown in parentheses are the number of subsamples testing positive for *S. homoeocarpa* contamination in each run

[4]Q-PCR detection was ceased after it was determined that PCR inhibitors in seed DNA samples rendered the assay ineffective

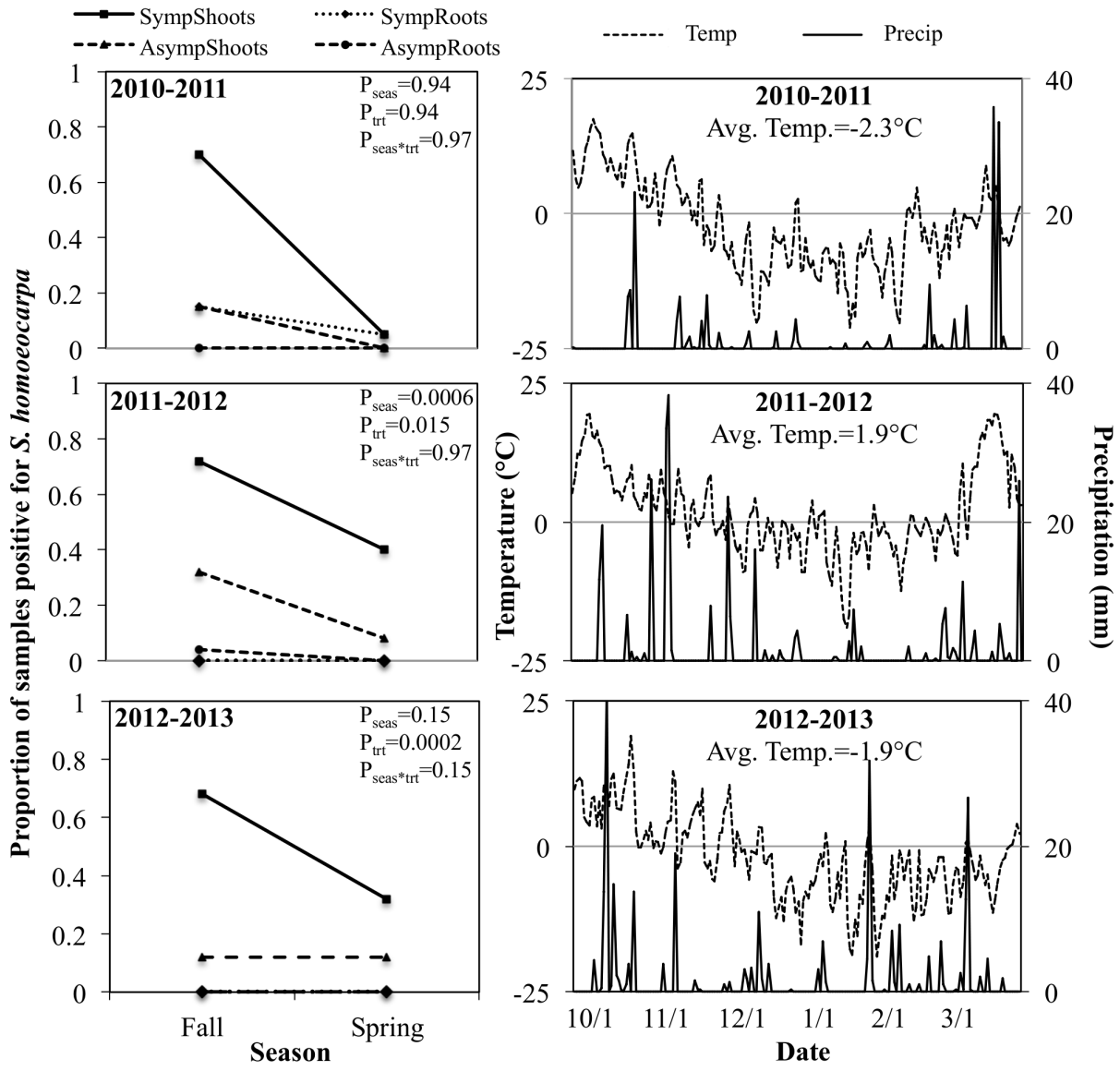

Figure. 1 *Sclerotinia homoeocarpa* isolation and weather data for pathogen overwintering studies. Roots and shoots were collected from symptomatic and and asymptomatic turf in the fall of each year to determine starting *S. homoeocarpa* populations. The same areas were resampled in the spring to assess pathogen survival. ANOVA values for season, treatment, and season by treatment effects are reported for each year. Weather data includes daily air temperature (dashed line) and precipitation (solid line) averages. The average temperature in 2011-2012 was c. 4°C higher than in the other two years and a major snow events occurred in early November 2011-2012 and early February of 2012-2013 (arrows).

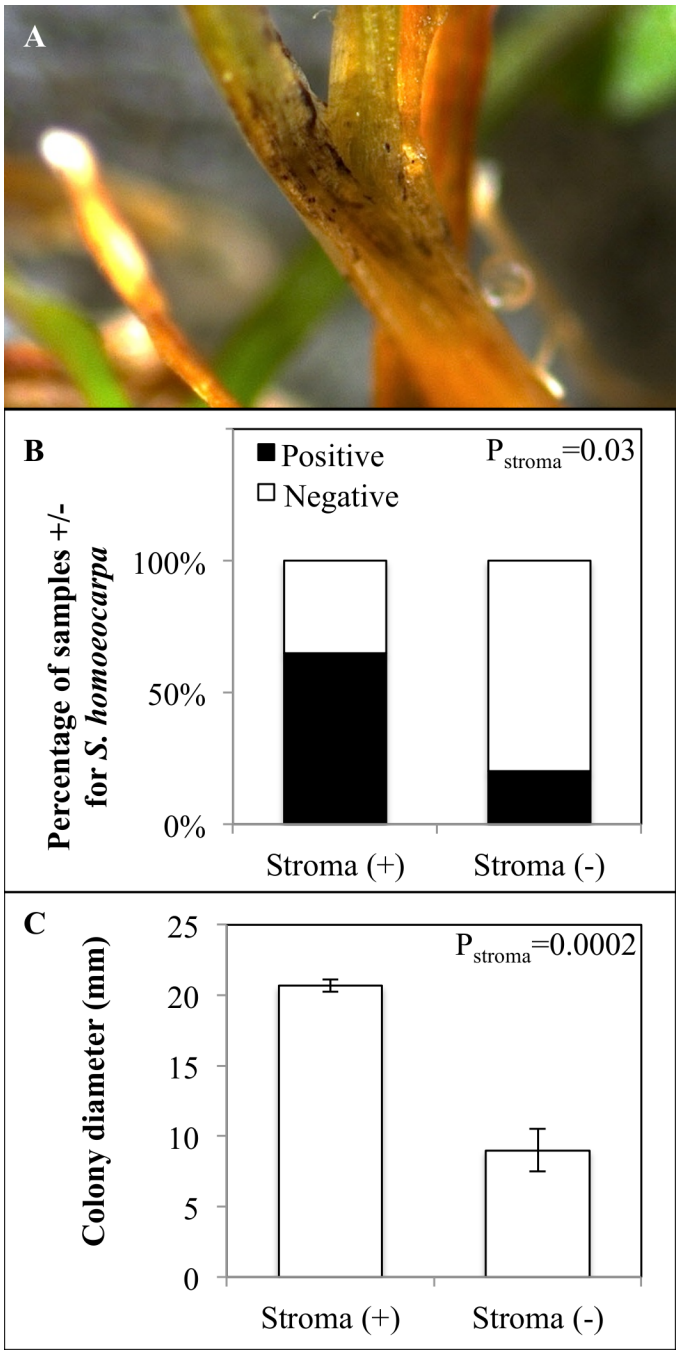

Figure. 2 Stroma on creeping bentgrass leaf blades and its effect on isolation on *S. homoeocarpa*. A, typical stroma-like material observed on a turfgrass leaf blade was brown in color and appeared as flaky, plate-like material. **B,** The percentage of CRB leaf blades with and without visible stroma from which *S. homoeocarpa* was isolated. P-value from ANOVA using the binary distribution for presence/absence of *S. homoeocarpa* and α=0.05. **C,** Average diameter of *S. homoeocarpa* colonies 48 h after plating leaf blades with or without visible stroma. P-value is from ANOVA with the normal distribution and α=0.05. Error bars represent ± one standard error of the mean (n=5).

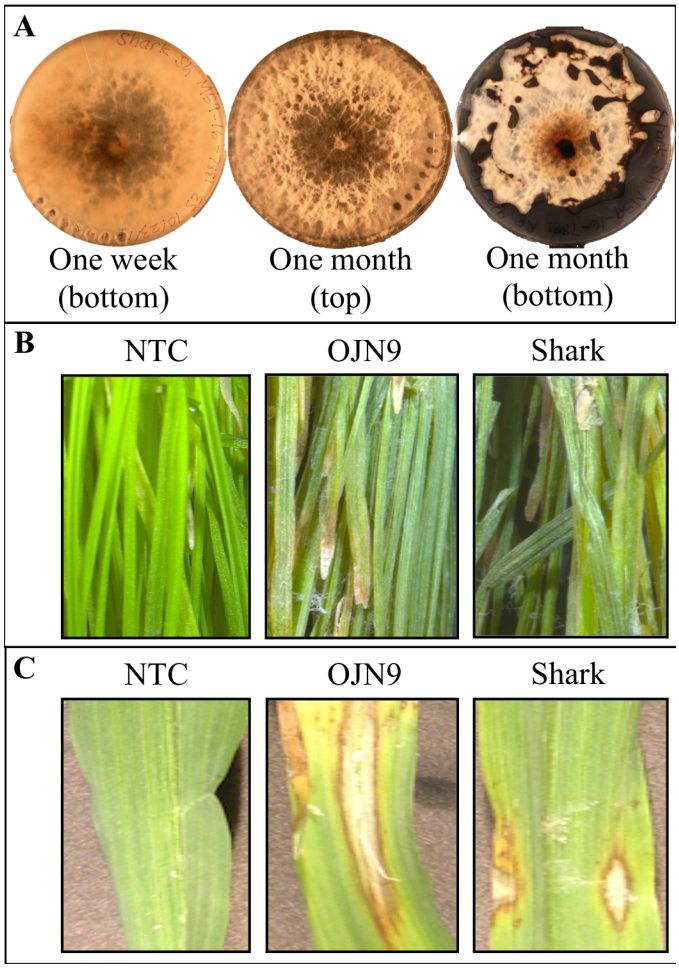

Figure. 3 *Sclerotinia homoeocarpa* isolate 'Shark' obtained from Shark Lot 1 using culture-based detection of semi-selective medium. A, Colony morphology of the isolate 'Shark' obtained from CRB cv. 'Shark' commercial seed lot 1. **B,** Symptoms produced by mock-inoculated control, virulent *S. homoeocarpa*, and seed *S. homoeocarpa* isolate 'Shark' on creeping bentgrass (cv. 'Penncross') at 5 dpi. Similar symptoms were produced for each treatment in six biological replicates. **C,** Symptoms produced by mock-inoculated control, virulent *S. homoeocarpa*, and seed *S. homoeocarpa* isolate 'Shark' on barley (cv. 'Parkland') at 5 dpi. Similar symptoms were produced for each treatment in six biological replicates.

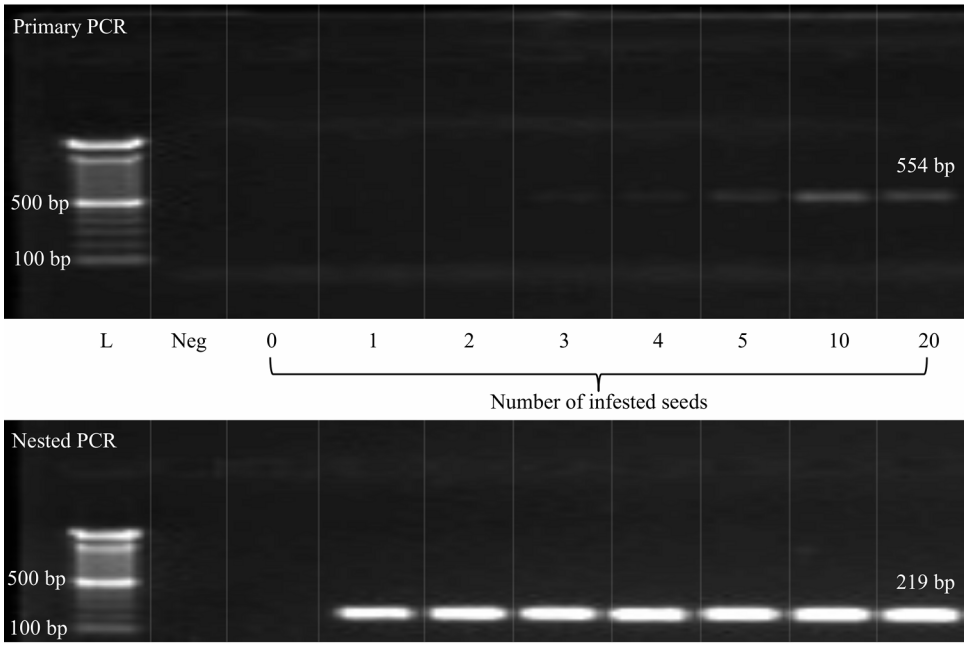

Figure. 4 Sensitivity of *Sclerotinia homoeocarpa*-specific primers in primary and nested PCR. Primary PCR was run with *S. homoeocarpa* specific primers EF1α_OuterF/ EF1α_OuterR, with an expected product size of 554 bp, and contained 2µL of seed DNA from seed samples spiked with the indicated number of artificially-infested CRB seeds prior to DNA extraction. Nested PCR was performed using *S. homoeocarpa* specific primers EF1α_F/EF1α_R, with a product size of 219bp internal to EF1α_OuterF/ EF1α_OuterR, and contained 2µL of primary PCR products diluted 1:30 in NFW. The negative control was treated exactly as samples except that 2µL of NFW replaced template DNA in the primary PCR. Five µL of sample were mixed with 1µL of DNA loading dye and run in a 1% agarose gel in TBE buffer along with 5µL of TrackIt 100bp DNA ladder (Invitrogen, Carlsbad, CA).

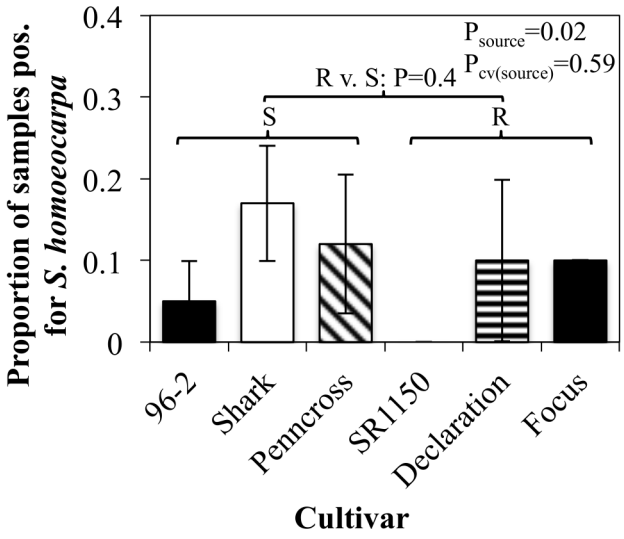

Figure. 5 Results of nested PCR detection of *Sclerotinia homoeocarpa* DNA in creeping bentgrass commercial seed lots. Columns reflect the average proportion of samples positive for *S. homoeocarpa* contamination across the two lots of each cultivar. Shading indicates cultivar source: ’96-2’, ‘Focus,’ and ‘SR1150’ were from a single source while ‘Shark,’ ‘Penncross,’ and ‘Declaration’ were from different seed distributors. Error bars represent ± one standard error of the mean. Source and cv(source) P-values are from ANOVA of the data with the normal distribution and α=0.05. The P-value for R versus S cultivars was obtained from a pre-planned orthogonal contrast.

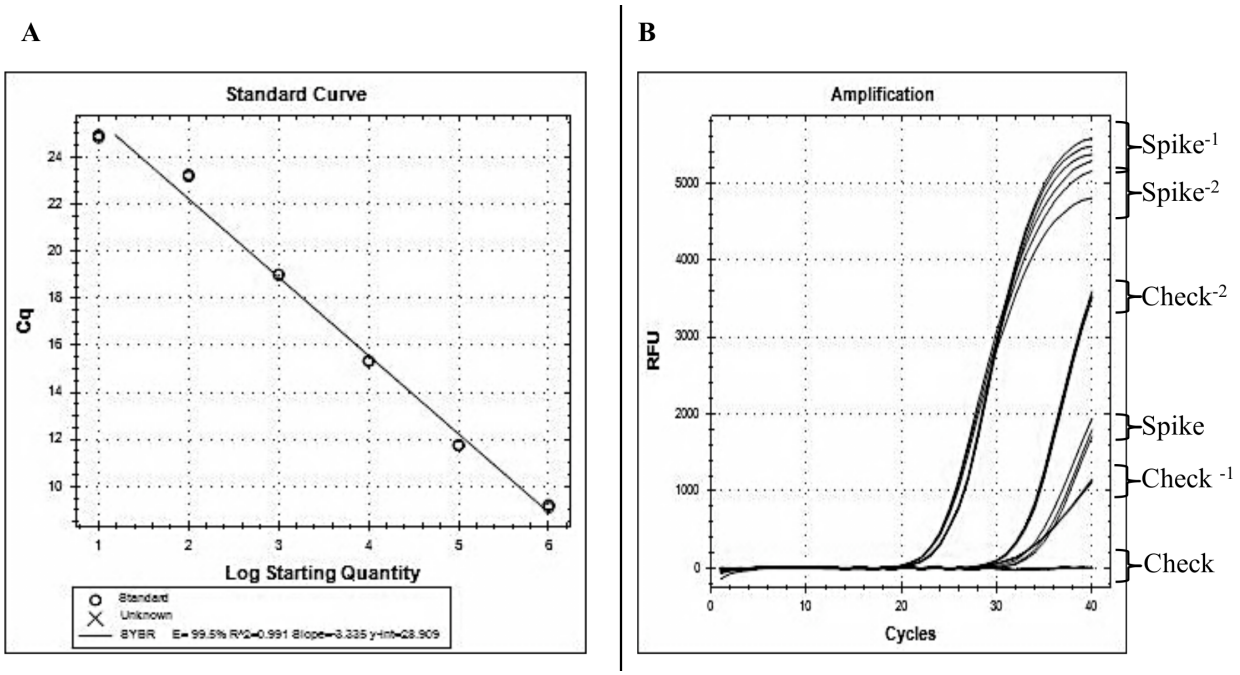

Figure. 6 Q-PCR for molecular detection of *Sclerotinia homoeocarpa* DNA in creeping bentgrass commercial seed lots. A, Standard curve of EF1α plasmid DNA with the primer set EF1α_NestF/EF1α_NestR indicating near 100% primer efficiency. **B,** Q-PCR run with various controls to reveal the presence of PCR inhibitors: Spike, Spike^{-1}, Spike^{-2}—Ten-fold dilution series of seed DNA mixed with 1/10 volume EF1α plasmid DNA at a starting concentration 1x10^{6}; Check, Check^{-1}, Check^{-2}—Ten-fold dilution series of a seed DNA sample positive by nested PCR but negative by Q-PCR. Decreasing Cq value with dilution indicates presence of inhibitors in the original DNA sample.

CHAPTER 4: INITIAL CHARACTERIZATION OF THE ROLE OF OXALIC ACID IN PATHOGENESIS OF *SCLEROTINIA HOMOEOCARPA* AND OF POTENTIAL MODEL HOSTS FOR STUDIES OF *S. HOMOEOCARPA*/HOST INTERACTIONS

This chapter is a preliminary version of the following manuscript:

Rioux, R.A., Willis, D.K., Kabbage, M., Bent, A.F., and J.P. Kerns. 2014. Oxalic acid is important for successful *Sclerotinia homoeocarpa* infection and is produced in response to specific signals. PLOS One. *In preparation.*

Contribution: R.A.R. was the primary author. She designed, performed, analyzed data for all experiments, and wrote the manuscript, In conjunction with other authors. D.K.W. assisted with design and implementation of RT-qPCR experiments. M.K., A.F.B., and J.P.K. contributed ideas for experimentation and experimental design.

Abstract

Sclerotinia homoeocarpa, the fungus that causes dollar spot of turfgrasses, produces oxalic acid but its role is unknown, largely due to lack of genetic resources for studying *S. homoeocarpa*/host interactions. In this study, whole plant inoculation assays were used to determine if various host plants could serve as a model system for *S. homoeocarpa*/host interactions. These assays revealed that both host endogenous oxalate content and pathogen-produced oxalic acid influence the timing and magnitude of symptom development. Endogenous oxalate concentrations in dollar spot resistant creeping bentgrass cultivars collected from field plots are lower than less resistant cultivars. Time-course expression analysis of oxalate oxidase and other defense-associated germin-like protein genes in creeping bentgrass showed strong upregulation starting at 48-72 hpi. This indicates that these genes are most likely involved in defense against the necrotrophic phase of this pathogen and demonstrates the importance of oxalic acid in *S. homoeocarpa* pathogenesis. Furthermore, we demonstrated using *in vitro* assays that *S. homoeocarpa* produces oxalic acid in response to host tissue and xylan monomers. In comparison, *Sclerotinia sclerotiorum* constitutively produced high amounts of oxalic acid. These results provide the first evidence that both host endogenous oxalate and pathogen-produced oxalic acid are important for *S. homoeocarpa* pathogenesis and that host cell wall components, in particular xylan, may induce oxalic acid production as *S. homoeocarpa* transitions to necrotrophy within its host.

KEYWORDS: *Sclerotinia homoeocarpa*, dollar spot, creeping bentgrass, oxalic acid

Introduction

Sclerotinia homoeocarpa is the causal agent of dollar spot, one of the most important diseases of amenity turfgrasses worldwide (42). Additionally, this pathogen has a broad host range that spans five plant families (71) and includes economically and ecologically important plants, such as switchgrass (70), perennial peanut (30), and tufted bulrush (32). Dollar spot is of particular concern in a golf course setting because the sunken, silver dollar-sized patches of diseased turf from which this disease earns its name are aesthetically unappealing and may affect ball roll. Conditions favoring dollar spot development are quite broad. The fungus can grow and cause disease at temperatures ranging from 14-35°C, if relative humidity remains above 70% (42). As a result, successful dollar spot management requires periodic fungicide applications throughout the golfing season, making dollar spot the most economically important disease of turf worldwide (19,25,42).

In recent years, increasing governmental regulation of broad-spectrum fungicides and concerns over the environmental and financial costs of chemically-intensive management programs for dollar spot have brought the sustainability of these practices into question. Additionally, development of resistance to fungicides with specific modes of action, is relatively common in *S. homoeocarpa* populations (37,38,45,61,72). These issues have necessitated the development of integrated management practices for this diseases (42). Such practices incorporate host resistance, cultural control, disease forecasting, and knowledge of pathogen biology to minimize the need for fungicide applications. Limited research on *S. homoeocarpa* pathogenesis mechanisms, however, has hindered development of management strategies that do not rely solely on fungicides.

Lack of a tractable host system for experiments investigating *S. homoeocarpa* pathogenesis and host resistance mechanisms contributes significantly to the limited research in this area. Popular turfgrass species that are prone to dollar spot epidemics, in particular creeping bentgrass (*Agrostis stolonifera,* L.), have complex, polyploid genomes and are highly outcrossing, making genetic characterization of dollar spot resistance difficult (8). To date, only a single study comparing global gene expression among inoculated and non-inoculated creeping bentgrass plants exist (58) and dollar spot resistance has been identified on only a few major quantitative trait loci (15). The small size of individual turfgrass plants also make it difficult to manipulate and visually assess disease phenotypes. An acceptable model host would provide a much-needed tool for molecular and phenotypic characterization of *S. homoeocarpa*/host interactions and may be applicable to other turfgrass pathogens.

The vast array of genetic resources for *Arabidopsis thaliana* make it a natural choice as a model host for any pathosystem. Further, *A. thaliana* has been successfully used in a number of studies on pathogenicity of *S. sclerotiorum* and *Botrytis cinerea*, both of which belong to the same fungal order as *S. homoeocarpa* (3,20,26,27,40,56). The preference of *S. homoeocarpa* for monocot hosts indicates that model grass species may be preferable to a dicot. Rice is considered the forerunner in cereal genetic resources (47), but is less closely related to creeping bentgrass and other major turfgrass species than *Brachypodium distachyon*, barley, and wheat (2,65). Studies of host/pathogen interactions and generous genetic resources are available for all three of these plants, indicating their potential utility as models for *S. homoeocarpa* pathogenesis. Consequently, one of the objectives of the present research was to compare *S. homoeocarpa* pathogenesis between the five aforementioned model hosts and the natural host creeping

bentgrass in order to determine if these plants could serve as a useful model system for *S. homoeocarpa*.

