POPULATION DIVERSITY AND FUNGICIDE SENSITIVITY OF *Gaeumannomyces graminis* var. *graminis* FROM ST. AUGUSTINEGRASS IN TEXAS

A Thesis

by

MATTHEW JOHN ZIDEK

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Young-Ki Jo
Committee Members,	Daniel J. Ebbole
	Richard H. White
Head of Department,	Leland S. Pierson III

August 2015

Major Subject: Plant Pathology

ABSTRACT

Gaeumannomyces graminis var. *graminis* (*Ggg*) is an ectotrophic root infecting fungus that causes root-decline of warm-season turfgrasses and black sheath rot of rice (*Oryza sativa*). A total of 76 *Ggg* isolates were collected from St. Augustinegrass (*Stenotaphrum secundatum*) throughout Texas. Variations of colony morphology, hyphopodia production, teleomorphic and anamorphic structures, and growth rate of isolates were examined on potato dextrose agar (PDA), and rice seedling pathogenicity assays were performed. A multilocus phylogeny reconstruction was developed to support phenotypic data and to resolve the phylogeny of *Ggg*. Fungicide sensitivities were also evaluated. Azoxystrobin 50% effective concentration (EC_{50}) values of nine isolates were determined in the presence and absence of salicylhydroxamic acid (SHAM) for determination of alternative respiration. Tebuconazole and azoxystrobin EC_{50} values from 15 and 20 isolates, respectively, were used to establish single discriminatory concentrations for resistance screening and EC_{50} determination of remaining isolates. Isolates were also screened for thiophanate-methyl resistance.

Three phenotypic groups were found on PDA: (i) highly melanized with round colony formation (termed M group), (ii) non to slightly melanized with round colony formation (termed L group), and (iii) highly melanized with irregular colony formation (termed H group). All isolates produced lobed hyphopodia and a *Phialophora*-like anamorph, where phialospore lengths were significantly longer among M and H groups compared to the L group. M group isolates were adapted to warmer temperatures

compared to L and H group isolates, and groups were differentiated based on growth rates at 35°C. The M group was most aggressive. A multilocus phylogeny reconstruction supported the differentiation between phenotypic groups and distinguished *Ggg* and *G. graminis* varieties *avenae* and *tritici*.

EC_{50} values from azoxystrobin-amended PDA were significantly lower in the presence of SHAM, indicating utilization of alternative respiration. Azoxystrobin and tebuconazole discriminatory concentrations were 0.1 µg a.i. ml^{-1} and were effective for rapid EC_{50} determination. Azoxystrobin EC_{50} values for 75 isolates ranged from <0.014 to 0.399. Tebuconazole EC_{50} values for 76 isolates ranged from 0.021 to 0.296 with a mean of 0.049. All isolates were completely inhibited by thiophanate-methyl at 500 µg a.i. ml^{-1}.

DEDICATION

This work is dedicated to my dearest family. My academic endeavors were driven by the life lessons taught by my parents. To my mom, Carol Zidek, thank you for your enlightening words and for always supporting my education. To my dad, John Zidek, from whom I learned a strong work ethic. To my sisters, Olivia and Emily Zidek, I hope that my achievements will inspire you to strive academically and never stop learning.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Young-Ki Jo. You saw something in me and gave me the opportunity to pursue a higher degree. Your dedication to research encouraged me to work hard towards my projects, and your creativity greatly contributed to my accomplishments as a young researcher. I also thank my committee members, Dr. Richard White and Dr. Daniel Ebbole, for your helpful guidance and input. Your intellect and words of advice also contributed to the success of this work.

Special thanks also go to Sheila McBride and Dr. Kevin Ong. Your enthusiasm for plant pathology and plant disease diagnostics enlightened me to further my education. You are both amazing teachers, and I am very grateful for the opportunity I had to work at the diagnostic clinic. Furthermore, thank you for providing me the majority of samples used during my research.

Thanks also go to the department faculty and staff, my professors, colleagues, and lab mates for making graduate school an enjoyable experience. To Dr. David Appel, I enjoyed your witty character and found your classes exciting. I looked forward to the thought-provoking conversations awaiting journal club meetings led by Dr. Marty Dickman. To the Scholthof Lab, thank you for the copious amounts of coffee that extended my hours of productivity. To Andrew Cromwell, Zhenzhong Guo, and Yuguo Yang, thank you for assisting with fungal isolations. To Maxwell Handiseni, your knowledge of statistics helped me to properly analyze and make conclusions about my data. To Lin Yu, you greatly contributed to my fungal sequencing analyses and taught

me much of what I know about phylogenetics. I enjoyed our conversations and learned more from you than you probably realize.