Identifying key pathogenicity mechanisms are an important aspect of understanding how *S. homoeocarpa* interacts with its hosts. Recently, researchers showed that this pathogen produces oxalic acid (68). Oxalic acid is a common phytotoxin and is produced by many important plant pathogens, particularly those in the *Sclerotiniaceae* family (1). *S. sclerotiorum*, a polyphagous necrotrophic plant pathogen with more than 400 hosts (6,7), relies heavily on production of oxalic acid for successful infection as oxalic acid deficient mutants of this fungus are non-pathogenic (24). The role of oxalic acid in *S. sclerotiorum* pathogenesis has been extensively studied (28,40,43,73); however, it is not clear if the role of oxalic acid in *S. sclerotiorum* pathogenesis is applicable to *S. homoeocarpa*. Despite its name, *S. homoeocarpa* is not a true a *Sclerotinia* species. It is morphologically and phylogenetically more closely related to fungal species in the *Rutstroemiaceae* family, a sister family to *Sclerotiniaceae* (13,55). Renaming and correct taxonomic placement of *S. homoeocarpa* are currently underway but have not been completed.

Differences among *S. homoeocarpa* and *S. sclerotiorum* host ranges indicate these pathogens may utilize oxalic acid differently during host colonization. *S. homoeocarpa* primarily infects monocot species within the family *Poaceae* (71). Conversely, *S. sclerotiorum* infects a broad range of dicot species, but is non-pathogenic on most monocots. Many grass species have oxalate oxidases that degrade oxalic acid, preventing successful infection by *S. sclerotiorum* (5,49). Transformation of dicot hosts with grass oxalate oxidase genes confer partial resistance to *S. sclerotiorum* (21,22,33). Consequently, if oxalic acid is an important pathogenicity factor for *S. homoeocarpa*, this fungus must utilize the phytotoxin in a manner that allows it to circumvent or

overcome oxalate oxidase-mediated plant defenses. Both oxalate oxidase activity (57) and up-regulation of oxalate oxidase encoding genes (58) occur during *S. homoeocarpa* infection of creeping bentgrass, indicating that oxalic acid is produced *in planta* during host colonization. A better understanding of the role of oxalic acid in host colonization by *S. homoeocarpa* will provide valuable insights on *S. homoeocarpa* pathogenesis that ultimately may lead to novel disease management strategies.

In the present research, we use infection and oxalate quantification assays to assess the utility of various plant species as model systems for *S. homoeocarpa*/host interactions and the importance of oxalic acid for successful host infection by *S. homoeocarpa*. We demonstrate that, endogenous oxalate content in monocots correlates with timing of symptom development and that disease severity is more closely correlated with low endogenous oxalate levels than high endogenous oxalate levels. Support for these findings is provided by comparing symptom severity and oxalate content over time in creeping bentgrass, a low oxalate content host, and *B. distachyon*, a high oxalate content host. Further, we show that an *S. homoeocarpa* isolate with reduced oxalic acid production *in vitro* has decreased aggressiveness on creeping bentgrass but not on *B. distachyon*. Comparison of endogenous oxalate levels and oxalate oxidase gene expression in creeping bentgrass cultivars with varying levels of resistance to dollar spot indicate that endogenous oxalate content may also be important for resistance to *S. homoeocarpa* in creeping bentgrass. Finally, KOA infiltration and *in vitro* oxalic acid induction assays demonstrated that oxalic acid is produced by *S. homoeocarpa*, but is not used in a fashion similar to *S. sclerotiorum*. These studies are the first to characterize the role of oxalic acid during host infection by *S. homoeocarpa*. Specifically, that host oxalate content and pathogen oxalic acid production contributes to success and extent of infection by this pathogen. Additionally, we

provide evidence that *S. homoeocarpa* and *S. sclerotiorum* produce and utilize oxalic acid in disparate ways.

Materials and methods

Biological materials. *Plant materials.* All monocots (Table S1) were grown from seeds and maintained in a growth room with a 14 h light period at 25±2°C and 10 h dark period at 22±2°C. *Arabidopsis thaliana* and *Nicotiana benthamiana* were grown under similar conditions, except growth chambers had an 18 h day-length and constant temperature of 26±2°C.

Fungal materials. All fungal isolates (Table S2) were maintained on potato dextrose agar and transferred weekly to maintain viability. Cultures were grown in the dark in an environmental chamber at ambient temperatures (22±2°C). Additionally, long-term storage stocks were made for all isolates by allowing fungal mycelia to colonize filter paper disks and storing dried, colonized disks in glass vials at -80°C.

Inoculation assays and disease rating. Monocots were inoculated using the previously described parafilm sachet method (36). The method differed according to plant species (Fig. S1). Dicots were inoculated by placing an agar plug mycelia side down on a fully expanded leaf surface. Flats containing inoculated dicots were covered with plastic domes to promote relative humidity >90%. Parafilm alone maintained high relative humidity for monocot inoculations and domes were not needed. Four-day-old cultures of *S. homoeocarpa* were used for all plant inoculations. Control plants were mock-inoculated with agar plugs from fresh PDA plates.

Symptom severity was rated every 24 h using a rating scale modified from the Horsfall-Barratt scale (Table S3) (31). This scale allowed for direct comparison of symptom severity between species with diverse physical characteristics and symptom phenotypes. To prevent the

possibility of rater-to-rater variability all symptom severity ratings were made by the same individual.

Inoculation experiments were repeated three times with three replicates of each treatment or treatment combination per experimental repetition. Data were analyzed with experimental repetition treated as a random blocking factor and blocks within each repetition treated as a random factor nested within experimental repetition. Plant species and fungal isolate were both considered fixed effects.

Trypan blue staining and microscopy. Inoculation and sample collection for all plant hosts and *S. homoeocarpa* isolates were performed in parallel. Scissors were used to excise plant material surrounding the site of inoculation and individual tissue samples were placed in 2mL microcentrifuge tubes. Staining and clearing of tissues were performed as previously described (16). All samples were visualized with an Axio Scope.A1 compound microscope (Zeiss, Thornwood, NY). Images were captured with an AxioCam MRc camera and processed with accompanying AxioVision Real 4.7.1 imaging software (Zeiss, Thornwood, NY). A minimum of three samples were examined for each time-point, isolate, and host combination.

Whole plant oxalate quantification. Plants for oxalate quantification were inoculated as previously described. For fresh plant samples, 0.25 g of tissue were ground in liquid nitrogen with a mortar and pestle. Ground plant material was then added to a 15 mL centrifuge tube containing 4 mL of 0.2 M KH_2PO_4 buffer (pH 6.5), in accordance with previously described methods (23). Tubes containing ground plant tissue were placed on their sides in an incubator shaker and mixed overnight with constant gentle shaking at room temperature (22±2°C). The following day, 2 mL of the liquid portion from each sample tube was transferred to a fresh 15

mL centrifuge tube and mixed with 2 mL of freshly prepared oxalate quantification kit sample diluent (Trinity Biotech, Jamestown, NY). The remainder of the oxalate assay was performed according to the manufacturer's instructions (Trinity Biotech, Jamestown, NY). Oxalate content was quantified based on absorption at 590 nm with a UV/Vis spectrophotometer (Beckman Coulter). A 0.5 mmol oxalate standard was included in each run.

Due to the high volume of samples in time-course oxalate studies, samples for this assay were harvested, weighed, flash frozen in liquid nitrogen, and stored at -80°C until use. Pilot studies indicated that flash freezing did not affect oxalate content in comparison with samples processed when fresh. Frozen samples were lightly ground in liquid nitrogen with a mortar and pestle, then transferred to Lysing Matrix A FastPrep tubes (MP Biomedicals, Santa Ana, CA) with 1.5 mL KH_2PO_4 buffer and ground in a FastPrep at 6.0 m/s for 40 s (MP Biomedicals, Santa Ana, CA). Homogenized samples were poured into 15 mL centrifuge tubes, and mixed with 1.5 mL sample diluent. Oxalate quantification was then performed as described above.

Germin-like protein gene expression analysis. Pots of one-week-old creeping bentgrass turfgrass cultivars '96-2' and 'Focus' were inoculated with two agars plugs colonized with *S. homoeocarpa* mycelia and covered to maintain humidity >90%. Cultivars '96-2' and 'Focus' were chosen for their low and high resistance to dollar spot in field trials, respectively (http://www.ntep.org). The experiment was arranged as a two treatment (inoculated and mock-inoculated) by two cultivar ('96-2' and 'Focus') factorial within a randomized complete block design with four replications. The same pots were resampled at 0, 24, 48, 72, and 96 hpi by harvesting 100 mg of tissue in the area surrounding the site of inoculation, removing roots and leaf blade tips, flash freezing in liquid nitrogen, and storing at -80°C until use.

Samples for RNA extraction were ground with a mortar and pestle in 500 µL frozen Trizol reagent and then transferred to 2 mL microcentrifuge tubes containing 500 µL Trizol reagent. RNA extraction was then performed with the Trizol Plus RNA Purification Kit, with optional on-column DNase step, according to the manufacturer's instructions (Life Technologies, Grand Island, NY). RNA quality was assessed with an Experion Automated Electrophoresis Station using the Experion RNA StdSens analysis kit (BioRad, Hercules, CA) and yield was quantified with an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Samples were stored at -80°C until further use.

Synthesis of cDNA and RT-qPCR were performed according to the specifications of the MIQE guidelines (12). Prior to cDNA synthesis, a no cDNA control RT-qPCR run was performed on pure RNA to confirm the absence of contaminating genomic DNA. RNA samples confirmed as free of genomic DNA were diluted to equivalent concentrations and subject to cDNA synthesis with iScript cDNA Synthesis Kit, according to the manufacturer's instructions (BioRad, Hercules, CA). All cDNA synthesis reactions for a single time-point were performed simultaneously to decrease any variability inherent in the synthesis process. cDNA quality was assessed by comparing Cq values for potential reference genes and reference genes were selected using BestKeeper Software (59). Creeping bentgrass actin (ACT7) and glyceraldehyde phosphate dehydrogenase (GAPDH) genes were selected as stable reference genes for all time-points in both cultivars.

Primers for RT-qPCR were designed from publicly available creeping bentgrass EST sequences with Primer3 primer design software (http://wwwgenome.wi.mit.edu/genome_software/other/primer3.html;Table S4) and validated be generating efficiency curves from a dilution series of cDNA pooled from all time-points. For

RT-qPCR, cDNA samples were diluted 10-fold and a final volume of 8 µL cDNA was used in each of three 20 µL technical replicates per reaction, along with 10 µL SsoFast EvaGreen Supermix (BioRad, Hercules, CA), 0.3 µM of each primer, and nuclease freee water. All reactions were run in hard-shell skirted 96-well plates with a white top and clear wells and sealed with Microseal 'B' adhesive film (BioRad, Hercules, CA). A CFX-96 detection system (BioRad, Hercules, CA) was used for quantification. PCR conditions were 98°C for 2 m; 40 cycles of 98°C for 2 s and 55°C for 5 s; followed by a dissociation curve with 80 cycles starting at 70°C for 10 s and increasing in 0.2°C increments per cycle. The dissociation curve was used to detect primer-dimers and amplification of a single product within the CFX detection system software (BioRad, Hercules, CA).

To calculate relative expression of target genes, transcript abundance was internally normalized to the two reference genes (actin and elongation factor 1-α) included on each experimental plate using the formula $2^{\Delta Cq(\text{target-reference})}$ (29). Relative expression ratios of target genes were then calculated using a method based on the previously described $2^{-\Delta\Delta Cq}$ method (29,60,64). Briefly, normalized target gene Cq values for infected plant tissue were divided by a calibrator value, which was calculated by averaging the normalized Cq values for all uninfected 96-2 samples from the same time point (29). This method allowed for comparison between all four treatment levels at each time-point. Statistical analysis was performed on resulting relative expression ratios using the proc mixed procedure in SAS v.9.3 (34). Orthogonal contrasts were used to compare relative expression ratios between mock-inoculated and inoculated samples within each cultivar and between the inoculated samples of the two cultivars.

KOA infiltration. KOA infiltrations for *Nicotiana bethamiana* were performed as previously described for *Sclerotinia sclerotiorum* (73) except that a needless 1 cc syringe was used to

perform infiltrations. Barley KOA infiltrations were performed by forcibly infiltrating 10 mmol KOA with a 1 cc needless syringe. Because infiltrations followed longitudinally along leaf veins and did not cross the central vein, both sides of the leaf blade were infiltrated. Negative controls were infiltrated with DI H_2O.

Medium preparation and oxalate quantification. Quarter-strength potato dextrose broth cultures were prepared according to the manufacturer's instructions (BD Difco, Franklin Lakes, NJ) but by dividing by the recipe by four. Plant amended media were prepared by adding 1 mg/mL of plant tissue prior to autoclaving. Cell wall component amended media was prepared by adding 1 g/L of the component (Table S5) to media prior to autoclaving. Media was either prepared in 250 mL flasks or 100 mL of media were pipetted into 250 mL flasks after autoclaving. Individual flasks were inoculated with a single agar plug taken from the advancing edge of a four-day-old *S. homoeocarpa* culture and incubated for one week at ambient temperature (22±2°C) with constant shaking. After one-week incubation, 2 mL of culture filtrate were collected from flasks and oxalate content was quantified as previously described.

Statistical analysis. Data analysis was performed using the MIXED, generalized linear mixed model (GLIMMIX), regression (REG) procedures in SAS version 9.3 (SAS Institute, Cary, NC). Initial models were fit including all random factors and interactions with fixed factors. Model fitting criteria (Akaike's Information Criterion) were then used to remove random factors and interactions not contributing to variability and identify the best model for each data set. Visual analysis of residual plots and output from PROC UNIVARIATE were used to assess normality of the data. Data were transformed as necessary to best approximate the normal distribution using the DIST= option in PROC GLIMMIX. Reported values were back-transformed using the ILINK option. The ANOVA F-test was used to determine contributions of fixed factors to

variability in the measured response variable(s). Additionally, pre-planned orthogonal contrasts were used to compare between specific fixed factors.

Use of the symptom severity rating scale resulted in data with a non-normal distribution that could not be corrected through common data transformations available within PROC GLIMMIX. Consequently, a Friedman's two-way analysis for block designs was employed (17). For each experimental repetition, the symptom severity for each data point (species by treatment) was ranked within blocks previously defined during experimental set-up. The resulting ranks were assessed for normality and agreement between experimental repetitions. The rank data from each experiment repetition was then pooled and analysis of variance was performed with the rank output as the response variable and both repetition and block(repetition) as random factors.

Results

Characterization of the S. homoeocarpa infection process on natural and model hosts. Microscopic analysis of the infection process at various time-points following inoculation revealed very similar events, regardless of host (Fig. 1). As early as six hours-post inoculation, *S. homoeocarpa* hyphae were observed growing along cell walls (Fig. 1A) and initiating host penetration via both infection pegs (Fig. 1A) and direct penetration through stomata (Fig. 1B). This resulted in extensive colonization of host tissue, particularly xylem, shortly following inoculation (Fig. 1C-E). Host cell penetration and colonization continued for at least 72 h. Dead host cells, as indicated by trypan blue staining outside of fungal hyphae, was observed only in later stages of infection (Fig. 1J-K). Possible potassium oxalate crystals were observed in the vascular tissue of barley 7 dpi (Fig. S2) but were not identified in any other species in these experiments. No differences in the infection process were noted between the four *S. homoeocarpa* isolates used in this study.

Symptoms of *S. homoeocarpa* infection were similar in monocot hosts, but were quite different in the dicot host *Arabidopsis thaliana* (Fig. 2). Inoculation of creeping bentgrass plants resulted in bleaching and necrosis of the inoculated leaf area (Fig. 2F). Within this area, individual leaf blades with hourglass lesions of white-gray tissue surrounded by reddish-brown borders were identified (Fig. 2E). Similar lesions were observed on the four model monocot hosts (Fig. 2A-D). Occasionally, complete wilting of infected leaves was observed, particularly in the later stages of infection. Symptoms on *A. thaliana* began as water-soaked lesions, with the affected areas eventually losing turgor pressure. These lesions were visually distinct from the necrotic lesions formed on *A. thaliana* following inoculation with *S. sclerotiorum* (40). In severe infections, the petiole would become necrotic, constrict, and, often, the symptomatic leaf would separate from the plant.

Analysis of area under the disease progress curve (AUDPC) values indicated no significant effect of isolate or isolate x species interaction based on a simple two-way ANOVA F-test at $\alpha=0.05$ level (Fig. 3A). Disease severity was influenced by species ($\alpha=0.05$). In particular, disease severity was different between rice and creeping bentgrass according to a Dunnett's test comparing mean AUDPC values for species ($P=0.0043$). Symptom severity differed between species at 48 hpi ($p=0.03$), when symptoms began to rapidly develop in *B. distachyon* and *O. sativa*, but not at any other time point. Greater symptom severity was observed in rice at 48, 72, and 96 hpi $P<0.05$) and in *B. distsachyon* at 48 hpi ($P=0.01$) when compared to creeping bentgrass. No additional differences in symptom severity between creeping bentgrass and other species were found. Throughout these experiments, symptom severity on mock-inoculated controls remained at or near zero.

Relationship between host endogenous oxalate content and symptom development. To explore the relationship between *S. homoeocarpa* pathogenicity and oxalic acid, the oxalate content of plants inoculated with *S. homoeocarpa* or mock-inoculated with PDA was compared. A positive relationship between oxalate content and symptom severity was detected in creeping bentgrass, barley, and wheat, with R^2 values ranging from 0.33 to 0.52 (Fig. 4). The relationship between oxalate content and symptom severity in rice and *B. distachyon* was also positive, but much lower when compared to the other species (0.10 and 0.01 respectively). A negative relationship between oxalate content and symptom severity was observed in *Arabidopsis thaliana*. Endogenous oxalate content of the six species differed significantly ($P<0.0001$) and comparisons against creeping bentgrass revealed that both rice and *B. distachyon* had higher oxalate levels ($P<0.0001$; Fig. 4).

Due to the resources needed to perform whole plant oxalate content analyses, it was necessary to decrease the number of species used in experiments to test this hypothesis. Creeping bentgrass was selected to represent species with low oxalate content and *B. distachyon* was selected to represent species with high oxalate content. *B. distachyon* was selected over *O. sativa* because symptom severity and genetics were more similar to creeping bentgrass (65). A time-course experiment was performed to compare symptom severity and oxalate content between *S. homoeocarpa*- and mock-inoculated during the progression of infection. Oxalate content in *S. homoeocarpa*-inoculated plants gradually increased over time and paralleled the development of symptoms (Fig. 5A). Oxalate content in mock-inoculated plants remained low throughout the experiment. Significant differences between the oxalate content in *S. homoeocarpa* and mock-inoculated plants developed at 48 hpi ($P=0.004$) and 120 hpi ($P<0.0001$), but not at 0 hpi ($P=0.738$). Conversely, oxalate content of inoculated and mock-inoculated *B. distachyon* plants

remained relatively stable over time (Fig. 5C). The oxalate content of inoculated and mock-inoculated *B. distachyon* only significantly differed at 120 hpi ($p=0.003$). Oxalate content correlated to symptom severity in both hosts ($P<0.0001$), but 75% of the variability in symptom severity was explained by oxalate content in creeping bentgrass, compared to only 28% in *B. distachyon*.

To further elucidate the influence of pathogen-produced oxalic acid on symptom development in creeping bentgrass and *B. distachyon*, the aggressiveness of *S. homoeocarpa* isolates producing varying levels of oxalic acid in culture was compared (Fig. 6). Isolates producing high, low, and moderate amounts of oxalic acid were selected based on color change of bromophenol blue amended medium (24) (Fig. S3). Orthogonal contrasts were used to compare symptom severity between the oxalate deficient *S. homoeocarpa* isolate and the two isolates with moderate to high oxalic acid production at each time point (Fig. 6). On creeping bentgrass, aggressiveness of isolates differed at 72 hpi and, from this point on, the oxalic acid deficient isolate was significantly less aggressive than that other two isolates ($p\leq0.05$; Fig. 6B). Aggressiveness among the *S. homoeocarpa* isolates did not vary on *B. distachyon* (Fig. 6C).

Comparison between endogenous oxalate content and oxalate oxidase expression in creeping bentgrass cultivars. To further test the hypothesis that host endogenous oxalate content influences infection by *S. homoeocarpa*, the oxalate content of samples collected from healthy turfgrass with varying resistance to dollar spot was quantified. In National Turfgrass Evaluation Program (NTEP; http://www.ntep.org) trials, two of these cultivars (Memorial and Declaration) had consistently lower dollar spot severity than the remaining six cultivars (Fig. S4). An orthogonal contrast comparing the oxalate content of the two resistant cultivars against the

remaining six cultivars revealed that Memorial and Declaration had significantly less endogenous oxalate than the other six cultivars (Fig. 7; P=0.008).

Various germin-like protein (GLP) genes, including oxalate oxidases, were some of the most up-regulated genes in the dollar spot susceptible cultivar 'Crenshaw' 96 hpi with *S. homoeocarpa* (58). In the present study, RT-qPCR was used to compare the expression of oxalate oxidase and other germin-like protein genes between cultivars considered resistant and susceptible to *S. homoeocarpa*. Four genes of interest were selected for RT-qPCR analysis of expression through sequence alignment and phylogenetic analysis of creeping bentgrass ESTs and known GLP genes from barley, rice, and *B. distachyon* (Fig. S5). Expression was tested by extracting total RNA from four independent pots of *S. homoeocarpa*-inoculated or mock-inoculated resistant and susceptible creeping bentgrass cultivars. All four of these genes were up-regulated in both resistant and susceptible cultivars by 96 hpi in *S. homoeocarpa* inoculated versus mock-inoculated control plants (Fig. 8. Fig. S5). The oxalate oxidase gene, AST_798, was up-regulated in the susceptible cultivar at 48, 72, and 96 hpi and its expression was stronger in the susceptible than in the resistant cultivar 48 and 72 hpi. AST_798 was only up-regulated in the resistant cultivar at 96 hpi (Fig. 8A). Similarly, AST_854, a creeping bentgrass gene grouping with defense-associated GLP genes from barley and rice in phylogenetic analysis, was up-regulated in the susceptible cultivar at 72 and 96 hpi, but only in the resistant cultivar at 96 hpi (Fig. 8B). Similar expression patterns were observed for two more creeping bentgrass GLP genes that did not group with known defense-associated GLPs in phylogenetic analysis (Fig. S5).

Comparison of the role of oxalic acid in pathogenicity of *S. homoeocarpa* and *S. sclerotiorum* and factors affecting production of oxalic acid in these two species. When *S. sclerotiorum* is deficient in oxalic acid production, as has been shown in the mutant isolate A2,

pathogenicity is severely impaired. Yet, infiltration of host tissue with KOA prior to inoculation restores pathogenicity of this mutant (73). Production of oxalic acid by *S. homoeocarpa* is much lower than that by *S. sclerotiorium* (1); consequently, we hypothesized that KOA infiltration would promote infection by *S. homoeocarpa* if these two pathogens utilize oxalic acid in the same way during host colonization. KOA infiltration of creeping bentgrass was not possible due the small size of leaves individual leaf blades. Barley was used as a substitute because it has broad leaf blades that can be efficiently infiltrated and the relationship between oxalate content and symptom severity was most similar to that of creeping bentgrass (Fig. 3). The experiment was also performed on the model dicot host *Nicotiana benthamiana*, which has previously been used for KOA infiltration studies with the *S. sclerotiorum* A2 mutant (73)

N. benthamiana plants were infiltrated with either H_2O or 10 mmol KOA (pH7) and then inoculated with PDA plugs colonized with one of two different *S. homoeocarpa* isolates or without mycelia. Orthogonal contrasts comparing the AUDPC for KOA and H_2O treatments indicated no treatment effect (P>0.5; Fig. 9A). Barley plants were infiltrated with H_2O, 10 mmol KOA (pH7), or 10mmol KOA (pH3) and inoculated with PDA plugs either with or without *S. homoeocarpa* mycelia. Infiltration treatment had no effect on AUDPC (P>0.5; Fig. 9B).

In vitro assays were used to determine if the presence of host material induces oxalic acid production by *S. homoeocarpa*. *S. sclerotiorum* was included in these assays to assess if oxalic acid induction was specific to *S. homoeocarpa* or a general phenomenon. When creeping bentgrass clippings were added to ¼-strength PDB, *S. homoeocarpa* culture filtrates contained more oxalic acid than when *S. homoeocarpa* was grown in ¼-strength PDB alone (P=0.02; Fig. 10A). No difference was observed in oxalic acid content of *S. sclerotiorum* culture filtrates with and without added creeping bentgrass tissue.

S. homoeocarpa only produced oxalic acid only in the presence of reproductively mature *N. benthamiana* leaves (Fig. 10B), which indicated that cell wall composition may affect oxalic acid production by *S. homoeocarpa*. To test this, pure forms of three cell wall components (pectin, lignin, and xylan) were added to liquid medium and the oxalate content of culture filtrates was quantified. Dunnett's test was used to compare the oxalate content of culture filtrates collected from cell wall component-amended media to a non-amended PDB control. Of the four cell wall components tested, only xylan stimulated oxalic acid production by *S. homoeocarpa* (P=0.05; Fig. 10C). Xylan did not promote oxalic acid production by *S. sclerotiorum* (P=0.98). Oxalic acid production by *S. sclerotiorum* was significantly lower on pectin-amended media (P=0.002) when compared to the other media. Although oxalic acid production by *S. homoeocarpa* was numerically the lowest in pectin-amended medium, it did not separate statistically (P=0.10) (Fig. 10C).

Discussion

One of the primary goals of the present research was to identify a host species that could be developed into a useful model system for molecular studies of *S. homoeocarpa* pathogenesis and host resistance. The lack of a significant species by isolate interaction in this research supports previous findings in turfgrass hosts that indicate a lack of race specificity in *S. homoeocarpa*/host interactions (14). Therefore a few model host cultivars or accessions with high and low resistance to *S. homoeocarpa* could be used to dissect molecular and physiological aspects of host resistance to this pathogen. We detected a difference in disease severity between species, indicating that not all species are equally suited as a model system for *S. homoeocarpa*. However, the correlation between symptom severity and endogenous oxalate shows that hosts with high oxalate content could still be developed into a useful model system, particularly if

accessions or cultivars with varying endogenous oxalate levels can be identified. Soybean cultivars (50) and spinach varieties (41) differ in oxalate content in the field, suggesting that natural variation in oxalate levels is common in plant species and should be readily identifiable in a chosen model host system. We are unaware of previous reports that plant endogenous oxalate levels influence resistance to fungal pathogens; however, oxalate is known to influence resistance to herbivory by some insects (46,74).

While infection progressed similarly on all species at the microscopic level (Fig. 1) and symptomology was similar on all monocots (Fig. 2), symptoms produced by *S. homoeocarpa* on *A. thaliana* were different from those produced on grass hosts (Fig. 2). Additionally, oxalate was not detected in symptomatic *A. thaliana* tissue (Fig. 4), indicating that infection of this host by *S. homoeocarpa* may depend on different mechanisms than those employed to infect monocot hosts. These findings demonstrate that *A. thaliana* is a poor model system for *S. homoeocarpa*. Given that *S. homoeocarpa* occurs predominantly on monocot hosts in nature, this finding may reflect a preference of this pathogen for certain physiological aspects of monocots that differ from dicots, such as cell wall composition (69) or leaf morphology (66). Interestingly, our *in vitro* assay results suggest that physical differences between reproductively mature and immature leaves may be important for induction of oxalic acid production by *S. homoeocarpa* (Fig. 9). Influence of leaf surface morphology on infection has previously been demonstrated for the rice blast fungus, *M. oryzae*, which responds to surface hydrophobicity and forms appressoria more readily on host tissue than artificial surfaces (35).

To our knowledge, a correlation between host endogenous oxalate content and susceptibility to *S. homoeocarpa* has not previously been demonstrated for creeping bentgrass or any other turfgrass species. Plants produce and use oxalate for a variety of purposes, including

defense, pH regulation, osmoregulation, and calcium homeostasis (reviewed in (48)); thus, oxalate content can vary widely between cultivars and environments. Correspondingly, resistance to dollar spot is inherited quantitatively and largely dependent upon environmental conditions (9,10,11,15). Since we found lower oxalate levels in cultivars considered resistant to dollar spot (Fig. 7), it is possible that oxalate content contributes to dollar spot resistance but additional research is needed. Bonos and colleagues (11) found a general, though not significant, tendency for higher turf density and larger trichome size in creeping bentgrass clones resistant to *S. homoeocarpa*. Since both trichomes and oxalic acid contribute to insect resistance, there could be a trade-off between these two defense mechanisms by which dollar spot resistant cultivars have low oxalate levels that lower their resistance to insect pests but are protected from herbivory by larger trichomes (18,46). It has also been reported that fertilizers high in ammonium content and low in nitrate are most effective at suppressing dollar spot in field trials (53). This is of note because nitrogen form, in particular nitrate, can affect plant oxalate levels (48). Promotion or reduction of turfgrass oxalate content could occur in response to different fertilization regimes and suggests a novel means by which fertility could be used to manage dollar spot. Further studies in the both the field and lab are needed to better understand the relationship between creeping bentgrass cultivar oxalate content and dollar spot resistance. Additionally, studies that investigate the effects of cultural practices, such as fertility, on both dollar spot resistance and plant oxalate content will be useful in identifying best management tactics for dollar spot suppression.

Germin-like proteins are largely thought to be an important part of grass basal defense mechanisms (14,25). Consequently, the slow increase in expression of oxalate oxidase and other GLP genes in this study was unexpected (Fig. 8). Orshinsky and colleagues (58) also found

strong induction of GLP genes in creeping bentgrass 96 hpi inoculation with *S. homoeocarpa*. Interestingly, the expression of these genes in creeping bentgrass parallels the timing of symptom appearance and increased more rapidly in the susceptible than the resistant creeping bentgrass cultivar in our study (Fig. 8). An explanation for this may be that these genes are turned on as *S. homoeocarpa* transitions from biotrophy to necrotrophy. This could be due to suppression of host basal defenses during early stages of infection (39) or a result of the host failing to recognize pathogen invasion. Alternatively, oxalate oxidase and other GLP genes may be expressed by creeping bentgrass only after detection of pathogen-produced oxalic acid. *Brassica napus* GLP genes are induced as early as 6 hpi with *S. sclerotiorum* but it is unclear if expression is related to detection of oxalic acid or other signs of pathogen attack (63). Transient induction of GLP gene expression could have occurred at time-points before the 24 h initial sample collection in this study or aspects of experimental design, such as the young age of the host or collection of tissue not yet in contact with *S. homoeocarpa*, could have interfered with our ability to detect meaningful changes in gene expression at early time points. Functional studies with plants overexpressing or lacking oxalate oxidase and other GLP genes will help to better identify the importance of these genes in host resistance to *S. homoeocarpa*.

S. sclerotiorum isolates deficient in oxalic acid production are hypovirulent or non-pathogenic (24,75). Similarly, hypovirulent isolates of the chestnut blight fungus, *Cryphonectria parasitica*, produce lower amounts of oxalic acid *in vitro* than their virulent counterparts (4). In this research, we found that an *S. homoeocarpa* isolate deficient in oxalic acid production had decreased aggressiveness on creeping bentgrass but not on *B. distachyon* (Fig. 6). This indicates that oxalic acid contributes to aggressiveness but is not a pathogenicity determinant for *S. homoeocarpa*. Similarly, the symptoms observed on *A. thaliana* were visibly distinct from those

observed on monocots and no increase in oxalate content was found in symptomatic *A. thaliana* leaves, indicating that other pathogenesis mechanisms are likely responsible for infection of this host. *S. homoeocarpa* may only produce oxalic acid during host infection in response to specific signals that are not produced in *A. thaliana*.

The fungal pathogen *Heterobasidium parviporum* upregulates oxalic acid production during infection only after encountering host defenses that create suboptimal conditions for nutrient uptake (52). It is plausible that a similar trigger is necessary for oxalic acid production by *S. homoeocarpa* and that this signal initiates transition from biotrophy to necrotrophy, as evidenced by symptom appearance only 48-72 hpi with this pathogen. The gradual increase in oxalic acid content and oxalate oxidase gene expression in *S. homoeocarpa*-infected host tissue support a pathogenesis model in which this fungus only produces oxalic acid after successful host colonization. Additionally, our results from KOA infiltration of *N. benthamiana* and barley prior to inoculation with *S. homoeocarpa* indicate that oxalic acid is not important for success of this fungus during early stages of infection, though experiments with an oxalic acid-deficient *S. sclerotiorum* isolate as a positive control are needed (Fig. 9). *S. sclerotiorum* however, uses oxalic acid to create an environment that suppress host oxidative burst during host infection (40,73). Differential use of oxalic acid during early host colonization events may be partially responsible for the prolonged biotrophic period of *S. homoeocarpa* in comparison with *S. sclerotiorum*. To more fully elucidate the role of oxalic acid in *S. homoeocarpa* pathogenesis, isogenic lines with and without the ability to produce oxalic acid are necessary and could be created through random mutagenesis, as has been done to study the importance of oxalic acid for virulence of *S. sclerotiorum* (24).

Further support for differential production and use of oxalic acid by *S. homoeocarpa* and *S. sclerotiorum* was provided by *in vitro* oxalate quantification assays. These experiments demonstrated that *S. homoeocarpa* increases oxalic acid production in response to host tissue and xylan monomers. Conversely, host tissue or cell wall component monomers affected *in vitro* oxalic acid production by *S. sclerotiorum* (Fig. 10). Based on these results, we believe that oxalic acid production by *S. homoeocarpa* is induced by detection or breakdown of host cell wall components, particularly xylan. Secondary cell walls of dicot species contain 15-25% more xylan than primary cell walls (69). Thus, our findings that oxalic acid was not produced by *S. homoeocarpa* during infection of *A. thaliana* and was induced only by reproductively mature *N. benthamiana* leaves support a connection between xylan degradation and oxalic acid production in pathogenicity of *S. homoeocarpa*. Orshinsky and colleagues (58) identified genes for xylan degradation as among those most highly upregulated by *S. homoeocarpa* in host tissue 96 hpi. Additionally, *S. homoeocarpa* had the most potent xylan degrading activity of 86 phytopathogenic fungi tested in a study to assess degradation of various cell wall substrates (44). Xylanase activity has been identified as an important virulence factor for pathogens infecting a number of cereals, including wheat, rice, and corn (51,54,62,67). Though all of these findings indicate a connection between xylan degradation and oxalic acid in pathogenicity of *S. homoeocarpa,* functional characterization of xylanase genes is necessary to support this theory.

Based on the present research, we believe that both host endogenous oxalate content and pathogen-produced oxalic acid affect interactions between *S. homoeocarpa* and its hosts. Our finding that creeping bentgrass cultivars with resistance to dollar spot have lower oxalate levels than susceptible cultivars is the first indication that this physiological characteristic is correlated with dollar spot resistance. Further studies on the relationship between oxalate content and

resistance to dollar spot are needed, but this has potential as a quantifiable trait for selection of resistant clones in creeping bentgrass breeding programs. We also provide evidence that xylan triggers oxalic acid production by *S.homoeocarpa*, indicating that degradation of xylan may be an important mechanism for pathogenicity of this fungus. A major limitation to our studies was the lack of genetic resources for *S. homoeocarpa* and for its natural host, creeping bentgrass; however, we were able to identify potential model systems for study of *S. homoeocarpa*/host interactions in this research. In the future, use of a model host system and development of functional genetics resources for *S. homoeocarpa* will enable elucidation of the importance of host oxalate, pathogen-produced oxalic acid, and xylanase activity for pathogenesis of this fungus.

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Table S1. Plants used in this research

Common Name	Scientific Name	Abbreviation	Line[1]	Source
Creeping bentgrass	*Agrostis stolonifera*	As	Penncross	Tee-2-Green
Creeping bentgrass	*Agrostis stolonifera*	As	Focus	Seed Research of Oregon
Creeping bentgrass	*Agrostis stolonifera*	As	96-2	Seed Research of Oregon
Thale cress	*Arabidopsis thaliana*	At	Col-0	Andrew F. Bent lab collection
Purple false brome	*Brachypodium distachyon*	Bd	Bd 21-3	USDA-GRIN[2]
Barley	*Hordeum vulgare*	Hv	Parkland	USDA-GRIN
Rice	*Oryza sativa*	Os	Nipponbare	USDA-GRIN
Wheat	*Triticum aestivum*	Ta	Odessa	USDA-GRIN
Wild tobacco	*Nicotiana benthamiana*	Nb	N/A	Andrew F. Bent lab collection

[1]Depending on species, 'line' may refer to cultivar, ecotype, accession, or variety.
[2]United States Department of Agriculture Germplasm Resources Information Network; http://www.ars-grin.gov/npgs/index.html

Table S2. Fungal isolates used in this research

Scientific Name	Isolate Name	Source	Use[1]
Sclerotinia homoeocarpa	HE-10G-19	NC State Collection	Fig. 6, S3
Sclerotinia homoeocarpa	2F92-1	UW-Madison Collection	Fig. 1-4,9, S3
Sclerotinia homoeocarpa	Heath1A	UW-Madison Collection	Fig. 1-4, S3
Sclerotinia homoeocarpa	OJN9	UW-Madison Collection	Fig. 1-5,9,10, S3
Sclerotinia homoeocarpa	A617	NC State Collection	Fig. 9, S3
Sclerotinia homoeocarpa	ML75	NC State Collection	Fig. 6, S3
Sclerotinia homoeocarpa	S10	UW-Madison Collection	Fig. 1-4,8, S3
Sclerotinia homoeocarpa	A1623	NC State Collection	Fig. 6, S3
Sclerotinia homoeocarpa	Shark	UW-Madison Collection	Fig. S3
Sclerotinia homoeocarpa	LWC40	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	WMA4	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	F12-F22	UW-Madison Collection	Fig. S3
Sclerotinia homoeocarpa	F12-S12	UW-Madison Collection	Fig. S3
Sclerotinia homoeocarpa	BC20	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	RE-12G-38	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	A1476	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	ML715	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	LFD8	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	A1619	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	ML729	NC State Collection	Fig. S3
Sclerotinia homoeocarpa	A1421	NC State Collection	Fig. S3
Sclerotinia sclerotiorum	SS#21	UW-Madison Collection	Fig. 10

[1]The figure(s) listed indicate the experiments for which each isolate was used

Table S3. Symptom severity rating scale used for *Sclerotinia homoeocarpa* infection of creeping bentgrass and models hosts

Rating	Percent symptom severity	Description of symptoms
0	0	No visible symptom apparent
1	1-10	Slight necrosis or small, defined lesions
2	11-25	Spreading lesion; most host tissue still green
3	26-50	Spreading lesion; chlorosis possible; nearly half of tissue symptomatic
4	51-75	Majority of leaf symptomatic and beginning to loose structural integrity; small green areas may remain
5	76-90	Nearly all tissue symptomatic; little to no green areas remaining; wilting or loss of structural integrity
6	91-100	No green tissue remaining near complete loss of structural integrity

Table S4. Primers used for RT-qPCR expression analysis of creeping bentgrass GLP genes

Primer name	Target GenBank Accession ID	Primer sequence	Product length
CRB_ACT7_F	DY543529	CCAGCAGCTTCCATACCAAT	162
CRB_ACT7_R	DY543529	AGCTCGCATATGTGGTCTT	162
CRB_GAPDH_F	GR280385	GGTTGTCATCTCAGCCCCTA	239
CRB_GAPDH_R	GR280385	CACCTCTCCAGTCCTTGCTC	239
AST_608_F	JU115988	TCGGATCAGATGCAATAG	130
AST_608_R	JU115988	GGACACAACAATACAACAA	130
AST_590_F	JU115794	ACACAACAAAGCCACAAA	83
AST_590_R	JU115794	AAGAGCGACAAGAAGGAA	83
AST_854_F	JU118678	GGACAACCACAATTAAATCAAT	152
AST_854_R	JU118678	ATCATCACAGAACAAGACAAT	152
AST_798_F	JU118061	ATTGCCTTTGGAAATACG	93
AST_798_R	JU118061	GACACTCTACCTTGGATT	93

Table S5. Cell wall components used for *in vitro* oxalic acid production assays

Component	Description	Product Number	Distributor
Cellulose	Cellulose	310697	Sigma Aldrich
Lignin	Lignin, alkali	370959	Sigma Aldrich
Pectin	Pectin	416862500	Acros Organics
Xylan	Xylan from beechwood	X4252	Sigma Aldrich

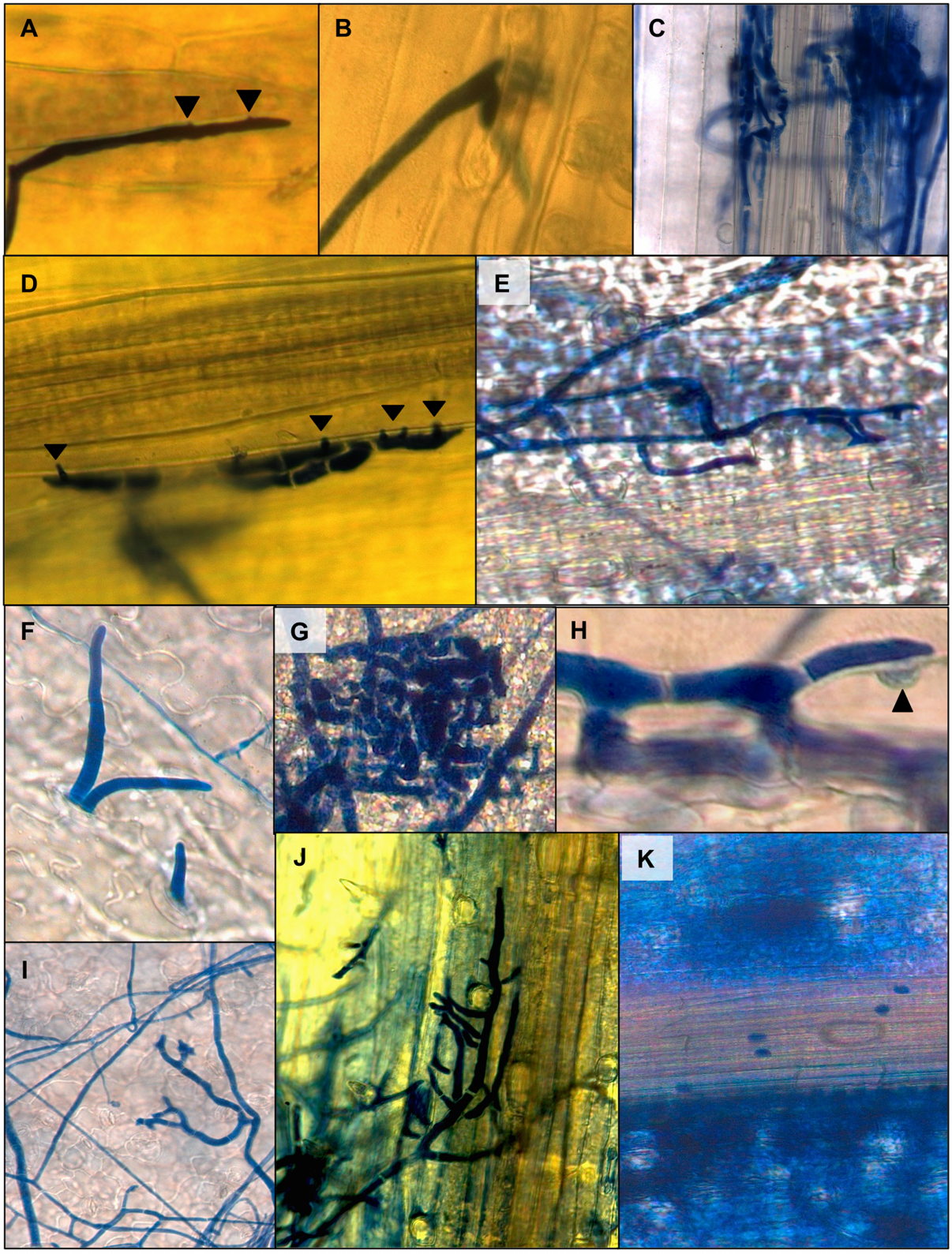
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Figure 1. Microscopic analysis of plant foliar tissue colonization by *Sclerotinia homoeocarpa*. In-tact plants were inoculated and plant samples were taken by excising the area around the site of inoculation. All samples were cleared of chlorophyll and stained with 0.01% trypan blue. Host penetration was observed as early as 6 hpi and included: **A.** Growth along cell walls and formation of infection pegs (arrowheads) projecting directly into host tissue; shown in creeping bentgrass. **B.** Direct penetration through stomata; shown in barley and **C.** Extensive colonization of host tissue with an affinity for vascular tissue; shown in barley. At 12 to 24 hpi fungal hyphae continued to grow along cell walls and form penetration pegs into vascular tissue, as observed in **D.** Creeping bentgrass at 12 hpi **E.** Wheat at 24 hpi and **G.** Rice at 24 hpi. **F.** *S. homoeocarpa* protruding from a stomate in *Arabidopsis thaliana* tissue colonized by fungal mycelia. **H.** Appressorium (arrowhead) formed by an *S. homoeocarpa* hypha on barley at 96 hpi. Extensive host cell death, as indicated by trypan blue staining outside of fungal hyphae, was not observed until 4-7 dpi regardless of host. **J.** Extensive cell death 7 dpi inoculation of *Brachypodium distachyon* with *S. homoeocarpa*. **K.** Extensive cell death outside of host vascular tissue at 7 dpi in wheat. Images are representative of similar observations made for all hosts and *S. homoeocarpa* isolates used in this study.


A
B
C
D
E
F
G

Figure 2. Symptoms produced by *Sclerotinia homoeocarpa* on natural and model host plants. *S. homoeocarpa* infected and produced visible symptoms on all host plants. Similar symptoms were observed on **A.** *Brachypodium distachyon* **B.** Wheat **C.** Barley and **D.** Rice. These symptoms included light tan to white lesion with reddish brown borders and resembled those observed on **E.** Creeping bentgras, a natural host. **F.** Creeping bentgrass stand symptoms resulting from our parafilm sachet inoculation method looked visibly similar to symptoms observed in the field. **G.** Foliar symptoms observed on *Arabidopsis thaliana* were distinct from those observed on monocot hosts and were characterized by water soaking and tissue collapse.