Finally, thanks to my family and friends for their support during my undergraduate and graduate careers. Time spent away from home is not easy, but you continued to encourage me to follow my ambitions. Your love and support made my education possible.

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CHAPTER I

INTRODUCTION

Gaeumannomyces graminis (Sacc.) von Arx & D. Olivier *var. graminis* (*Ggg*) is the etiological agent of take-all root rot (TARR) of St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) (Elliott et al. 1993), diseases of other warm-season turfgrasses, (Elliott 1991; McCarty and Lucas 1989; Wilkinson 1994; Wilkinson and Kane 1993), and crown (black) sheath rot of rice (*Oryza sativa* L.) (Walker 1981). Other varieties of *G. graminis* have been recognized, which include *Gaeumannomyces graminis* var. *avenae* (E.M. Turner) Dennis (*Gga*) and *Gaeumannomyces graminis* var. *tritici* Walker (*Ggt*) (Walker 1972), both of which infect a broad range of wild grasses, wheat (*Triticum aestivum* L.), and other cereals (Walker 1981). *Gga* is primarily associated with take-all of oats (*Avenae sativa* L.) and causes take-all patch of turfgrasses, while *Ggt* is often associated with take-all of wheat and barley (*Hordeum vulgare* L.) (Walker 1981). In addition to pathogenicity, *G. graminis* varieties are distinguished by ascospore size and hyphopodia morphology, where *Gga* and *Ggt* produce simple hyphopodia, and *Ggg* produces lobed hyphopodia (Walker 1981). *G. graminis* varieties also produce *Phialophora*-like anamorphs and comprise part of the *Gaeumannomyces-Phialophora* (G-P) complex (Bryan et al. 1995). Furthermore, genetic variations have differentiated members of the G-P complex, and many reports indicate variability within *Ggg* (Elliott et al. 1993; Fouly 2002; Fouly et al. 2011; Fouly et al. 1997; Fouly et al. 1996; Ward and Akrofi 1994; Ward and Bateman 1999).

Previous reports have also indicated the separation of *Ggt* into groups based on wheat and rye pathogenicity assays (Hollins and Scott 1990) and multiple genetic approaches (Augustin et al. 1999; Bateman et al. 1997; Bryan et al. 1995; Bryan et al. 1999; Daval et al. 2010; Freeman et al. 2005; Lebreton et al. 2004; O'Dell et al. 1992). Resolution of the intravarietal complexities within *Ggg* could indicate the existence of specific groups, which is important from a biological perspective, as well as an applied standpoint, considering development of proper diagnostic procedures and disease management programs.

Chemical control is a current option for TARR and root-decline management. Penetrant fungicides, such as methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs), have been widely used for turfgrass disease management since the 1970s (Martin 2003). Fungicides of these chemical groups are also labeled for residential and commercial use for controlling root-decline. To date, several reports have indicated increased resistance of fungal turfgrass pathogens to the MBC, DMI, and QoI fungicides (Avila-Adame et al. 2003; Golembiewski et al. 1995; Jo et al. 2006; Vincelli and Dixon 2002); however, fungicide sensitivity distributions of *Ggg* are not currently understood, and proper *in vitro* fungicide sensitivity assays need to be developed.

Fungi possess the ability of bypassing the effects of QoIs by induction of an alternative respiration pathway (Avila-Adame and Köller 2002; Joseph-Horne et al. 2001; Köller et al. 2002). For proper *in vitro* assessment of QoI sensitivities, growth media is often amended with salicylhydroxamic acid to inhibit alternative respiration

(Avila-Adame et al. 2003; Kim et al. 2003; Olaya and Köller 1999; Vincelli and Dixon 2002; Wong et al. 2007). The ability of *Ggg* to utilize alternative respiration is currently unknown, and determining whether this pathway is induced is critical for the development of proper screening assays.

Fungicide sensitivities are typically represented by EC_{50} values, the effective concentration that inhibits growth by 50% (Brent 1988; Jo et al. 2006; Köller and Scheinpflug 1987). Traditional methods of EC_{50} determination require the preparation of synthetic media at serial fungicide concentrations, which can be time-consuming and laborious. Alternatively, EC_{50} values may be predicted from relative growth at a single discriminatory dose, a more efficient method that has been previously used (Golembiewski et al. 1995; Jo et al. 2006; Miller et al. 2002; Wong et al. 2007). A rapid and high-throughput *in vitro* method for fungicide resistance screening and EC_{50} determination of *Ggg* should be developed for processing large sample collections.