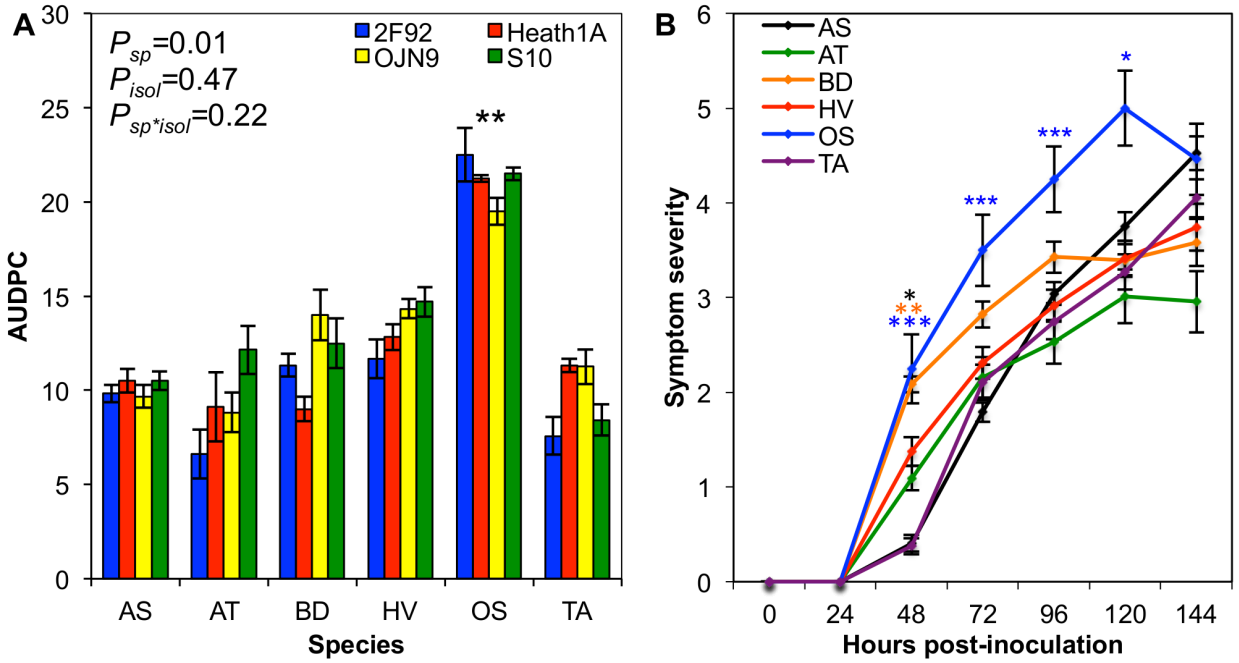

Figure 3. Symptom severity of *Sclerotinia homoeocarpa* infection on natural and model hosts. All plants were inoculated in the same time in a split-plot RCBD with host as the whole-plot factor and *S. homoeocarpa* isolate as the sub-plot factor. Three experimental repetitions were conducted and treated as a random blocking factor in experimental analyses. Blocks within experimental repetitions were considered random factors nested within repetition. Host, isolate, and host x isolate interaction were fixed effects. **A.** AUDPC for each *S. homoeocarpa* isolate on the six host plants. Colored bars represent four different *S. homoeocarpa* isolates. P-values presented are from a two-way ANOVA with $\alpha = 0.05$. Asterisks indicate significant difference from the natural host creeping bentgrass (AS) based on a Dunnett's test with $\alpha = 0.05$. Only the AUDPC for rice (OS) differed from that of creeping bentgrass. N≥9 for each species*isolate combination. **B.** Time-course progression of infection by *S. homoeocarpa* on all host species. The black asterisk indicates an overall significant effect of species on symptom severity at 48 hpi, as assessed by one-way ANOVA. Blue and orange asterisks indicate significant differences between rice and *B. distachyon* (BD), respectively, and creeping bentgrass at the corresponding time-points based on analysis with Dunnett's test. N≥30 for each species. Abbreviations for both figures are as follows: AS=*Agrostis stolonifera* (creeping bentgrass); AT=*Arabidopsis thaliana*; BD=*Brachypodium distachyon*; HV=*Hordeum vulgare* (barley); OS=*Oryza sativa* (rice); and TA=*Triticum aestivum* (TA). For significance codes, *=P≤0.05; **=P≤0.01; ***=P≤0.001. All error bars represent ± one standard error of the mean.

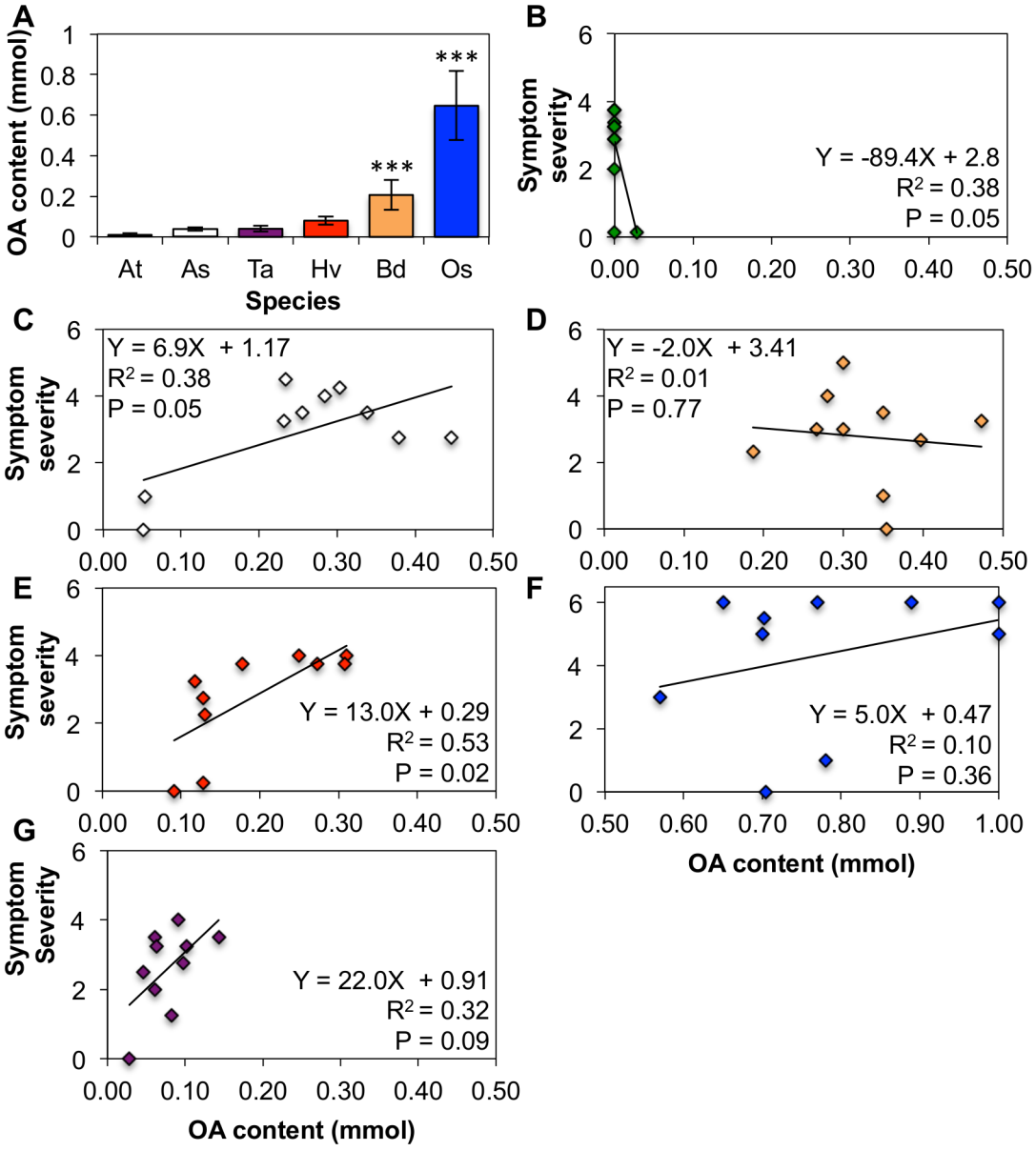

Figure 4. Correlation between host endogenous oxalate content and symptom severity. A. Endogenous oxalate content of all host species used in these study. Asterisks indicate significant difference from creeping bentgrass (AS) at the α=0.05 level using Dunnett's test. Error represent ± one standard error of the mean. Correlation between oxalate content and symptom severity of *S. homoeocarpa* infection were fit a linear regression model for **B.** *A. thaliana* **C.** Creeping bentgrass **D.** *Brachypodium distachyon* **E.** Barley **F.** Rice and **G.** Wheat. N=10. All regression equations, R^2, and P-values were obtained from models fit with SAS proc reg. Abbreviations for both figures are as follows: AS=*Agrostis stolonifera* (creeping bentgrass); AT=*Arabidopsis thaliana*; BD=*Brachypodium distachyon*; HV=*Hordeum vulgare* (barley); OS=*Oryza sativa* (rice); and TA=*Triticum aestivum* (TA). For significance codes, *=P≤0.05; **=P≤0.01; ***=P≤0.001. All error bars represent ± one standard error of the mean.

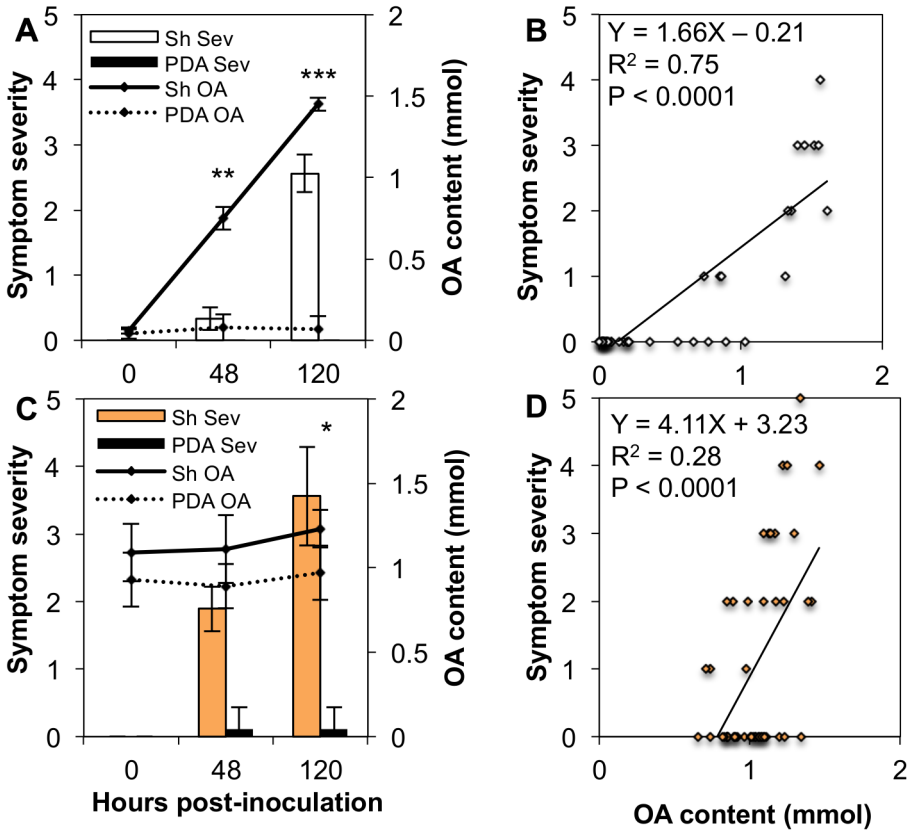

Figure 5. Time-course for the relationship between symptom severity and oxalate content in creeping bentgrass and *Brachypodium distachyon.* A. Time-course of symptom severity and oxalate content in creeping bentgrass. Asterisks indicate difference between oxalate content in *S. homoeocarpa* and mock-inoculated plants by one-way ANOVA with a cut-off value of α=0.05. Errors bars represent ± one standard error of the mean. **B.** Scatter plot showing the correlation between oxalate content and symptom severity for creeping bentgrass. Regression line calculated from simple linear regression in SAS proc reg. **C.** Time-course of symptom severity and oxalate content in *B. distachyon*. Asterisks indicate difference between oxalate content in *S. homoeocarpa* and mock-inoculated plants by one-way ANOVA with a cut-off value of α=0.05. Errors bars represent ± one standard error of the mean. **B.** Scatter plot showing the correlation between oxalate content and symptom severity for *B. distachyon*. Regression line calculated from simple linear regression in SAS proc reg. For significance codes, *=P≤0.05; **=P≤0.01; ***=P≤0.001. All error bars represent ± one standard error of the mean.

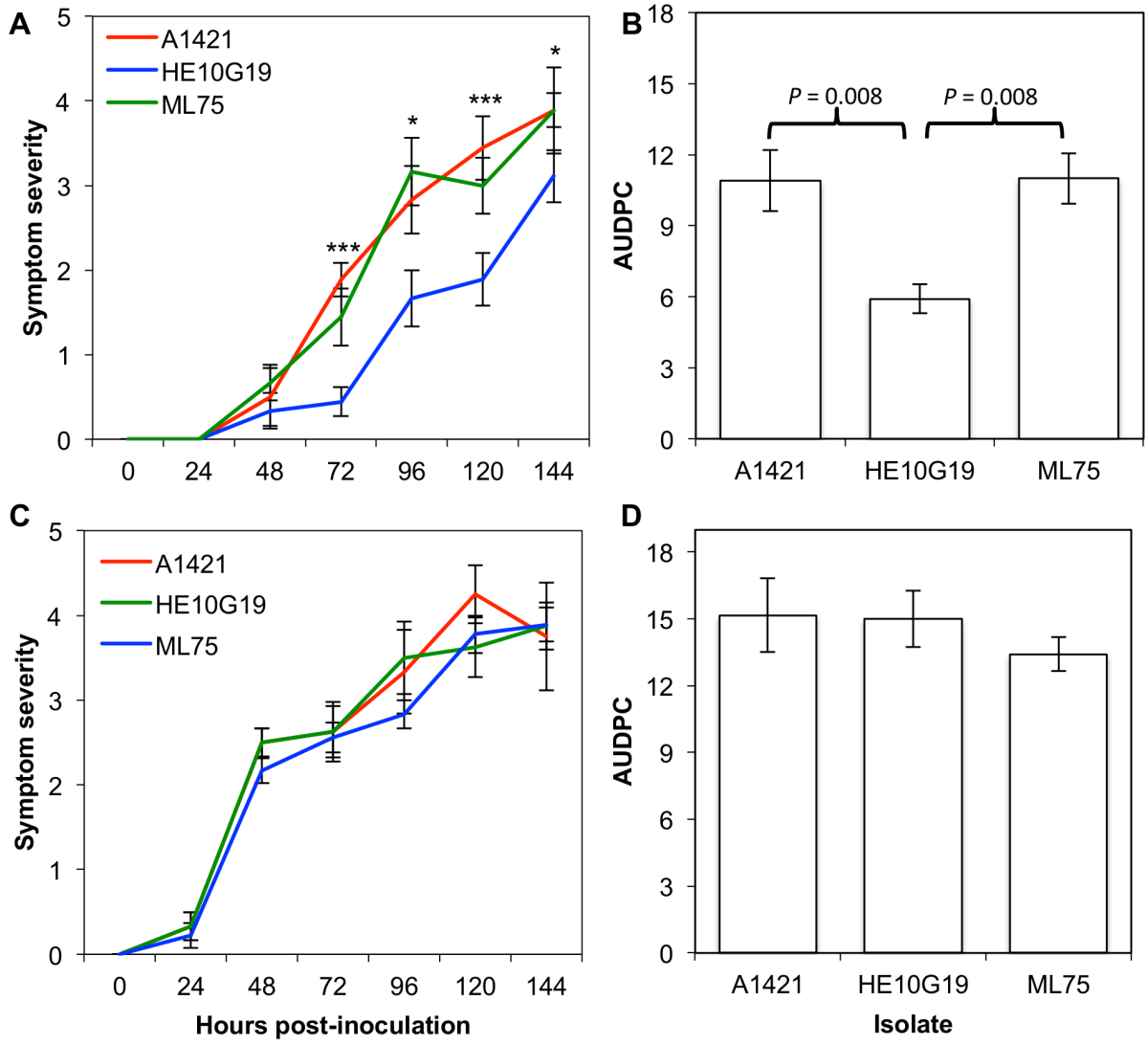

Figure 6. Progression of infection on creeping bentgrass and *Brachypodium distachyon* by *Sclerotinia homoeocarpa* isolates with varying oxalic acid production capacities. Isolates producing high, moderate, and low amounts of oxalic acid were selected based on their ability to produce a color change in pH indicator-amended media (Fig. S3). In line graphs, red, green, and blue lines indicate isolates with high, moderate, and low oxalic acid production abilities, respectively. Creeping bentgrass and *B. distachyon* experiments were performed in tandem. Values shown in these graphs represent the means from the three experimental repetitions. **A.** Time-course of symptom development and **B.** AUDPC of symptom severity on creeping bentgrass. **C.** Time-course of symptom development and **B.** AUDPC of symptom severity on *B. distachyon.* Asterisks in **A** represent a significant effect of isolate in one-way ANOVA with α=0.05. P-values in **B** were obtained through orthogonal contrasts comparing the AUDPC means between the isolates indicated. No difference between isolates was detected for *B. distachyon*.

For significance codes, *=P≤0.05; **=P≤0.01; ***=P≤0.001. All error bars represent ± one standard error of the mean.

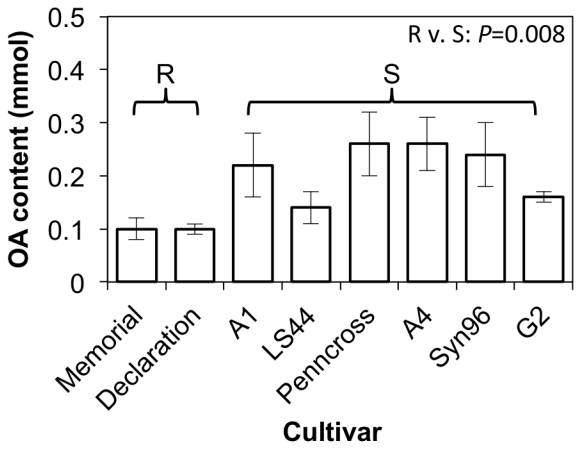

Figure 7. Endogenous oxalate content of creeping bentgrass cultivars collected from the field. Samples were collected in July, 2013 from a National Turfgrass Evaluation Program bentgrass variety trial located at the O.J. Noer Turfgrass Research Facility in Verona, Wisconsin. Oxalate content was quantified within 24 h of sampling. The field trial was arranged as a randomized complete block design with four replications. Samples were collected from each block and statistical analysis of sample oxalate concentration followed the field experimental design. Designation of cultivars as resistant ('R') or susceptible ('S') is based on dollar spot severity in three years of field trials (Fig. S4). An orthogonal contrast was used to compare endogenous oxalate content of resistant and susceptible cultivars. Columns represent the mean oxalate content from the four replicate samples for each cultivar and error bars represent ± one standard error of the mean.

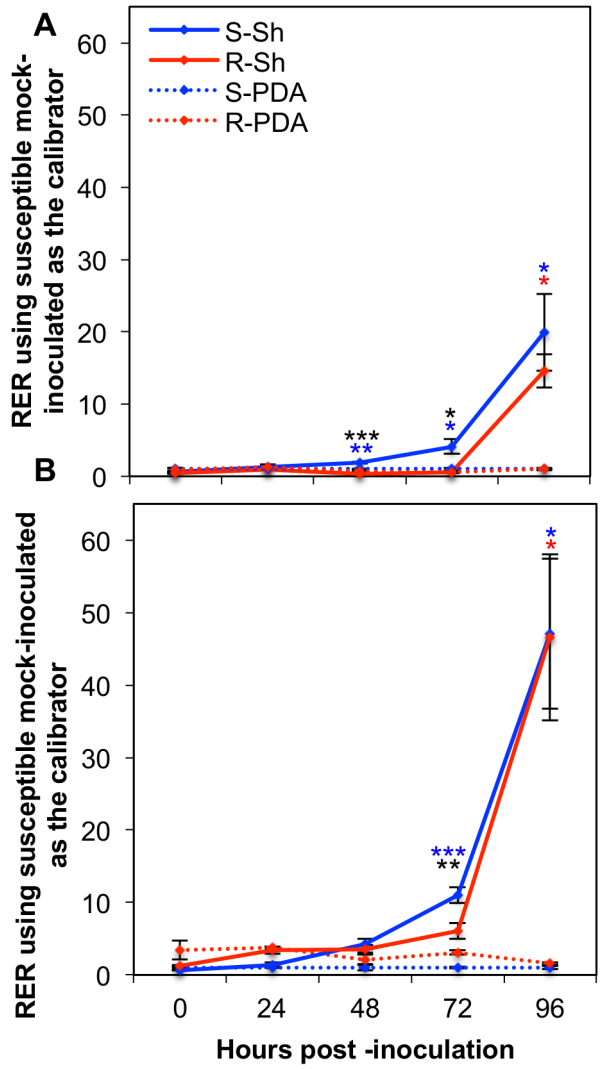

Figure 8. Time-course expression of oxalate oxidase and another germin-like protein gene in resistant and susceptible creeping bentgrass cultivars. A. Time-course expression of the creeping bentgrass oxalate gene AST_798. **B.** Time-course expression for the creeping bentgrass GLP GER4 subfamily gene AST_854. Relative expression ratios shown were normalized the constitutively expressed creeping bentgrass ACT7 reference gene. Similar results were obtained with the creeping bentgrass GAPDH gene. In the legend for both **A** and **B**, 'S' and 'R' represent the 'susceptible' and 'resistant' cultivar, respectively; 'Sh' and 'PDA' indicate '*S. homoeocarpa*-inoculated' and 'PDA mock-inoculated' samples, respectively. Orthogonal contrasts were used to compare relative expression ratios between specific treatment pairs at the α=0.05 level. Black asterisks indicate a significant difference between relative expression ratios for the *S. homoeocarpa*-inoculated resistant and susceptible cultivar; blue asterisks indicate a significant difference the *S. homeoocarpa* and mock-inoculated samples for the susceptible cultivar; red asterisks indicate a significant difference between the *S. homoeocarpa* and mock-inoculated

control for the resistant cultivar. For significance codes, *=P≤0.05; **=P≤0.01; ***=P≤0.001. All error bars represent ± one standard error of the mean.

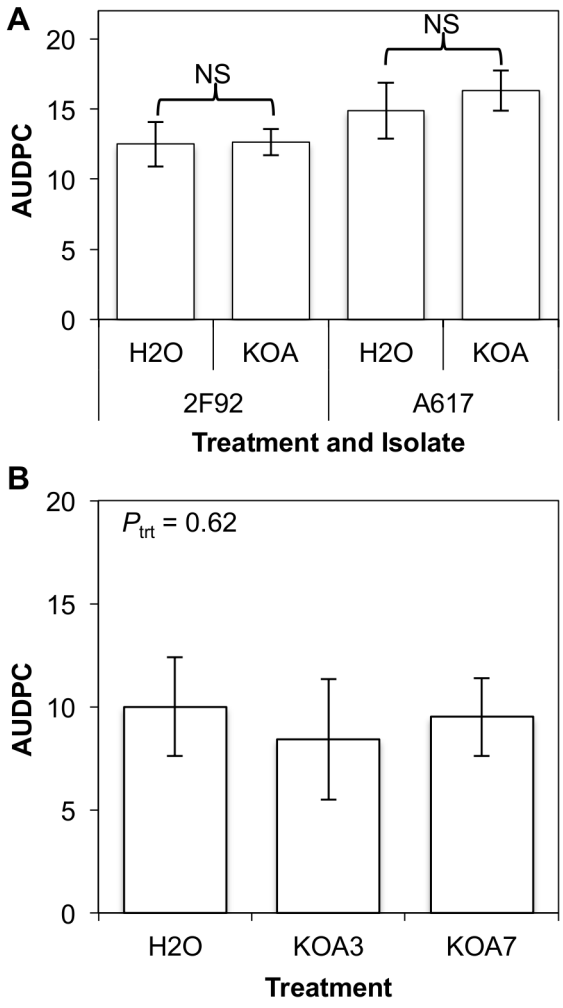

Figure 9. KOA infiltration of *Nicotiana benthamiana* and barley. A. AUDPC of *N. benthamiana* leaves infiltrated with H2O or 10mmol KOA (pH7)prior to *S. homoeocarpa* inoculation. Orthogonal contrasts between the indicated treatment pairs were not significant at the α=0.05 level. **B.** AUDPC of barley leaves infiltrated with H2O, 10 mmol KOA (pH7), 10 mmol KOA (pH3) prior to *S. homoeocarpa* inoculation. Overall one-way ANOVA indicated no difference in AUDPC between treatments at the α=0.05 level. Columns represent AUDPC means from three pooled experiments, with experimental repetition treated as a random blocking factor in statistical analyses. Errors bat represent ± one standard error of the mean.

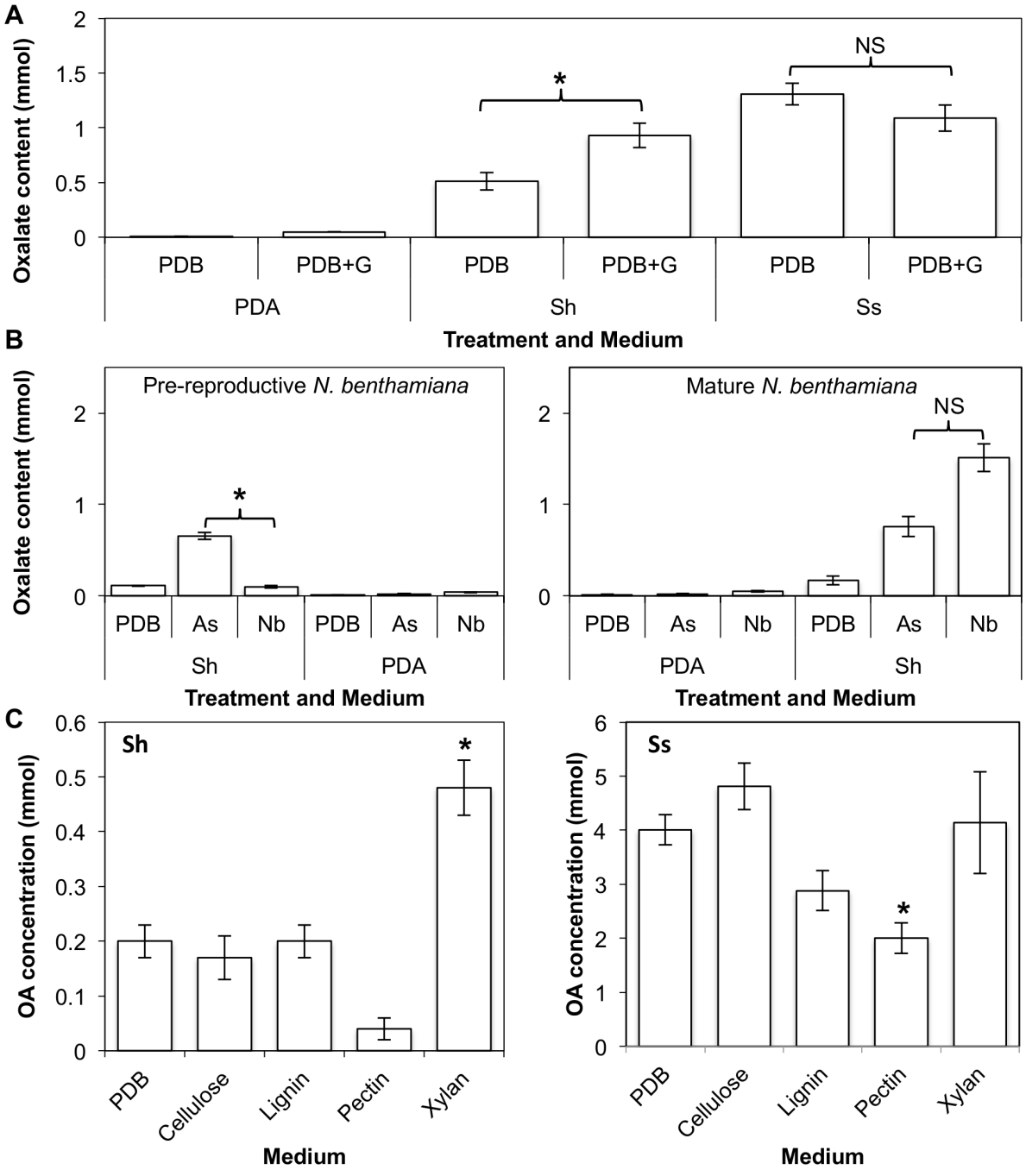
A
Oxalate content (mmol)
2
1.5
1
0.5
0
*
NS
PDB
PDB+G
PDB
PDB+G
PDB
PDB+G
PDA
Sh
Ss
Treatment and Medium
B
Oxalate content (mmol)
Pre-reproductive N. benthamiana
Mature N. benthamiana
*
NS
PDB
As
Nb
PDB
As
Nb
Sh
PDA
PDA
Sh
Treatment and Medium
Treatment and Medium
C
OA concentration (mmol)
Sh
Ss
*
*
PDB
Cellulose
Lignin
Pectin
Xylan
Medium
Medium

Figure 10. Factors affecting oxalic acid production by *Sclerotinia homoeocarpa* and *S. sclerotiorum in vitro*. A. Oxalic acid content from culture filtrates of *S. homoeocarpa* or *S. sclerotiorum* grown in medium with (PDB+G) or without (PDB) grass amendments. Asterisks indicate a significant difference at the α=0.05 level. **B.** Oxalic acid content of culture filtrates from *S. homoeocarpa* (Sh) grown in unamended media (PDB), media amended with creeping bentgrass (As), or medium amended with *N. benthamiana* (Nb). Mock-inoculated (PDA) controls are also shown for each medium. The first panel represents experiments in which medium was amended with pre-reproductive leaf tissue. The second panel represents experiments in which *N. benthamiana* leaves were collected from reproductively mature plants. Significance values were obtained from orthogonal contrast comparing the indicated at treatment and medium combinations at the α=0.05 level. **C.** Oxalic acid content of filtrate collected from cultures of *S. homoeocarpa* ('Sh'; left panel) or *S. sclerotiorum* ('Ss'; right panel) grown in medium with indicated cell wall component monomer amendments. Results from the two fungi were separated due to the much higher oxalic acid production by *S. sclerotiorum*. A Dunnett's test was used to compare oxalic acid production in cell wall component-amended media against the unamended PDB control. Bars with asterisks above them were significantly different from the PDB control at the α=0.05 level. Error bars in all graphs represent ± one standard error of the mean.

Figure. S1. Methods used for inoculation of various hosts with *Sclerotinia homoeocarpa.* A. Inoculation of *Arabidopsis thaliana* leaves with single agar plugs placed mycelia-side down on leaf surfaces. Inoculated plants were covered with a humidity dome to maintain high relative humidity. **B.** *Brachypodium distachyon* plants were inoculated by placing an agar plug colonized with *S. homoeocarpa* mycelium-side down against foliar material and wrapping with parafilm. **C.** Pots of creeping bentgrass were inoculated by collecting leaf blades in the center of the pot into a bundle and placing a single agar plug against the foliar tissue, then wrapping with parafilm. **D.** The most recent fully expanded leaf of barley, rice, and wheat plant were inoculated by placing a single agar plug colonized with *S. homoeocarpa* mycelia side down on the adaxial side of the leaf , then wrapping with parafilm. Mock-inoculations were performed similarly for all species but *S. homoeocarpa* colonized plugs were replaced with fresh PDA plugs.

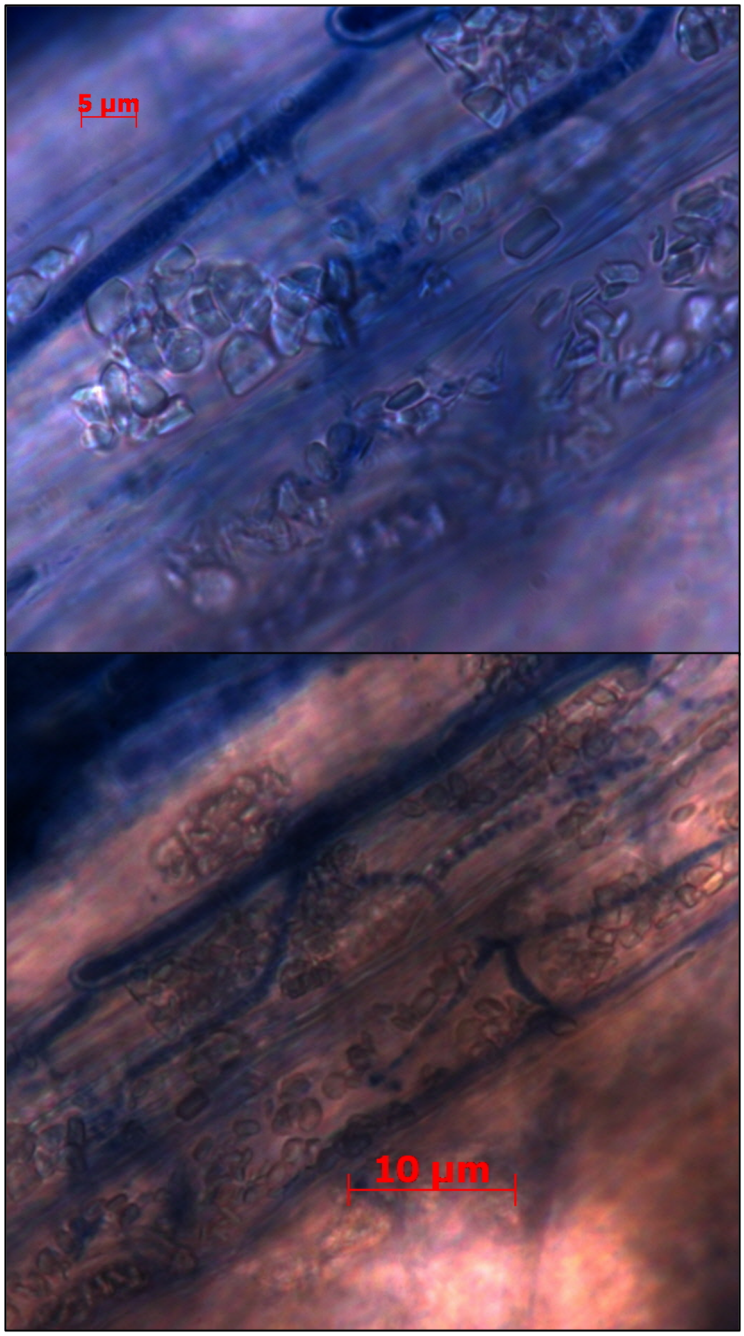

Figure. S2. Possible calcium oxalate crystals in barley 7 dpi with *S. homoeocarpa.* Top. 400X. **Bottom.** 200X. Leaf samples were stained with trypan blue and visualized with an Axio Scope.A1 compound microscope. Images were captured with a AxioCam MRc camera and processed with AxioVision Real 4.7.1 imaging software. No similar structures were observed in uninoculated hosts.

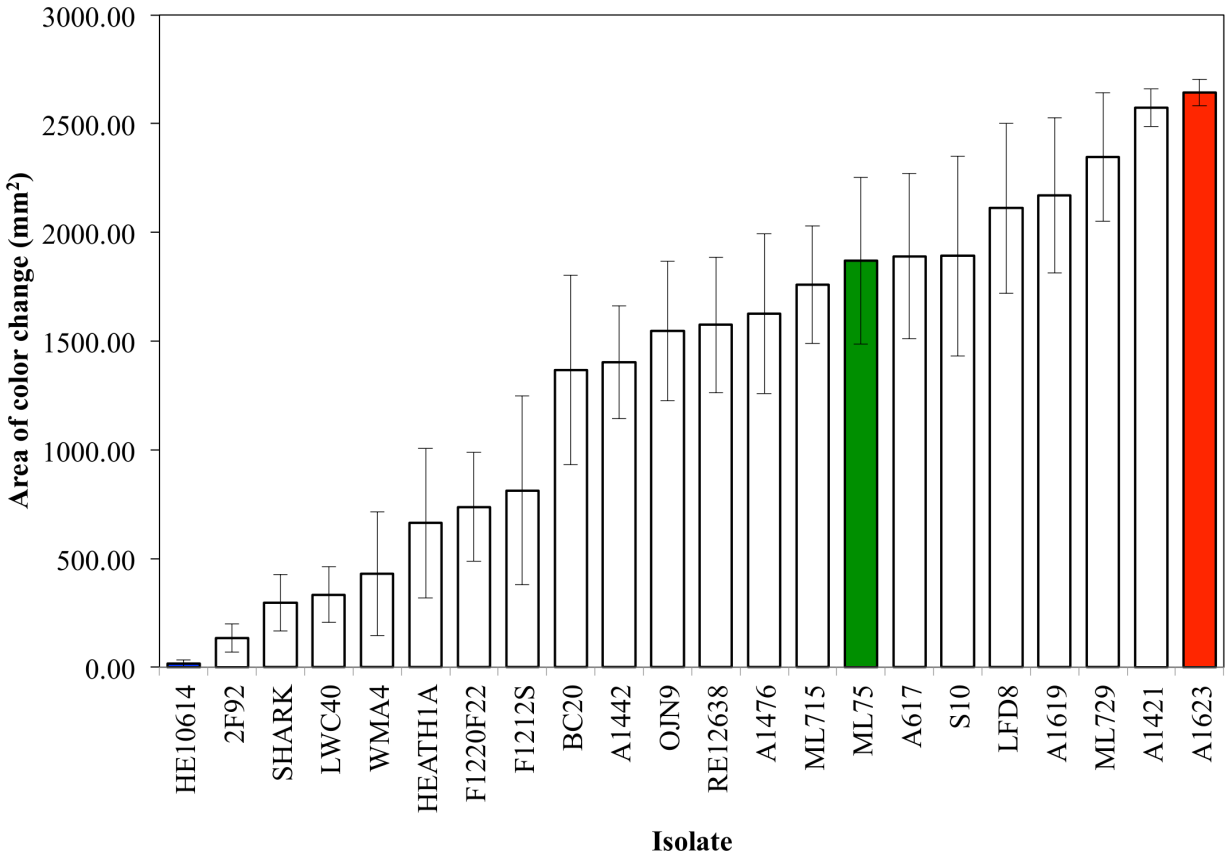

Figure. S3. Oxalic acid production by *S. homoeocarpa* isolates. Oxalic acid production was measured *in vitro* using bromophenol blue amended medium, which changes from purple to yellow in color as a result of medium acidification. The area of color change was measured by averaging two perpendicular contrasts across the yellow areas. When areas of color change were diffuse, all were measured and the average measurements were combined. Columns represent the pooled means from three experimental repetitions with three replicates per isolate in each repetition (n=9). Error bars represent ± one standard error of the mean. A1623 (red), ML75 (green), and HE10G14 (blue) were selected as isolates with high, moderate, and low oxalic acid production capacities, respectively.

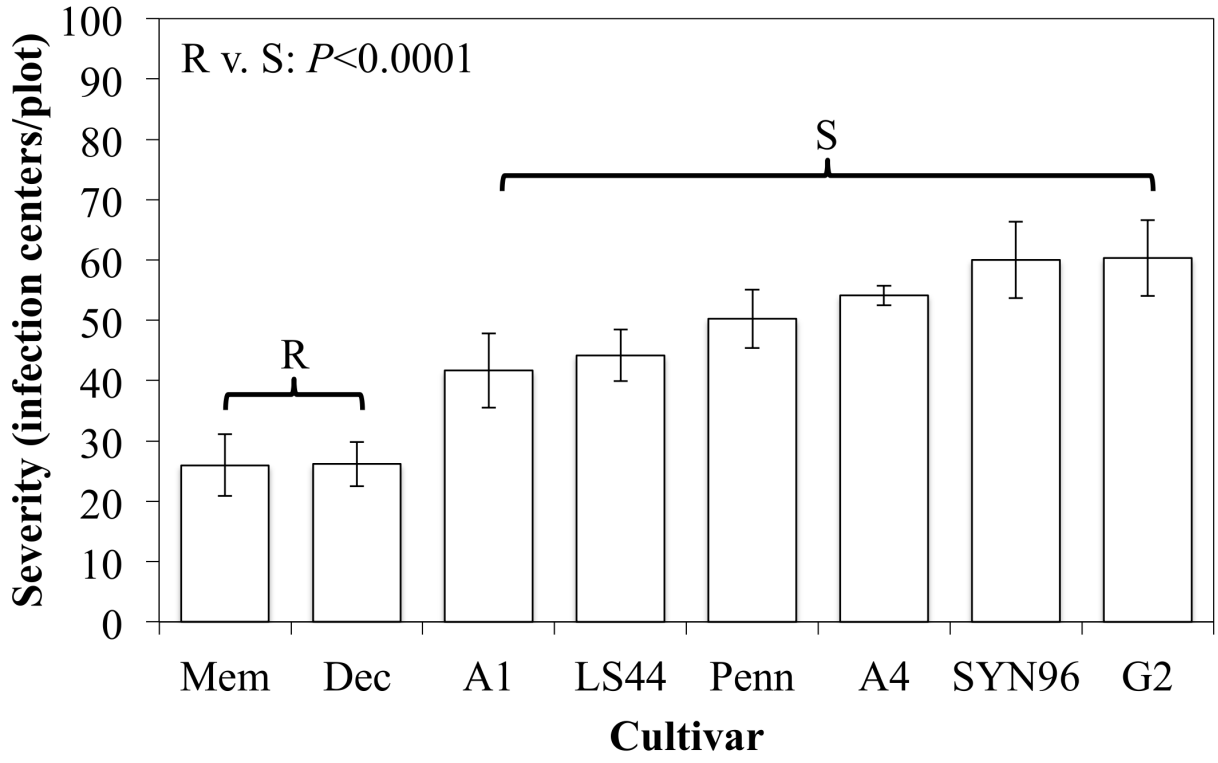

Figure. S4. Dollar spot severity for eight creeping bentgrass cultivars from an NTEP creeping bentgrass fairway variety trial at the OJ Noer Turfgrass Research Facility in Verona, WI. Disease severity data were collected during the summers of 2009-2011. Columns represent mean disease severity over the three years of data collection. The trial was arranged as an RCBD with four replications. For statistical analysis, blocks were treated as nested within years. An orthogonal contrast was used to compare disease severity between the two cultivars considered resistant to dollar spot and the remaining six cultivars with a significance cut-off value of α=0.05. Error bars represent ± one standard error of the mean.

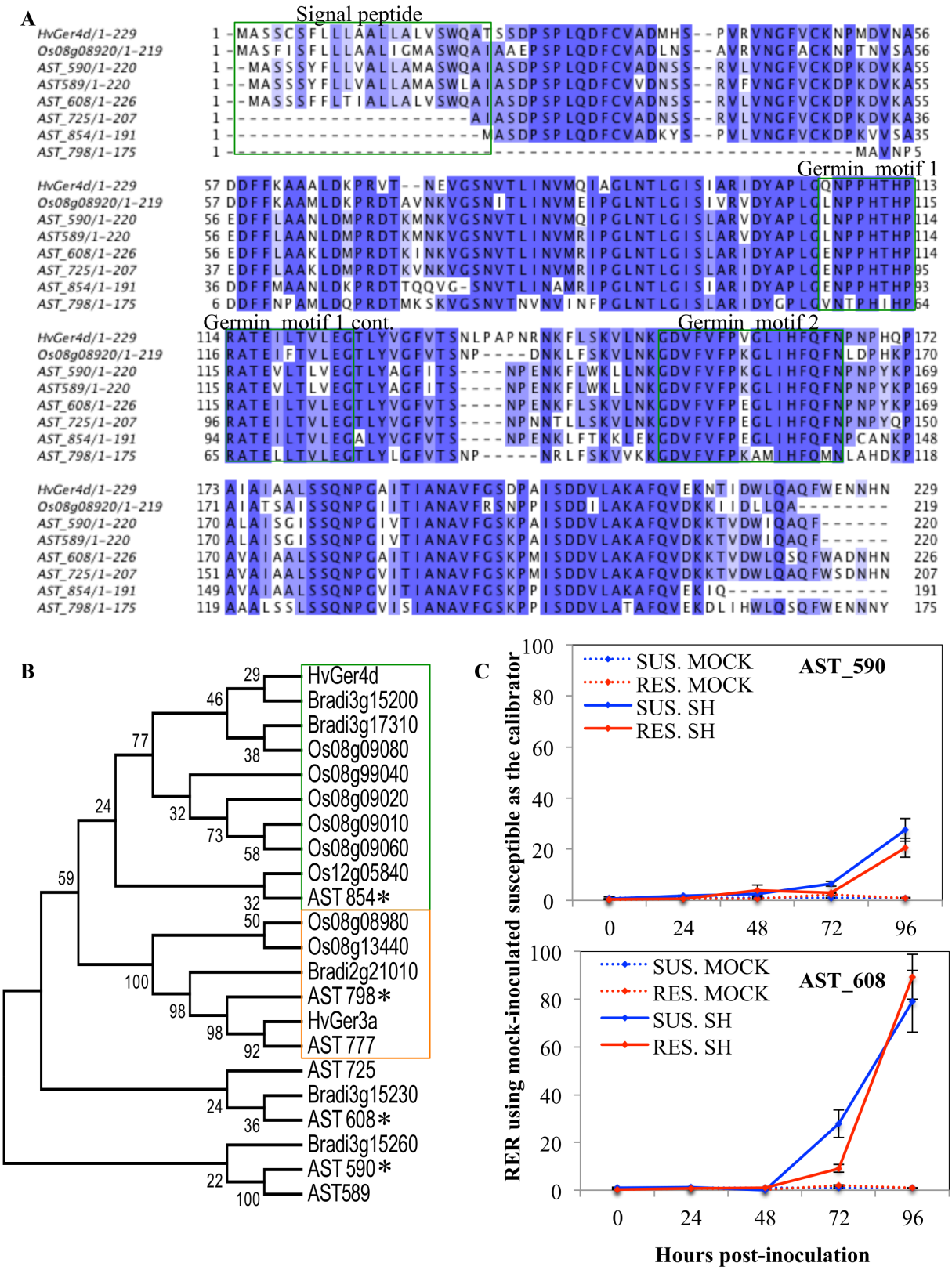
A
Signal peptide
HvGer4d/1-229
Os08g08920/1-219
AST_590/1-220
AST589/1-220
AST_608/1-226
AST_725/1-207
AST_854/1-191
AST_798/1-175
Germin_motif 1
Germin_motif 1 cont.
Germin_motif 2
B
HvGer4d
Bradi3g15200
Bradi3g17310
Os08g09080
Os08g99040
Os08g09020
Os08g09010
Os08g09060
Os12g05840
AST 854*
Os08g08980
Os08g13440
Bradi2g21010
AST 798*
HvGer3a
AST 777
AST 725
Bradi3g15230
AST 608*
Bradi3g15260
AST 590*
AST589
C
AST_590
SUS. MOCK
RES. MOCK
SUS. SH
RES. SH
AST_608
RER using mock-inoculated susceptible as the calibrator
Hours post-inoculation

Figure. S5. Selection of candidate oxalate oxidase and germin-like protein genes. A. CRB (AST) and representative barley (HvGer) and rice (Os) GLP amino acid sequences from rice and barley were aligned using ClustalW. JalView was used to color sequence alignment according to percent identity. Key features of GLP genes are annotated with green boxes and black text. Blue arrowheads mark the start and end of the GLP cupin domain. 'AST' nucleotide sequences were originally identified by Orshinsky et al. 2012. **B.** Maximum parsimony phylogenetic tree (100 bootstraps with sequence order re-arranging over 10 data sets) of CRB and select grass GLP-genes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Rice and barley defense-associated GLP genes are indicated by the green box. Oxalate oxidase-type CRB GLP-genes are indicated by the orange box. RT-qPCR analysis of expression was performed for creeping bentgrass genes marked by an asterisk. **C.** Time-course expression analysis for creeping bentgrass GLP-genes AST_590 and AST_608. Both genes were significantly up-regulated in the *S. homoeocarpa*-inoculated susceptible cultivar relative to the *S. homoeocarpa*-inoculated resistant cultivar and mock-inoculated susceptible cultivar at 72 hpi ($P<0.05$). At 96 hpi, both genes were upregulated in the *S. homoeocarpa*-inoculated susceptible and resistant cultivars relative to the mock-inoculated controls for each cultivar ($P<0.05$). There was no difference in expression of either gene between the *S. homoeocarpa*-inoculated susceptible and resistant cultivars at 96 hpi ($P>0.10$).

CHAPTER 5: *BRACHYPODIUM*: A POTENTIAL MODEL HOST FOR FUNGAL PATHOGENS OF TURFGRASSES

This chapter is a preliminary version of the following manuscript:

Rioux, R.A., Van Ryzin, B.J., and J.P. Kerns. 2014. *Brachypodium*: A potential model host for fungal pathogens of turfgrasses. BMC Plant Biology. *In preparation.*

Contribution: R.A.R. was the primary author. She designed experiments, performed *S. homoeocarpa* inoculations on Bd 21-3, collected analyzed data, and wrote the manuscript. B.V.R. performed *S. homoeocarpa* inoculations on *Brachypodium* sp. accessions as well as *R. solani* and *M. nivale* inoculations and collected data. J.P.K. helped with experimental design and drafting of the manuscript.

Abstract

Background: Dollar spot, brown patch, and *Microdochium* patch, are three of the most economically devastating fungal diseases of amenity turfgrasses worldwide. Management of these diseases is achieved through frequent fungicide applications, which are undesirable for a number of reasons. Disease resistant turfgrass cultivars are a viable management alternative, but progress in this realm is hampered by a limited understanding of how turfgrass pathogens interact with their hosts. *Brachypodium distachyon* is a C3 grass with an assortment of genetic resources and, due its genetic similarity to many cool-season turfgrasses, is an attractive model host system for studying pathogenicity factors of major turfgrass pathogens. The aims of this study were i) to evaluate pathogenicity of three major fungal turfgrass pathogens towards *B. distachyon* inbred line Bd 21-3 ii) to perform an in-depth study of pathogenesis by the dollar spot fungus, *Sclerotinia homoeocarpa*, on Bd 21-3 and iii) to evaluate natural variation of *Brachypodium* sp. ecotypes in response to *S. homoeocarpa* isolates.