The objectives of this study were to characterize (i) morphological variations in terms of colony morphology, hyphopodia production, and anamorphic and teleomorphic structures, (ii) physiological variations in terms of growth rate and pathogenicity, and (iii) genetic variations based on a multilocus phylogeny reconstruction using a set of 76 *Ggg* isolates infecting St. Augustinegrass throughout Texas.

For better understanding fungicide sensitivities, objectives were to (i) determine if *Ggg* can utilize alternative respiration for circumventing respiration inhibition by azoxystrobin, (ii) determine single discriminatory concentrations of azoxystrobin and tebuconazole for rapid screening and prediction of EC_{50} values, and (iii) determine

azoxystrobin, tebuconazole, and thiophanate-methyl sensitivity distributions from the same set of isolates evaluated for population diversity.

CHAPTER II

RESOLVING INTRAVARIETAL COMPLEXITIES OF *Gaeumannomyces graminis* var. *graminis* ISOLATED FROM ST. AUGUSTINEGRASS IN TEXAS

Introduction

Gaeumannomyces graminis (Sacc.) von Arx & D. Olivier *var. graminis* (*Ggg*) is a soil-borne, ectotrophic root infecting fungus that is the etiological agent of take-all root rot (TARR) of St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) (Elliott et al. 1993). During early stages of TARR, symptoms are characterized by chlorotic patches of turfgrass resulting from necrosis of the infected root system. As the disease progresses, infected areas will begin to thin and eventually turn bare, where recovery is difficult. *Ggg* is also associated with root diseases of other warm-season turfgrasses, such as bermudagrass (*Cynodon dactylon* (L.) Pers. × *transvaalensis* Burtt-Davy) decline (Elliott 1991), spring dead spot of bermudagrass (McCarty and Lucas 1989), and root rot of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) (Wilkinson 1994) and zoysiagrass (*Zoysia japonica* Steud.) (Wilkinson and Kane 1993). Together, these diseases are termed as root-decline of warm-season turfgrasses (Smiley et al. 2005). Furthermore, *Ggg* is the etiological agent of crown (black) sheath rot of rice (*Oryza sativa* L.) (Walker 1981).

Other varieties of *G. graminis* have been recognized, which include *Gaeumannomyces graminis* var. *avenae* (E.M. Turner) Dennis (*Gga*) and *Gaeumannomyces graminis* var. *tritici* Walker (*Ggt*) (Walker 1972), both of which can

infect a broad range of wild grasses, wheat (*Triticum aestivum* L.), and other cereals (Walker 1981). *Gga* is primarily associated with take-all of oats (*Avenae sativa* L.) and causes take-all patch of turfgrasses (Walker 1981). *Ggt* is often associated with take-all of wheat and barley (*Hordeum vulgare* L.), an important disease that reduces yield worldwide (Walker 1980; Walker 1981). *Gga*, *Ggg*, and *Ggt* all exhibit *Phialophora*-like anamorphs and comprise part of the *Gaeumannomyces-Phialophora* (G-P) complex (Bryan et al. 1995). In addition to pathogenicity, *G. graminis* varieties are distinguished by ascospore size and hyphopodia morphology, where *Gga* and *Ggt* produce simple hyphopodia, and *Ggg* produces lobed hyphopodia (Walker 1981).

Analyses of nuclear ribosomal DNA (rDNA) have been used for differentiating members of the G-P complex, and many reports indicate variability within *Ggg* (Elliott et al. 1993; Fouly 2002; Fouly et al. 2011; Fouly et al. 1997; Fouly et al. 1996; Ward and Akrofi 1994; Ward and Bateman 1999). Restriction fragment length polymorphisms (RFLPs) of internal transcribed spacer (ITS) and 5.8S rDNA differentiated *G. graminis* varieties, and RFLP patterns were variable within *Ggg* (Bryan et al. 1999; Fouly et al. 1997; Ward and Akrofi 1994). Lengths of an amplified portion of 18S rDNA were also similar between *Gga* and *Ggt*; however, banding patterns varied for *Ggg* (Fouly et al. 1997). Randomly amplified polymorphic DNA (RAPD) markers were also used for identifying each of the *G. graminis* varieties, and *Ggg* showed a greater diversity of banding patterns than *Gga* and *Ggt* (Bryan et al. 1999; Fouly et al. 1996). Sequence analyses of rDNA have also been used for making phylogenetic comparisons between and within members of the G-P complex. A phylogenetic analysis based on the ITS

region indicated that *Gga* and *Ggt* formed monophyletic groupings, while *Ggg* appeared to be polyphyletic (Fouly et al. 1997). In a separate study, a phylogenetic analysis based on the ITS region showed that isolates of *Ggg* were variable and formed two groups, where each group was either closely or distantly related to *Gga* and *Ggt* (Fouly 2002). Two sets of sequence insertions within the small subunit (SSU) of rDNA were identified for members of the G-P complex, distinguishing the varieties of *G. graminis* and supporting that *Ggg* isolates were variable and complex (Fouly et al. 2011).