Results: Infection assays with two or more isolates of the casual agents of dollar spot, brown patch, and *Microdochium* patch resulted in compatible interactions with Bd 21-3. The symptoms produced by these pathogens on Bd 21-3 closely resembled those observed on the natural turfgrass host, creeping bentgrass, in the lab and frequently observed on other turfgrass species in the field. Histological studies of Bd 21-3 infection by *S. homoeocarpa* also revealed colonization similar to that observed on creeping bentgrass, including formation of appressoria and penetration through stomata. When challenged with *S. homoeocarpa*, all the natural *Brachypodium* sp. ecotypes were susceptible, but disease severity varied among ecotypes. No ecotype by isolate interaction was detected, indicating a lack of race-specific interactions.

Conclusions: *B. distachyon* is susceptible to the fungal pathogens that cause dollar spot, brown patch, and *Microdochium* patch on turfgrasses. The symptoms produced by each causal agent are distinct from one another on *B. distachyon,* yet similar to the symptoms produced by each pathogen on turfgrasses. Similarly, colonization of Bd 21-3 by *S. homoeocarpa* at the microscopic level closely resembles colonization of creeping bentgrass by this pathogen. Taken together, these findings demonstrate that *B. distachyon* can be used as a model host system for these turfgrass diseases. Additionally, the differences in susceptibility of *Brachypodium* sp. ecotypes indicate genetic differences that can be leveraged to better understand the molecular components of host resistance to *S. homoeocarpa*.

Keywords : turfgrass, *Brachypodium distachyon*, dollar spot, snow mold, host-pathogen interactions

Background

The turfgrass industry generates over $40 billion of revenue in the United States annually and makes significant contributions to the global economy (18) Though generally not thought of as an important ‘crop’ plant, turfgrass covers roughly 35 million acres of land in the United States alone (18) and was recently identified as the largest irrigated crop in the United States (44). Additionally, turf provides many functional, recreational, and ornamental benefits to human activities, making it an important provider of ecosystem services (9). Professional turfgrass sectors include golf courses, sod production, institutional facilities, and management services, such as lawn care and irrigation. Of these, the golf course industry is the single largest contributor, generating $33.2 billion in gross annual revenues and providing hundreds of thousands of jobs (18,32).

High quality turf is essential in the golf course industry and is generally achieved through frequent applications of pesticides for weed, insect, and disease control. It is estimated that fungicide applications for disease management cost the golf industry $174.40 million per year in the United States (39)). A single golf course may spend $20,000-50,000 on fungicide applications for putting greens, a small but intensively managed part of the golf course, in a single year. In addition to the economic costs associated with frequent fungicide applications, non-target effects of fungicides can be hazardous to the environment (5,8) and the development of fungicide resistance in target pathogens is a common result of repeated exposure to a single active ingredient (41)). Biological and cultural controls offer alternatives to frequent fungicide applications, but fail to consistently achieve adequate disease suppression comparable to that of chemical management tactics (40)).

Breeding popular turfgrass species for better disease resistance is the primary goal for sustainable disease management in golf course settings (15). Due to the lack of genomic sequence data and other genetic tools for major turfgrass species, breeding for disease resistance has largely been based on traditional methods and has proceeded at a slow, albeit steady, pace (11,14,15,63). A biologically relevant model host for important turfgrass pathogens would enable precise identification of key genes involved in disease resistance or susceptibility. These genes could then be specifically targeted in turfgrass breeding efforts to produce a more streamlined approach towards development of disease resistant cultivars (62). Unfortunately, an ideal model host for turfgrass pathogenesis studies has not previously been identified.

The genus *Brachypodium* includes C3 grass species in the subfamily Pooideae and is closely related to the major cool season grass tribes Avenae, Bromae, Poeae, and Triticeae (38). Just over a decade ago, a member of this genus, *Brachypodium distachyon* (L.) P. Beauv., was

proposed as a novel model system for both turf-type/forage grasses and temperate cereals (26). More recently, the biofuels industry has adopted *B. distachyon* as a model due to its similarities with key bioenergy crops, including *Miscanthus* and switchgrass (31,70). Much like the dicot model plant *Arabidopsis thaliana*, *B. distachyon* is small, fast growing, amenable to inbreeding, and has a simple diploid genome (26). Genetic resources for *B. distachyon* have steadily increased since its emergence as a model species, including the release of a high-quality, annotated complete genomic sequence in 2010 (30). The availability of a genomic sequence and accompanying genetic resources (17,28)(http://brachybase.org; http://www.phytozome.net www.brachypodium.org) have encouraged wide-spread use of *B. distachyon* as a model for surveys of evolutionary genetics in grasses and comparisons of grass genomes (16,30). *B. distachyon* has also been successfully used in multiple cases to generate and refine linkage maps for cool-season grasses (21,69,70).

Multiple options for functional genetics studies are also available for *B. distachyon*. Draper and colleagues (26) first described an efficient transformation system for *B. distachyon* using microprojectile transformation technology and hygromycin selection. More recently, various methods employing *Agrobacterium*-mediated T-DNA transformation of *B. distachyon* have been developed and refined to achieve consistently high rates of success (2,42,65). Thole and colleagues (64) first described high throughput generation and characterization of 1000 T-DNA mutant lines in the inbred line Bd21. Later, this process was refined and used to generate a much larger collection of T-DNA mutants in Bd 21-3, which now comprises the publicly available Western Regional Research Center *Brachypodium* insertional mutant population (17)(http://brachypodium.pw.usda.gov). These mutant collections, along with the recently described BRACHYTIL chemical mutagenesis platform (23), can be used to identify and

functionally characterize specific genes important for *B. distachyon* phenotypic responses, such as resistance to pathogens. Additionally, the development and validation of tools for RT-qPCR (33) and virus-induced gene silencing (24,53,68) in *B. distachyon* can be used to further investigate the expression and function of target genes.

Host-pathogen interactions between *B. distachyon* and plant pathogens have previously been described for a number of economically important monocot diseases (4,7,27,54,55,56,59). The first of these studies focused on the interaction between *B. distachyon* and the model pathogen *Magnaporthe grisea*, causal agent of blast disease on rice and other grasses (59). Not only did this research prove the utility of *B. distachyon* as a model for host-pathogen interactions, it also demonstrated the *B. distachyon/M. grisea* interaction closely mirrored interactions of this pathogen with rice at both the histological and molecular levels, including the presence of resistance conferred by a single dominant gene (59). Parker and colleagues (54) built upon this research by describing a highly reproducible infection assay for studying *B. distachyon*/*M. grisea* interactions. Similarly, *B. distachyon* has been proposed as an optimal model host for studying *Fusarium* diseases of wheat because infection of *B. distachyon* is remarkably similar to the infection process observed in wheat, including infection of *B. distachyon* spikes by the Fusarium head blight pathogens *F. graminearum* and *F. culmorum* (55). Compatible interactions have also been described between *B. distachyon/Oculimacula* sp. and *B. distachyon*/*Ramularia collo-cygni*, the causal agents of eyespot and Ramularia leaf spot of cereal, respectively (56). Multiple researchers have characterized the interactions between *Brachypodium* sp. and various cereal rust pathogens, including *P. graminis, P. brachypodii, P. triticina*, and *P. striiformis* (4,7,27). Collectively, these studies have demonstrated a range of responses to rust pathogens in different

Brachypodium sp. ecotypes and the utility of *Brachypodium* sp. as a model system for studying molecular components of incompatibility and non-host resistance to rust pathogens.

The present research aims to extend the body of work on *Brachypodium*/cereal pathogen interactions by evaluating the compatibility of *Brachypodium distachyon* with three major fungal pathogens of turfgrass, *Sclerotinia homoeocarpa*, *Microdochium nivale*, and *Rhizoctonia solani*. In addition, this research explores the genetic diversity present in wild-ecotypes in response to *S. homoeocarpa* isolates and paves the way for widespread use of *Brachypodium* sp. as a model for turfgrass pathogens.

Materials and methods

Biological Materials

All pathogen isolates (Table 1) were isolated from symptomatic turfgrass using standard methods or obtained from colleagues. Briefly, turfgrass blades displaying symptoms characteristic for dollar spot, Microdochium patch and brown patch were surface disinfested in 10% Clorox solution and rinsed with distilled water. Individual blades were then excised and placed on antibiotic or pH-amended media and incubated for at least 48 h. Hyphal tips of colonies resembling target pathogens were transferred to fresh media to obtain pure cultures. Positive identification was achieved through observation of colony morphology, spore production, and sequencing of the ITS region using conserved fungal primers (67). Cultures were maintained in the dark at 21±2°C on potato dextrose agar (PDA) plates and transferred weekly to fresh media. Additionally, long-term stocks of all isolates were stored at -80°C on filter paper disks.

Seed for Bd inbred line Bd 21-3 was obtained from researchers at Cornell University. Bd accessions (Table 2) were ordered from the USDA National Plant Germplasm System

(http://www.ars-grin.gov/npgs) and have previously been assessed for stem rust susceptibility (7). Bd was cultivated in 8 cm diameter pots filled with a 50:50 (v:v) mixture of calcine clay (Turface; Buffalo Grove, IL) and potting mix. Pots were placed into plastic flats to allow for bottom watering and a single seed was placed in each pot. To promote germination, a propagation dome was placed over pots for the first 3-5 days after sowing. This was then removed to allow for plant growth. Adequate plant nutrition was maintained by bottom-watering once weekly with ¼-strength MiracleGro. For the first three weeks of growth, all plants were maintained in a growth room with a 14 h day-length at 24±2°C. In the fourth week, plants for *R. solani*, *P. aphanidermatum*, and *M. nivale* inoculations were moved to growth chambers at inoculation temperatures, described below, to allow time for acclimation prior to pathogen challenge. Movement to different environments had no observable effects on plant growth.

Bd 21-3 Growth Chamber Infection Assays

Infection assays of Bd 21-3 with all four fungal pathogens were set up as an RCBD with 3 replicates and repeated three times. Each experimental repetition was treated as a random blocking factor in statistical analysis. The majority of inoculations were performed using the parafilm sachet method (37). Briefly, a 6 mm diameter agar plug was excised from the advancing edge of fungal colonies with a sterile cork borer and adhered to the plant, mycelium side down, with a thin strip of parafilm. Plugs were applied 2-4 cm from the base of the plant. Control plants were mock-inoculated with fresh PDA plugs. Due to differing growth rates of the fungal pathogens used in these studies, the age of cultures when used for inoculation varied, as described below.

S. homoeocarpa inoculations were performed in a growth room held at 24±2°C with a 14 h day-length. Five-day-old cultures were used for inoculation. The parafilm sachet method alone

was adequate for maintaining relative humidity conducive for consistent symptom development. Disease severity, the percentage of tissue affected, was rated every 24 h using the Horsfall-Barratt scale (34).

A growth chamber at 28±4°C with a 10 h day-length was used for *R. solani* inoculations. *R. solani* cultures were five-days-old at the time of inoculation. To achieve consistent disease pressure and symptom development, relative humidity was maintained at or near 100% by covering flats with humidity domes. Disease severity was rated at five day post-inoculation as described for *S. homoeocarpa* above. The experiment was conducted as an RCBD with four replicates for each treatment within each of three experimental repetitions, for a total of 12 data points per isolate.

Due to the slower growth of *M. nivale*, inoculations were performed using 7 day-old cultures. Inoculated plants were placed in a growth chamber held at a constant temperature of 21°C with a 14 h day-length. The lower temperature mimicked the cool conditions under which *M. nivale* typically infects. A humidity dome was placed over inoculated plants to maintain high relative humidity and disease severity ratings were performed at five days post-inoculation, as described above. Similar to *R. solani*, there were four treatment replicates within each of three experimental repetitions.

Following the five-day disease incubation period and rating of symptom severity, all plants were photographed to document and compare symptoms. Pathogens were re-isolated from symptomatic material to confirm presence of the inoculated pathogen. This was done by briefly surface disinfesting diseased *B. distachyon* foliage in 10% Clorox solution, rinsing with sterile deionized water, and plating small pieces of the infected material on antibiotic medium. Plates

were incubated at room temperature for up to one-week to allow time for the target fungi to grow and identity was confirmed by colony morphology.

Brachypodium Natural Ecotype by *S. homoeocarpa* Isolate Infection Assays

Twelve natural ecotypes, as well as the inbred line Bd 21-3, and three Sh isolates were used for ecotype x isolate interaction infection assays. *Brachypodium* ecotypes differed in ploidy level and included representatives from the species *B. distachyon* and *B. hybridum* (Table 4). Four-week old plants were used for infection assays and plants were maintained as previously described. Infection assays were performed in a growth room held at 24±2°C with a 14 h day-length. The experiment was designed as a four treatment (three *S. homoeocarpa* isolates and PDA control) x 13 *Brachypodium* accession factorial within an RCBD with three replications. Treatments and accessions were randomly assigned within each block and the entire experiment was repeated three times. Blocks were treated as nested within experimental repetitions for statistical analysis. Inoculations were carried out as previously described for *S. homoeocarpa*. At 5 days post-inoculation, disease severity on individual plants was rated with the Horsfall-Barratt scale and plants were photographed to allow for comparison of infection type and symptom severity.

Statistical Analysis

All data was analyzed using SAS v.9.3 (35). Horsfall-Barratt ratings were converted to the geometric mean of the percentage range associated with each rating index, which resulted in normal distribution of the data as judged by residual plots and tests for normality within PROC UNIVARIATE. Bd 21-3 infection assays with *S. homoeocarpa*, *R. solani*, and *M. nivale* were analyzed using the PROC MIXED procedure. For analysis of Bd 21-3 data, treatment was considered a fixed main effect and experimental replicate was considered a random blocking

factor. ANOVA was used to compare treatment means and single degree-of-freedom contrast statements were used to make pre-planned comparisons between isolates and inoculated versus mock-inoculated controls. Studentized residual plots and model fitting criteria (AIC) were generated and assessed to confirming appropriateness of the models used. When not significant, random factors and interactions were removed from the model used for final analysis if the model fitting criteria indicated a better fit in the absence of these terms.

S. homoeocarpa isolate x *B. distachyon* accession data were analyzed with the PROC GLIMMIX procedure to allow for the use of slicing to compare levels of one main factor within the different levels of the other. Isolate and accession were both treated as fixed main effects. Initially, block and block(repetition) were treated as random effects. Block(repetition) did not contribute significantly to experimental variability and was excluded from the final analysis. ANOVA was used to compare means for main effects and single degree-of-freedom orthogonal contrast statements were used to make pre-planned means comparisons between specific treatments. The SLICE option was used to allow for orthogonal contrast statements comparing levels of one factor within those of the other.

Results

S. homoeocarpa symptoms, disease severity, and time-course of infection on Bd 21-3

All *S. homeoocarpa* isolates were pathogenic on Bd 21-3 (Fig. 1A). Symptoms were similar to those observed on creeping bentgrass in the lab and other turfgrass species under field conditions, including tan lesions with reddish brown borders (Fig.2A). Occasionally, hourglass-shaped lesions, a hallmark symptom of *S. homoeocarpa* infection, were observed on stems, sheaths, and leaves (Fig 2B). In addition to characteristic lesions, necrotic flecking, possibly indicative of hypersensitive response, was frequently observed in the early stages of infection

(24-48 hpi; Fig. 2C). Chlorosis, often leading to necrosis, was occasionally present along leaf margins (Fig. 2D). Five days following infection, individual leaf blades would appear wilted and necrotic (Fig. 2E). In severe infections, the entire plant would become necrotic and wilted (Fig. 2F).

Microscopic and macroscopic time-course infection studies were performed to compare *S. homoeocarpa* infection on creeping bentgrass and *B. distachyon*. For microscopic comparison of infection, creeping bentgrass turfgrass was inoculated and stained in parallel with *B. distachyon* (Fig. 3A, C, E, G, I, K). Similar penetration and colonization of host tissue was observed with both *B. distachyon* and creeping bentgrass. *S. homoeocarpa* actively grew from PDA plugs onto *B. distachyon* tissue within six hours of inoculation and initiated the formation of infection structures, primarily over stomata, as early as 12 hours post-inoculation (hpi; Fig. 3B, D, F, H). By 24 hpi, *S. homoeocarpa* hyphae extensively colonized host tissue by growing along and between cells (Fig. 3J). Little to no host cell death, as visualized by trypan blue staining outside of fungal hyphae, was observed at this time. These microscopic observations correspond with macroscopic ratings of whole plants, which remained symptomless until after 24 hpi (Fig. 1B). At later time points, *S. homoeocarpa* hyphae continued to grow between host cells and dead host tissue, which was stained blue, was observed outside of hyphal boundaries (Fig. 3L). Symptom severity ratings on whole plants also indicated the appearance of necrotic host tissue by 48 hpi. Symptom severity increased rapidly from 48-96 hpi and had generally peaked by 5 dpi (Fig. 1B). *S. homoeocarpa* isolate had no significant effect on symptom severity or the rate at which infection progressed, indicating a lack of race-specific interactions (Fig. 1B). Additionally, symptoms observed were consistent between isolates, indicating similar mechanisms of pathogenicity for all.

ROS production by BD 21-3 following inoculation with *S. homoeocarpa*

The formation of ROS is a characteristic of host defense activation and can be visualized by staining for various molecules. To provide a general overview of host-defense activation following *S. homoeocarpa* inoculation, Bd 21-3 leaf segments from inoculated whole plants were harvested 24 hpi and stained with 3,3'-diaminobenzadine (DAB), which forms a brown precipitate in the presence of H_2O_2. DAB staining results indicated that both C3 and C4 isolates of *S. homoeocarpa* resulted in the production of H_2O_2 by 24 hpi (Fig. 4A). Creeping bentgrass turfgrass samples were inoculated, harvested, and stained in parallel and also produced significant amounts of H_2O_2 by 48 hpi (Fig. 4A). Brown precipitate was also detected in negative controls, indicating some H_2O_2 was produced in response to the inoculation method itself; however, quantitative analysis revealed that staining was both more extensive and more intense in inoculated plants (Fig. 4B).

***Brachypodium* ecotype x Sh isolate interactions**

To determine the presence of natural resistance to *S. homoeocarpa* in *Brachypodium* sp., thirteen *Brachypodium* sp. ecotypes were inoculated with three isolates of *S. homoeocarpa* collected from both C3 and C4 turfgrass hosts and symptom severity was compared (Fig. 5). The recent separation of polyploid (2n=30) *B. distachyon* accessions into the new species *B. hybridum* resulted in this study including three *B. distachyon* ecotypes, nine *B. hybridum* ecotypes, and one inbred *B. distachyon* line (Bd 21-3).

Both accession and isolate had strong effects on symptom severity (P=0.0002 for both) but an accession by isolate interaction was not detected (P=0.4965), indicating that different *S. homoeocarpa* isolates produced similar symptom severity across the *Brachypodium* sp. ecotypes assessed in this study (Table 3). Orthogonal contrasts revealed that the C3 isolates were more

aggressive than the C4 isolate used in this assay *(P*=0.004) and that disease severity was greater in *B. hybridum* ecotypes than *B. distachyon* ecotypes (*P*<0.0001) (Table 4). Bd 21-3 is an inbred line developed from the *B. distachyon* wild-type accession PI 254867; consequently, the response of these accessions to *B. distachyon* was of interest but no difference was found between these two lines (*P*=0.3553). To test for differences in resistance among individual *B. distachyon* accessions to *S. homoeocarpa*, single degree of freedom orthogonal contrasts were used to compare the two *B. distachyon* ecotypes, PI 245730 and PI 639818, to the inbred line Bd 21-3 (Table 4). Disease severity was was similar between PI 639818 and Bd 21-3, but a moderate difference in severity was detected between PI 245730 and Bd 21-3 (*P*=0.02). Additional orthogonal contrasts between individual *B. distachyon* ecotypes were performed but no further differences in susceptibility to *S. homoeocarpa* were detected (Table 2).

The majority of *Brachypodium* sp. ecotypes do not differ in their relative resistance to *S. homoeocarpa* (Fig. 5A; Table 3). PI 245730 and PI 639818 were more resistant to *S. homoeocarpa* isolates than PI 227011. PI245730 was also more resistant than PI239713; otherwise, resistance to *S. homoeocarpa* did not differ between ecotypes. Since isolate was significant in the overall analysis, means separations were also performed within individual isolates (Fig. 5B-C). Similar to the overall test, PI227011 and PI239713 had the highest disease severity for both C3 *S. homoeocarpa* isolates in this study (Table 5). PI245730 had significantly lower symptom severity than the two least resistant ecotypes for both C3 *S. homoeocarpa* isolates. PI227011 and PI239713 were not significantly different from PI245730 in symptom severity when inoculated with the C4 *S. homoeocarpa* isolate (Table 5). PI245730 again had the lowest symptom severity, along with PI 226629. Only PI 287783 had greater symptom severity than these two ecotypes (Table 5).

Single degree of freedom orthogonal contrast statements revealed differences between *B. distachyon* and *B. hybridum* ecotypes in susceptibility to C3 *S. homoeocarpa* isolates (Table 4). This difference was detected regardless of whether Bd 21-3 was included as a *B. distachyon* accession. No difference in symptom severity was detected between *B. distachyon* and *B. hybridum* accessions inoculated with the C4 *S. homoeocarpa* isolate LFD8. There were also no significant differences in symptom severity between the inbred line Bd 21-3 and natural *B. distachyon* accessions or between *B. distachyon* accessions, regardless of the host type from which the *S. homoeocarpa* isolate was originally collected.

***Rhizoctonia solani* symptom development and severity on Bd 21-3**

Both *R. solani* isolates used in this experiment were pathogenic on Bd 21-3 and there was no difference in aggressiveness between the two isolates (Fig. 6A). Symptoms produced on Bd 21-3 were similar to those produced on creeping bentgrass under artificial conditions and to those reported in the field on many turfgrass species. Plant symptoms included tan to gray lesions along leaf blades with dark brown borders. Chlorosis spreading along leaf blades frequently accompanied these lesions (Fig. 6B-C). Lesion phenotype varied from small and circular to long and irregularly-shaped (Fig. 6B-C). Tips of severely infected leaf blades occasionally became necrotic and tissue surrounding the site of inoculation was frequently damaged (Fig. 6C). Symptoms were primarily observed on leaf blades and rarely advanced to stems and sheaths. Sparse white mycelia were occasionally produced in infected areas of host tissue.

***Microdochium nivale* symptom development and severity on Bd 21-3**

M. nivale isolates used in this study were pathogenic on Bd 21-3, but differed significantly in aggressiveness (Fig. 7A). Symptoms produced by BH7 generally included

necrosis of leaf blades that occasionally spread to ligules, sheaths, and culms. Chlorosis was observed spreading along infected leaf blades (Fig. 7B). Sparse white mycelia were frequently observed on infected plants (Fig. 7B). The more aggressive isolate, MN5, produced large, dark brown necrotic lesions affecting blades, ligules, sheaths, and culms (Fig. 7C). Chlorosis was frequently noted in leaf blades and spread beyond areas of necrosis (Fig.7D). Severely affected leaf blades were completely necrotic and occasionally appeared wilted (Fig. 7C). As with isolate BH7, sparse white mycelia were formed in areas of necrotic tissue (Fig. 7C).

Discussion

The aim of the present study was to determine the potential for use of *Brachypodium distachyon* as a model host for three major fungal pathogens of amenity turfgrasses. Additionally, this research investigated the pathogenesis and ecotype by isolate interactions of various *Brachypodium* sp. accessions with the dollar spot pathogen *S. homoeocarpa*. The results of these studies clearly demonstrate compatible interactions between Bd 21-3 and *S. homoeocarpa*, *M. nivale,* and *R. solani*. Time-course microscopy assays and DAB staining on Bd 21-3 revealed that *S. homoeocarpa* pathogenesis on this inbred line is similar to that on the natural turfgrass host creeping bentgrass. Additionally, screening of diverse *Brachypodium* sp. ecotypes with C3 and C4 isolates of *S. homoeocarpa* indicated that genetic variability exists within *Brachypodium* sp. accessions that can be leveraged for studies of host-pathogen interactions for *S. homoeocarpa* and other prominent fungal pathogens of turfgrass.

Aside from dollar spot, brown patch is one of the most frequently encountered diseases on amenity turfgrass (12) and pink snow mold is the primary cool-season disease of turfgrasses throughout the globe (61). In the present research, isolates of both *R. solani* and *M. nivale* caused symptoms on *B. distachyon* inbred line Bd 21-3 under temperature and humidity conditions that

are ideal for infection of turfgrass hosts by these pathogen. This suggests that both pathogens have similar requirements for infection on *B. distachyon* as on turfgrasses. *R. solani* lesions on *B. distachyon* closely matched those observed previously on creeping bentgrass in the lab and other turfgrass species in the field, including the formation of irregularly shaped tan lesions with dark red to brown borders between diseased and healthy tissue (61). Due to the lack of empirical data on development of brown patch symptoms on turfgrass in controlled settings, it is difficult to make additional direct comparisons; however, further studies of *R. solani* pathogenesis using *B. distachyon* as a model will contribute to better understanding of the physiological and molecular mechanisms of turfgrass infection by this pathogen. Additionally, *R. solani* AG-2-2IIIB, the primary turfgrass-infecting anastomosis group, affects other hosts in the *Poaceae* family (49). Information gained from the *B. distachyon* pathosystem can be extended to enhance understanding of *R. solani* pathogenesis in these hosts as well.

In addition to being a major turgrass pathogen, *M. nivale* is the causal agent of pink snow mold, also known as Microdochum patch, on winter wheat, rye, barley, and oat (57,60). Some research on *M. nivale*/host interactions has been performed in the wheat pathosystem, but the relatedness of *M. nivale* isolates from turf and cereal crop plants is not well understood (36,43,46,60). Consequently, a model system for studying *M. nivale*/host interactions will be helpful for turfgrass pathology and may extend to additional economically important crops, such as wheat, barley, and ryegrass. On individual turfgrass blades, symptoms of *M. nivale* infection are non-distinct with generalized water soaking and necrosis, as well as the presence of tufts of aerial white mycelia (61). The symptoms observed on *B. distachyon* plants artificially inoculated with agar plugs of *M. nivale* were remarkably similar (Fig. 7B-C). Dark, water-soaked lesions formed around the site of inoculation both upward into leaf blades and downward into culms.

Similar to turfgrass infections, fluffy white mycelia were observed protruding from the border between healthy and diseased tissue. The name 'pink snow mold' derives from the presence of pinkish mycelia and sporodochia that appear on infected turf, giving symptomatic areas a pinkish coloration. *M. nivale* sporulation is sunlight dependent, therefore, we did not see observe this phenomenon on *B. distachyon* but it may be possible with the right environmental conditions (61). *M. nivale* that was reisolated from symptomatic *B. distachyon* plants produced abundant conidia in culture, indicating no negative effects on the fungus from exposure to *B. distachyon.*

In light of growing concerns over extensive fungicide use for dollar spot control and increasing issues with resistance to common fungicide groups in *S. homoeocarpa* populations (19,48,50,58), there has been a recent resurgence in research on *S. homoeocarpa* pathogenesis. Orshinsky and colleagues (51) used histological methods and microscopy to study the infection process of virulent, hypovirulent, and asymptomatic isolates of *S. homoeocarpa* on detached creeping bentgrass leaves. In these studies, plugs from five-day-old PDA cultures of *S. homoeocarpa* were placed on leaf blades over moistened filter paper in parafilm sealed petri plates. Similar to our studies with intact plants, appressorium, particularly in association with stomata, and intercellular hyphal growth were observed by 48hpi (51). The present research indicates that both appressorium formation and intercellular hyphal growth of *S. homoeocarpa* initiated as early as 12 hpi on attached turfgrass and *B. distachyon* leaves. Similarly, in our study extensive ROS production was detected by DAB staining at 24 hpi in both *B. distachyon* creeping bentgrass, while Orshinsky and colleagues did not report ROS production in detached creeping bentgrass leaves until 48 hpi. It is not certain if the differences in timing noted between these two studies were due to earlier time points not being included in the previous research, or differences between detached and attached leaf tissue. We performed detached leaf assays but

they were deemed unsuccessful because symptoms produced on detached leaves were dissimilar from those on intact plants. Moreover, *B. distachyon* leaves tended to curl and turn brown within a few days after detachment (data not shown).

White, hourglass shaped lesions with reddish-brown borders are the hallmark symptom of *S. homoeocarpa* infection on most turfgrass species (1,66). The presence of these lesions on *S. homoeocarpa*-infected *B. distachyon* in these experiments (Fig. 2B) was a compelling sign for the utility of *B. distachyon* as a model host for *S. homoeocarpa*. Other symptoms observed on *B. distachyon*, including round to oval shaped lesions with reddish-brown borders, tip dieback, and necrosis along leaf edges have previously been described on various turfgrass hosts (1,61,66). Distinct lesions and other isolated symptoms were generally observed within the first three to four days following infection. At later time points, individual lesions or other distinct symptomatic areas would often coalesce, leaving entire leaf blades with a bleached, or wilted and necrotic appearance. This blighting of the entire leaf blade in advanced *S. homoeocarpa* infections is common in turfgass as well (22), further demonstrating the strong similarity between the development of *S. homoeocarpa* infection on natural hosts and the model *B. distachyon*.

The *Brachypodium* sp. ecotype by *S. homoeocarpa* isolate research in this study included 12 natural *Brachypodium* sp. accessions representing both the diploid species *B. distachyon* and the allotetraploid species *B. hybridum*, as well as the inbred *B. distachyon* line Bd 21-3, which was used for all other studies. Though the genetics of wild accessions are variable and inbred lines offer a more reliable phenotypic response to pathogen inoculation, natural ecotypes were selected for this research because no prior knowledge of *Brachypodium* sp. responses to *S. homoeocarpa* was available (28). Additionally, the accessions used in this study had previously

been characterized for their response to various cereal rust pathogens (7). PI 227011, which had the highest overall disease severity in this research, was also the only accession in the previous study to support sporulation of the stripe rust pathogens *Puccinia striiformis* f. sp. *hordei* and *P. striiformis* f. sp. *bromi.* Given the taxonomic and pathogenic life style differences between *S. homoeocarpa* and *P. striiformis,* it is intriguing that PI 227011 is particularly susceptible to both pathogens and further investigation into the mechanisms of susceptibility of this particular accession are warranted.

No accession by isolate interaction was noted in this research, which indicates that *S. homoeocarpa* employs a generalist pathogenesis strategy and that resistance/avirulence gene interactions do not occur between *S. homoeocarpa* and *B. distachyon*. This corresponds with interactions that have been reported for turfgrass hosts and is part of the reason that breeding for dollar spot resistant turfgrass cultivars presents such a challenge for turfgrass breeders (13). Orthogonal contrasts comparing specific groups within *Brachypodium* sp. accessions and *S. homoeocarpa* isolates did reveal some interesting differences. The isolates collected from C3 grasses (2F92-1 and OJN9) caused significantly greater symptom severity than the isolate collected from a C4 grass (LFD8), though all isolates produce similar symptom severity on creeping bentgrass (data not shown). Experiments with more isolates of *S. homoeocarpa* from both C3 and C4 grasses are needed to validate this observation, but it is of note because recent studies have revealed marked differences between *S. homoeocarpa* isolates collected from warm and cool season grasses (45).

An even more striking find was that *B. hybridum* accessions experienced significantly higher symptom severity than *B. distachyon* accessions overall and when inoculated with the two C3 *S. homoeocarpa* isolates. It is possible that morphological differences between the two

Brachypodium species contributed to differences in *S. homoeocarpa* symptom severity; however, *B. hybridum* is generally larger than *B. distachyon* (20). It is more likely that smaller, less bushy plants would be most susceptible to *S. homoeocarpa* infection, which indicates that genetic differences, and not morphology, are more likely the cause for greater symptom severity in *B. hybridum*. The observation that no difference in susceptibility was observed between the two *Brachypodium* species when inoculated with the C4 *S. homoeocarpa* isolate is further evidence of a genetic rather than a physical determinant of susceptibility to *S. homoeocarpa*. The taxonomic relationship of *B. distachyon* and *B. hybridum* was recently deciphered and it was determined that *B. hybridum* is an allotetraploid derived from hybridization of 2*n*=10 *B. distachyon* and 2*n*=20 *B. stacei* (20). It is an intriguing possibility that the enhanced *S. homoeocarpa* susceptibility observed in *B. hydridum* accessions is due to contributions from the *B. stacei* parent. Further studies that compare susceptibility of these three *Brachypodium* species will be important for deciphering the genetic basis of susceptibility to *S. homoeocarpa* and determining the best way to use *Brachypodium* sp. as a model to study *S. homoeocarpa*/host interactions at the molecular level. Based on previous studies by our lab, it is possible that certain physiological characteristics, such as oxalate content and cell wall content, and the genes controlling them may contribute to the differences in *S. homoeocarpa* susceptibility between *B. distachyon* and *B. hybridum* accessions. Evaluations of these host characteristics are ongoing.

Previous studies have demonstrated the utility of *B. distachyon* as a model host for various cereal rust pathogens that affect turfgrass species (4,7,10,27). The present research extends the applications of *B. distachyon* as a tool for studying host-pathogen interactions in turfgrasses to three additional major fungal diseases of these hosts: Dollar spot, brown patch, and pink snow mold. No genomic sequence data is publicly available for popular turfgrass species

and large-scale transcriptomic analyses of endophyte or pathogen-infected turfgrass hosts are limited (3,52). Additionally, the only genetic tools currently available for genetic manipulation of turfgrass species is *Agrobacterium*-mediated transformation, which is time consuming and complicated by the complex genomes of most turfgrass species (6,25,29,47). On the contrary, the multitude of public genetic resources and molecular tools available for *B. distachyon,* as well as the ease with which this species can be cultivated, inbred, and inoculated, make it an ideal model for studying the molecular aspects of host resistance to major turfgrass pathogens. In this research, *Brachypodium* sp. accessions with significant differences in resistance to *S. homoeocarpa* were identified. Inbred lines derived from these accessions and selected for resistance or susceptibility to *S. homoeocarpa* will provide useful tools for identification of genes involved in host resistance to this pathogen. In the future, similar studies that evaluate the resistance of *Brachypodium* sp. accessions to *R. solani* and *M. nivale* will be needed to identify the best accessions to use for dissection of host resistance mechanisms to these pathogens.

Conclusions

In this research, we demonstrate that *B. distachyon* inbred line Bd 21-3 is a compatible host to the causal agents of turfgrass dollar spot, brown patch, and pink snow mold diseases. These are three of the most economically important diseases of turfgrass and account for the vast majority of fungicide sprays in high-value turf settings. Symptoms of these diseases produced on Bd 21-3 mimic those observed on natural turfgrass hosts and indicate that *B. distachyon* is a useful model pathosystem to study molecular host-pathogen interactions of these diseases. The use of *B. distachyon* as a model will accelerate the pace at which key genes underlying host resistance or susceptibility to these causal agents of these diseases are identified and will enhance breeding efforts for disease resistant turfgrass cultivars. Targeted breeding efforts will decrease the time

needed to produce turfgrass cultivars resistant to dollar spot, brown patch, and pink snow mold and will significantly decrease dependence upon chemical control, resulting in more sustainable and environmentally friendly turfgrass disease management.

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Authors' contributions

RR and JK designed experiments. RR and BVR performed experiments and collected data. RR performed statistical analyses. RR, BVR, and JK participated in drafting the manuscript. All authors have read and approved the final manuscript.

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Table 1. Fungal isolates used for *B. distachyon* inoculations

Species	Isolate Code	Collection Site	Host Species
Rhizoctonia solani	Rs	North Carolina	Creeping bentgrass
Rhizoctonia solani	SRSE	North Carolina	Creeping bentgrass
Microdochium nivale	BH7	Wisconsin	Creeping bentgrass
Microdochium nivale	MN5	Illinois	Creeping bentgrass
Pythium aphanidermatum	P1	Pennsylvania	Perennial ryegrass
Pythium aphanidermatum	P40	Pennsylvania	Perennial ryegrass
Sclerotinia homoeocarpa	2F92-1	Wisconsin	Creeping bentgrass
Sclerotinia homoeocarpa	Heath1A	Wisconsin	Creeping bentgrass
Sclerotinia homoeocarpa	LFD8	North Carolina	Bermudagrass
Sclerotinia homoeocarpa	OJN9	Wisconsin	Creeping bentgrass
Sclerotinia homoeocarpa	S10	Wisconsin	Creeping bentgrass

Table 2. *Brachypodium* sp. accession used for fungal inoculations and accession x isolate interaction evaluations

Species[1]	Accession ID	Ploidy Level	Origin	Inbred Lines
Brachypodium distachyon	Bd 21-3	Diploid	Iraq	-
Brachypodium distachyon	PI 245730	Diploid	Turkey	Bd18-1
Brachypodium distachyon	PI 254867	Diploid	Iraq	Bd21, Bd 21-3
Brachypodium distachyon	PI 639818	Diploid	Ukraine	Bd29-1
Brachypodium hybridum	PI 226629	Allotetraploid	Iran	Bd11-1, Bd11-2
Brachypodium hybridum	PI 227011	Allotetraploid	Iran	Bd12-1, Bd12-2
Brachypodium hybridum	PI 233228	Allotetraploid	Israel	Bd13-1, Bd13-2
Brachypodium hybridum	PI 239713	Allotetraploid	Iran	Bd14-1, Bd14-2
Brachypodium hybridum	PI 239714	Allotetraploid	Iran	Bd15-1, Bd15-2
Brachypodium hybridum	PI 254868	Allotetraploid	Iraq	Bd22-1, Bd22-2
Brachypodium hybridum	PI 287783	Allotetraploid	Spain	Bd23-1, Bd23-2
Brachypodium hybridum	PI 372187	Allotetraploid	Uruguay	Bd26-1, Bd26-2
Brachypodium hybridum	PI 533105	Allotetraploid	Australia	Bd28

[1] Species designations are based on the reclassification of *Brachypodium* sp. based on ploidy level and other characteristics by Catalan and colleagues (2011)

Table 3. Overall ANOVA for *Brachypodium* sp. ecotype x *S. homoeocarpa* isolate interactions

Source	DF	SS	MS	F-value	P-value
Isolate	2	6740	3370	8.69	0.0002
Accession	12	15020	1552	3.23	0.0002
Isolate x Accession	24	9104	379	0.98	0.4956
Error	289	112102	388	---	---

Table 4. Specific comparisons for *Brachypodium* sp. ecotype x *S. homoeocarpa* isolate interactions

Sh Isolate(s)	Grp. 1	Grp. 2	Grp. 1 Mean (SE)	Grp. 2 Mean (SE)	F-value	P-value
All						
	C3	C4	50.46 (2.01)	43.73 (1.80)	8.57	0.004**
	Bd ecotypes[1]	Bh[2]	39.54 (2.88)	50.76 (1.80)	15.91	<0.0001***
	All Bd[3]	Bh	42.55 (2.64)	50.76 (1.80)	12.61	0.0004***
	Bd 21-3	PI 254867	48.33 (5.16)	43.70 (2.57)	0.69	0.4085
	Bd 21-3	PI 245370	48.33 (5.16)	35.37 (4.04)	5.37	0.0215*
	Bd 21-3	PI 639818	48.33 (5.16)	42.78 (4.75)	0.99	0.3214
	PI254867	PI 245730	43.70 (2.57)	35.37 (4.04)	2.36	0.1257
	PI 254867	PI 639818	43.70 (2.57)	42.78 (4.75)	0.03	0.8646
	PI 639818	PI 245730	42.78 (4.75)	35.37 (4.04)	1.86	0.1733
2F92-1						
	Bd ecotypes	Bh	37.04 (4.27)	50.36 (3.11)	8.10	0.0054**
	All Bd	Bh	37.93 (3.15)	50.36 (3.11)	8.45	0.0045**
	Bd 21-3	PI 254867	40.63 (9.23)	43.33 (6.45)	0.07	0.7897
	Bd 21-3	PI 245370	40.63 (9.23)	28.89 (7.4)	1.34	0.2492
	Bd 21-3	PI 639818	40.63 (9.23)	38.89 (5.58)	0.03	0.8642
	PI254867	PI 245730	43.33 (6.45)	28.89 (7.4)	2.16	0.1447
	PI 254867	PI 639818	43.33 (6.45)	38.89 (5.58)	0.20	0.6519
	PI 639818	PI 245730	38.89 (5.58)	28.89 (7.4)	1.86	0.1733
OJN9						
	Bd ecotypes	Bh	44.81 (3.83)	57.24 (2.73)	7.44	0.076**
	All Bd	Bh	48.14 (4.29)	57.24 (2.73)	4.88	0.0296*
	Bd 21-3	PI 254867	58.13 (5.08)	39.44 (5.86)	3.40	0.0682
	Bd 21-3	PI 245370	58.13 (5.08)	42.78 (6.88)	2.30	0.1330
	Bd 21-3	PI 639818	58.13 (5.08)	52.22 (8.04)	0.34	0.5614
	PI254867	PI 245730	39.44 (5.86)	42.78 (6.88)	1.12	0.1352
	PI 254867	PI 639818	39.44 (5.86)	52.22 (8.04)	1.69	0.1966
	PI 639818	PI 245730	52.22 (8.04)	42.78 (6.88)	0.92	0.3389
LFD8						
	Bd ecotypes	Bh	40.00 (4.24)	44.70 (2.17)	1.44	0.2323
	All Bd	Bh	41.56 (3.38)	44.70 (2.17)	0.78	0.3800
	Bd 21-3	PI 254867	46.25 (7.06)	48.33 (5.65)	0.06	0.8073
	Bd 21-3	PI 245370	46.25 (7.06)	34.44 (5.56)	1.92	0.1691
	Bd 21-3	PI 639818	46.25 (7.06)	37.22 (5.72)	1.12	0.2920
	PI254867	PI 245730	48.33 (5.65)	34.44 (5.56)	2.82	0.0961
	PI 254867	PI 639818	48.33 (5.65)	37.22 (5.72)	1.81	0.1820
	PI 639818	PI 245730	37.22 (5.72)	34.44 (5.56)	0.11	0.7357

[1] 'Bd ecotypes' includes the three *B. distachyon* wild-type accessions but not Bd 21-3
[2] 'Bh' indicates all *B. hybridum* accessions
[3] 'All Bd' includes the three *B. distachyon* wild-type accessions and Bd 21-3

Table 5. Means separation for symptom severity of *Brachypodium* species accessions inoculated with *S. homoeocarpa*

	Means Separation[1]			
Accession	**All Isolates**	**2F92-1**	**OJN9**	**LFD8**
PI 227011	A	A	A	AB
PI 239713	AB	AB	A	AB
PI 287783	ABC	BDC	ABC	A
PI 533015	ABC	BDC	ABC	AB
PI 254868	ABC	ABC	ABC	AB
Bd 21-3	ABC	BDC	ABC	AB
PI 372187	ABC	BDC	AB	AB
PI 226629	ABC	BDC	AB	B
PI 239714	ABC	BDC	ABC	AB
PI 233228	BC	BDC	BC	AB
PI 254867	BC	BDC	C	AB
PI 639818	BC	DC	ABC	AB
PI 245730	C	D	BC	B

[1] Accessions with the same letter are not significantly different from each other by Fisher's protected LSD, $\alpha = 0.05$

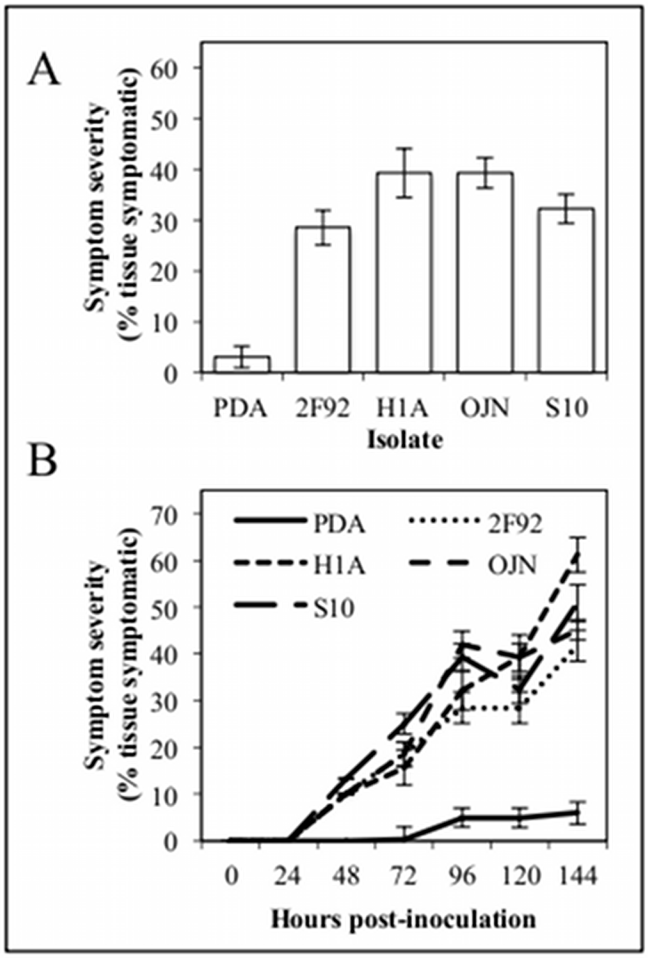

Figure 1. **Disease severity of *S. homoeocarpa* isolates on Bd 21-3 at 5 dpi. (A)** Average disease severity rating of whole *B. distachyon* plants five days following inoculation with one of four *S. homoeocarpa* isolates or a PDA plug. Disease severity ratings represent the mean severity from three experimental repetitions with three plants per experiment (n=9). Error bars represent ± one standard error of the mean. **(B)** Progression of infection on Bd 21-3 by four isolates of *S. homoeocarpa* and the mock-inoculated PDA control. Symptom severity was scored visually every 24 hours for six days. Each data points represents the mean severity from three experimental repetitions with three plants were experiment (n=9). Error bars represent ± one standard error of the mean.

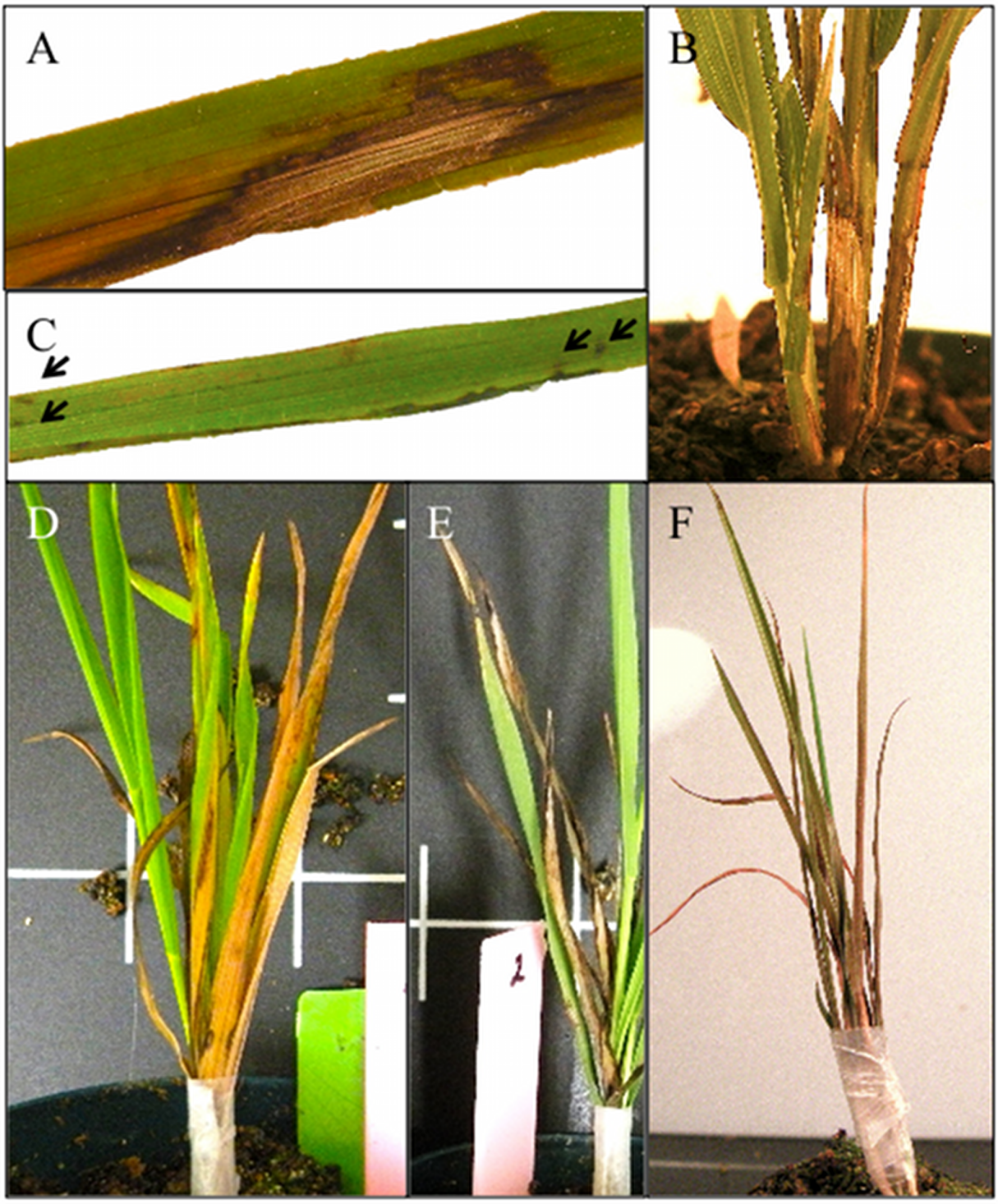
A
B
C
D
E
F

Figure 2. Symptoms of *S. homoeocarpa* infection on Bd 21-3. **(A)** A characteristic individual lesion, with white center and reddish brown border, frequently observed on infected foliage. **(B)** Hallmark hourglass-shaped lesion on Bd 21-3 stem and sheath. **(C)** Necrotic flecking frequently observed early in infection and possibly indicative of a hypersensitive-type response to infection. **(D)** Mild infection resulting in chlorosis of affected foliage and mild necrosis. **(E)** Moderate infection of Bd 21-3 by *S. homoeocarpa* at 5 dpi resulting in necrosis and wilting of some leaf blades while others remain relatively unaffected **(F)** Widespread necrosis and wilting of leaf blades with spread of symptoms to sheaths and stems.

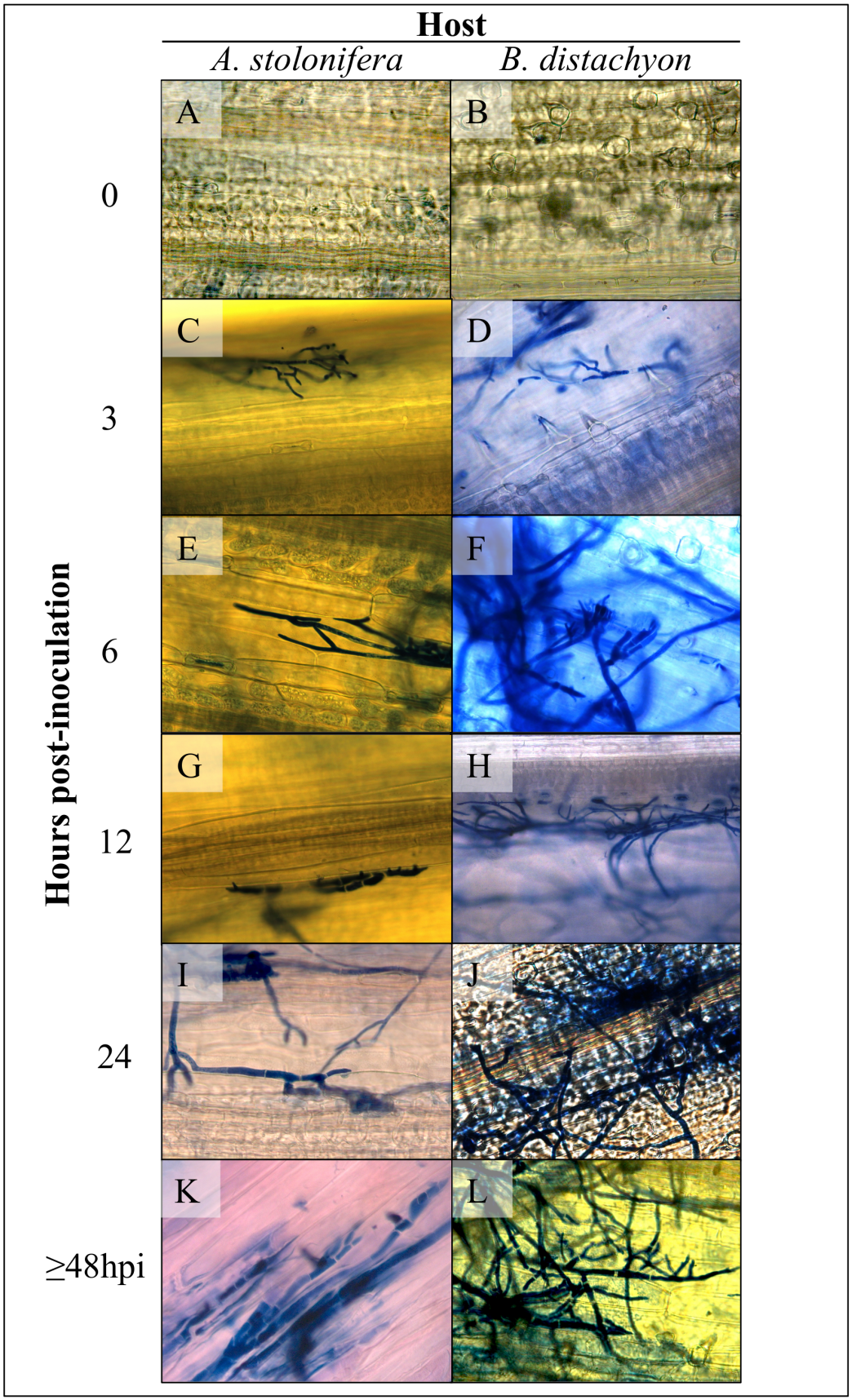
Host
A. stolonifera
B. distachyon
Hours post-inoculation
0
3
6
12
24
≥48hpi
A
B
C
D
E
F
G
H
I
J
K
L

Figure 3. Time-course histology of infection of *A. stolonifera* and *B. distachyon* inbred line Bd 21-3 *by S. homoeocarpa*. (A, C, E, G, I, K) Progression of infection on the natural host *A. stolonifera* at 0, 3, 6, 12, 24, and ≥48 hpi, respectively. **(B, D, F, G, H, J, L)** Progression of infection on *B. distachyon* inbred line Bd 21-3 at 0, 3, 6, 12, 24, and ≥48 hpi, respectively. All inoculation, sample collection, and staining was carried out in parallel for each host species. Samples were cleared with acetic acid:ethanol mixtures and stained overnight with 0.01% tryphan blue in lactophenol then washed with sterilized 60% glycerol. Images represent inoculation with various *S. homoeocarpa* isolates. A minimum of three samples per species x isolate combination were examined for each time point and no significant variation was observed between host species, *S. homoeocarpa* isolate, or individual replications with each combination.

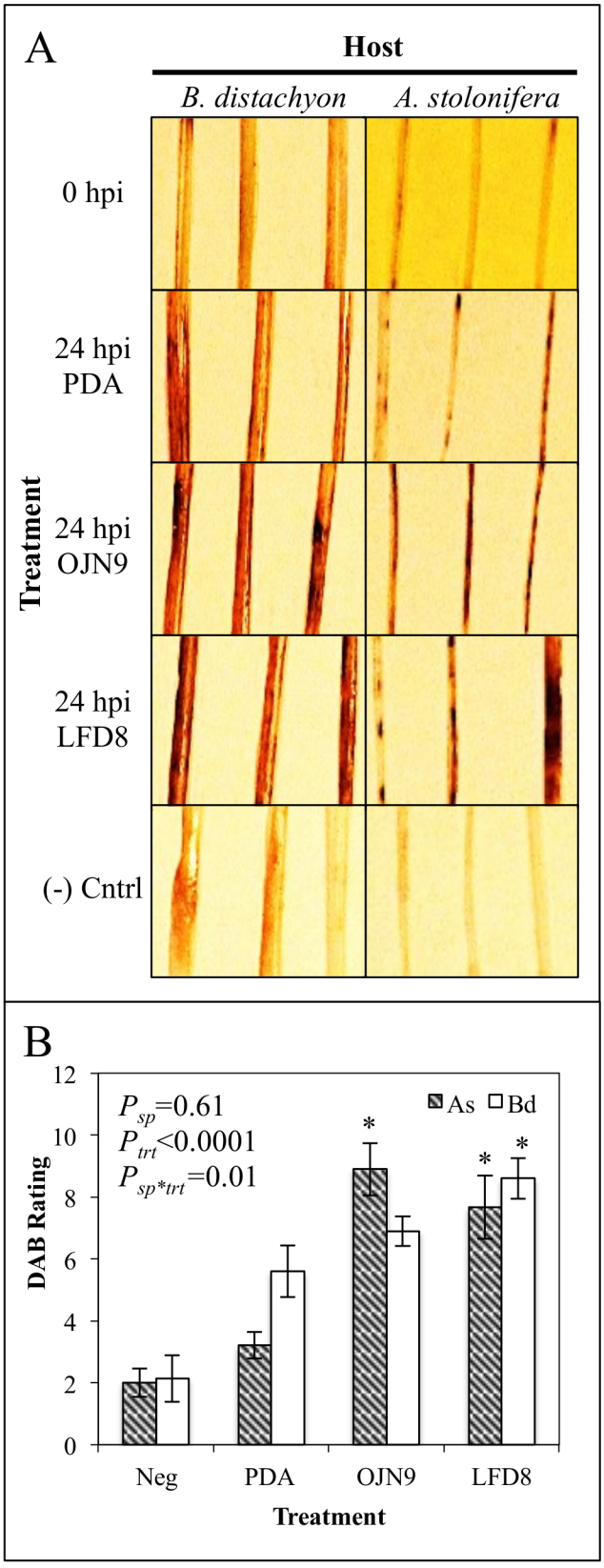
A
Host
B. distachyon
A. stolonifera
0 hpi
24 hpi PDA
24 hpi OJN9
24 hpi LFD8
(-) Cntrl
Treatment
B
P_{sp}=0.61
P_{trt}<0.0001
P_{sp*trt}=0.01
As
Bd
DAB Rating
0
2
4
6
8
10
12
Neg
PDA
OJN9
LFD8
Treatment

Figure 4. ROS formation in *A. stolonifera* and *B. distachyon* inbred line 21-3 following inoculation with *S. homoeocarpa*. (A) Images of representative leaf blades stained with diaminobenzidine (DAB) for ROS detection either before or 24 hours after pathogen inoculation. DAB is visualized as a brown precipitate indicative of the presence of hydrogen peroxide and staining was performed according to previously described procedures. Leaf blades are from a single representative experiment. The full experiment was repeated three times with similar results. **(B)** Quantification of ROS production. DAB staining was rated on a Horsfall-Barratt scale with rating based on the amount of leaf tissue in which brown precipitate was present. No differences in DAB staining intensity were present between the two host species. Asterisks represent a significant difference in staining intensity between the PDA control and pathogen-inoculated samples for the respective host with a significance cut-off of $\alpha=0.05$. Bars represent the mean of three replicated experiments with three leaf blades per treatment x host combination per experiment (n=9). Error bars represent ± standard error of the mean.

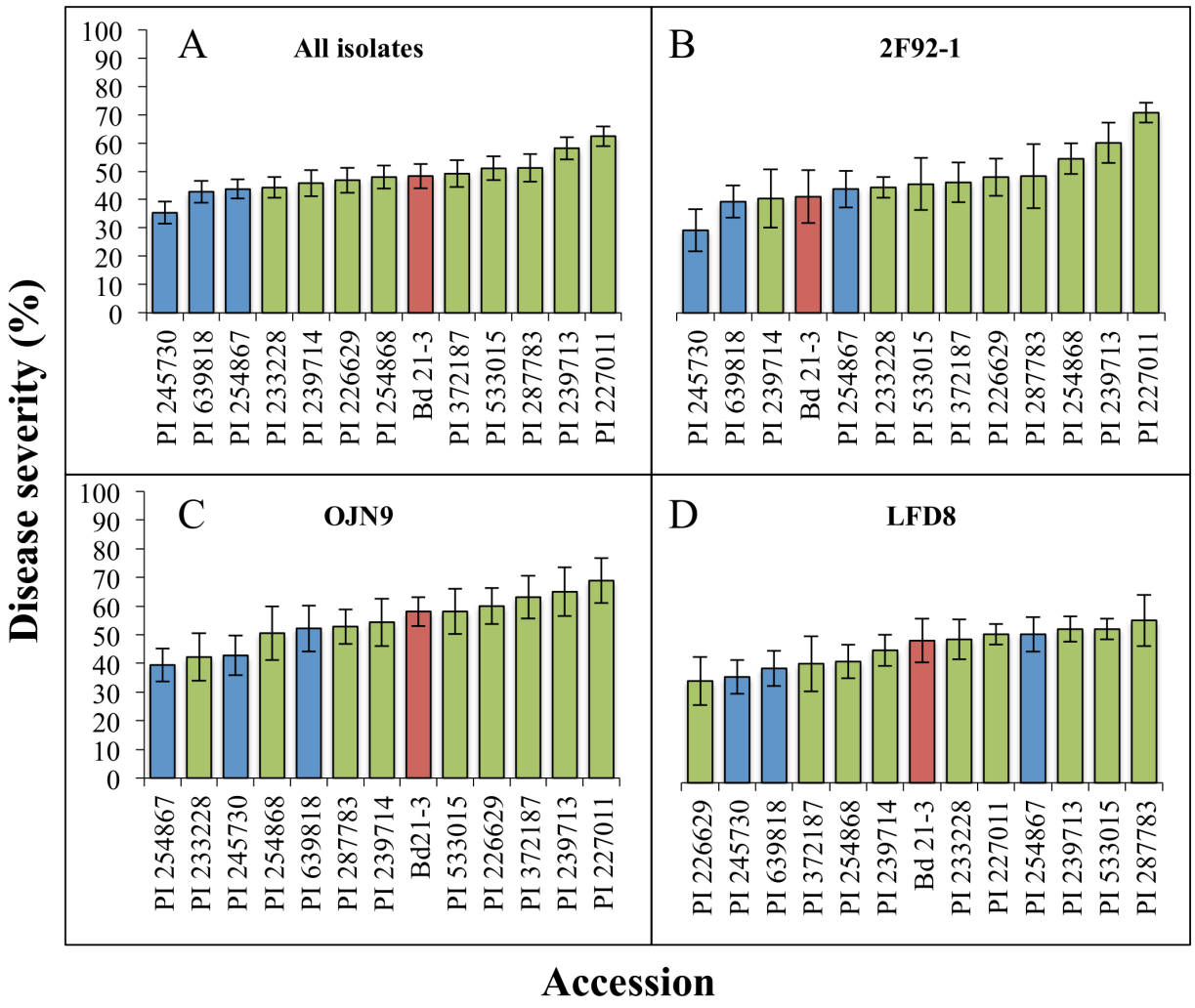

Figure 5. Symptom severity on *Brachypodium* species accessions five-days post-inoculation with *S. homoeocarpa*. (A) Mean symptom severity across all isolates tested. (**B)** Mean symptom severity for C3 isolate 2F92-1 **(C)** Mean symptom severity for C3 isolate OJN9. **(D)** Mean symptom severity for C4 isolate LFD8. Blue bars represent *B. distachyon* wild-type accessions; green bars represent *B. hybridum* wild-type accessions; and red bars represent *B. distachyon* inbred line Bd 21-3. Bars represent the mean of three replicate experiments with 2-3 plants per experiment (n=7-9) and errors bars represent ± one standard error of the mean.

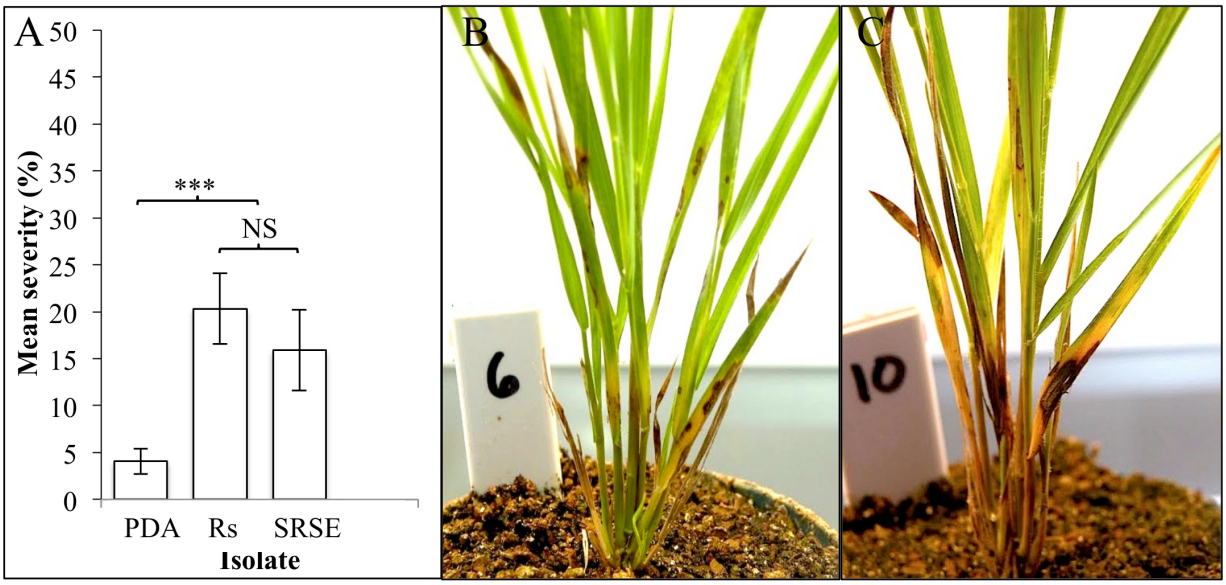

Figure 6. Infection of *B. distachyon* inbred line Bd 21-3 with *Rhizoctonia solani*. (A) Mean symptom severity of Bd 21-3 five days following inoculation with *R. solani* isolate Rs or SRSe, or with a PDA plug. Single degree-of-freedom orthogonal contrast statements were used to compare between *R. solani* isolates and between *R. solani* isolates and the PDA control at α=0.05. NS = no significant difference detected and *** = $P < 0.0001$. **(B)** Mild symptoms of *R. solani* infection on Bd 21-3 including chlorosis and small, necrotic lesions. **(C)** Moderate infection of Bd 21-3 by *R. solani* with necrotic leaf tips, severe necrosis surrounding the site of inoculation, and the presence of sparse mycelia near the site inoculation.

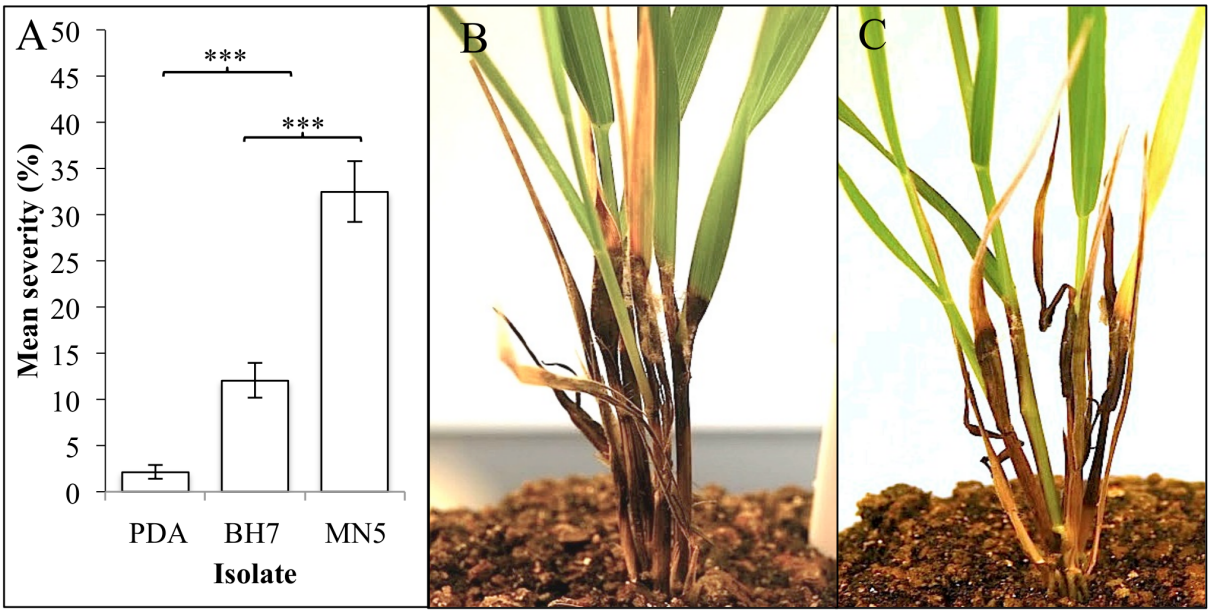

Figure 7. Infection of *B. distachyon* inbred line Bd 21-3 with *Microdochium nivale* (A) Mean symptom severity of Bd 21-3 five days following inoculation with *M. nivale* isolate BH7 or MN5, or with a PDA plug. Single degree-of-freedom orthogonal contrast statements were used to compare between *M. nivale* isolates and between M. Nivale isolates and the PDA control at α=0.05. *** = $P < 0.0001$. **(B)** Symptoms of less aggressive *M. nivale* isolate BH7 on Bd 21-3. **(C)** Moderate infection of Bd 21-3 by *M. nivale* isolate MN5, including necrosis, wilting, and production of sparse mycelia at the site of infection.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this dissertation provides valuable new insights on the biology and pathogenicity mechanisms of the fungus responsible for the most economically important disease of turfgrass. This research and future investigations based on my findings will facilitate the development and implementation of sustainable integrated disease management practices for dollar spot. To better understand dollar spot epidemiology, I assessed both overwintering of *S. homoeocarpa* and presence of this fungus on commercial creeping bentgrass seed. Overwintering studies indicated that *S. homoeocarpa* can survive the winter months in both symptomatic and asymptomatic creeping bentgrass shoots. Though *S. homoeocarpa* isolation rates were similar in all years of this study, spring isolation rates varied between years. This finding suggests that environmental factors such as temperature and snowfall influence *S. homoeocarpa* overwintering, but additional research focusing on the influence of these climatic variables on winter survival of this pathogen are needed. Additional research is also needed to determine if early season dollar spot outbreaks are influenced by overwintering inoculum and if population dynamics of this pathogen shift from the fall to the spring of a given winter.

A single viable isolate of *S. homoeocarpa* was obtained from commercial creeping bentgrass seed using culture-based detection methods. To facilitate more sensitive, higher-throughput screening, I developed a molecular method employing nested PCR to test for the presence of pathogen DNA in seed. With this method, 75% of commercial seed lots tested were positive for the presence of *S. homoeocarpa* DNA. This research provides the first report of culture-based or molecular detection of *S. homoeocarpa* in commercial seed of any turfgrass species. Further research is needed to demonstrate that the detection of this pathogen in seed actually translates to introduction of inoculum that leads to dollar spot epidemics into the field. If this is found to be the case, additional studies will be needed to develop high-throughput

detection methods that can be used by commercial seed production companies and to facilitate the development of best practices for preventing or controlling introduction of dollar spot inoculum into new locations.

Quantification of oxalate content in *S. homoeocarpa*-inoculated plants and expression analysis of oxalate oxidase and related genes indicated that oxalic acid is produced by *S. homoeocarpa* after initial host colonization and is likely associated with the transition of this pathogen from biotrophy to necrotrophy. Further, *in vitro* studies comparing oxalic acid production between *S. homoeocarpa* and *S. sclerotiorum* revealed that both host material and xylan monomers induce production of oxalic acid by *S. homoeocarpa*. Based on these results, I propose that degradation of xylan is an important aspect of early host colonization by *S. homoeocarpa* and is related to the induction of oxalic acid and transition to necrotrophy during infection by this pathogen. Mutant resources and a stable transformation system for this fungus are needed and can be used to functionally confirm the importance of xylan degradation and oxalic acid production in pathogenesis of *S. homoeocarpa*. Additionally, the finding that host endogenous oxalate content influences resistance to dollar spot offers a possible trait for selection in creeping bentgrass breeding programs. However further research is needed to confirm this connection beyond the correlative evidence I have provided.

Brachypodium distachyon is becoming a common model system for cereal genetics and pathology. Three major fungal pathogens of turfgrass species (*Sclerotinia homoeocarpa, Rhizoctonia solani,* and *Microdochium nivale*) infected *B. distachyon* and produced symptoms similar to those observed on the natural turfgrass host creeping bentgrass. In-depth studies with *S. homoeocarpa* revealed similar progression of infection at the microscopic and macroscopic levels on *B. distachyon* and creeping bentgrass. Further, differences in resistance to *S.*

homoeocarpa were found between *Brachypodium* species and accessions that could be leveraged to determine genetic determinants of resistance to this pathogen. While this research lays the initial groundwork for development of *B. distachyon* as a model system for fungal turfgrass pathogens, genetic studies are now needed to confirm that *B. distachyon* and natural turfgrass hosts respond similarly to infection by *S. homoeocarpa* at the molecular level.