Furthermore, intravarietal variability of *Ggt* has been well studied. Hollins et al. (Hollins and Scott 1990) first separated *Ggt* into N and R groups based on pathogenicity assays using wheat and rye seedlings. Similar groups were also distinguished by genetic approaches such as RFLP (Bryan et al. 1999; O'Dell et al. 1992) and RAPD (Bryan et al. 1999) analyses and by comparison of ITS rDNA sequences (Bryan et al. 1995). RFLP and RAPD analyses in other studies have separated *Ggt* into two genetically distinct groups, such as T1/T2 (Bateman et al. 1997), A/B (Freeman et al. 2005), and A1/A2 (Augustin et al. 1999). *Ggt* populations from several wheat crops during a single year were also separated into two groups, G_1/G_2, according to RAPD and amplified fragment length polymorphism (AFLP) analyses, where isolates from the G_2 group were more aggressive than those belonging to G_1 (Lebreton et al. 2004). Daval et al. (Daval et al. 2010) also described two genetically distinct groups of *Ggt* based on sequence data of two genes from a worldwide collection of isolates.

The relationships between and within *G. graminis* varieties *avenae*, *graminis*, and *tritici* are complex, although distinct groups of *Ggt* have been well-resolved.

Currently, the intravarietal complexities associated with *Ggg* are not well-understood; therefore, a need exists for characterizing the diversity of this pathogen and for better understanding the phylogenetic relationships between *Ggg* and other members of the G-P complex. The objectives of this study were to characterize (i) morphological variations of colony morphology, hyphopodia production, and anamorphic and teleomorphic structures, (ii) physiological variations of growth rate and pathogenicity, and (iii) genetic variations based on a multilocus phylogeny reconstruction using a set of 76 *Ggg* isolates from St. Augustinegrass throughout Texas.

Materials and Methods

Collection and isolation of *Ggg*. A total of 76 isolates of *Ggg* were previously obtained from St. Augustinegrass, where samples were collected from Texas A&M University at College Station, Texas A&M AgriLife Research Center at Beaumont, and various residential lawns throughout Texas. Samples were also provided by the Texas Plant Disease Diagnostic Laboratory (TPDDL).

Stolons bearing ectotrophic hyphae and lobed hyphopodia were cut and washed under running tap water for approximately 5 min. Thin slices of infested stolon tissue were excised to a length no longer than 5 cm and were surface sterilized in several volumes of 0.57% NaClO and rinsed once with sterile distilled water. Surface sterilized tissues were dried on sterile 7.5-cm filter paper, and three pieces of tissue were subsequently placed on each of two plates of *Ggg* semi-selective medium containing the following: potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) amended

with mefenoxam (Mefenoxam 2 AQ; Makhteshim Agan of North America, Inc., Raleigh, NC, USA), flutolanil (ProStar 70 WDG; Bayer Environmental Science, Montvale, NJ, USA), and iprodione (Ipro 2SE; Makhteshim Agan of North America, Inc.), each at 10 µg a.i. ml^{-1}, and streptomycin sulfate at 100 µg ml^{-1}. Plates were incubated at ambient temperature and were monitored for hyphae that curled back at the edges of fungal colonies, one of the cultural characteristics of *Ggg* (Tomaso-Peterson et al. 2000). Hyphal tips were transferred to PDA and were allowed to grow at 25°C for isolation. Long-term storage of each isolate was achieved according to a method previously described by Elliott (Elliott 2005), where eight plugs of actively-growing mycelium were cut using a 5-mm cork borer and transferred to a 4-ml clear glass screw cap vial containing 1.5 ml of sterile distilled water. Vials were sealed with Parafilm M (Structure Probe, Inc., West Chester, PA, USA) and kept in the dark at ambient temperature.

Cultural characterization. All isolates obtained for this study were evaluated for variation of colony morphology. Actively-growing mycelium belonging to each isolate was transferred to PDA using a sterile 5-mm cork borer. After one and two weeks of growth at 25°C, all isolates were evaluated and grouped based on morphological features such as melanization and colony formation. Percentages of isolates belonging to various phenotypic groups were determined in relation to the entire collection of *Ggg*. Five representative isolates belonging to each of three morphological groups observed in this study were randomly selected for further evaluations of growth rate, pathogenicity, and phylogenetic analysis. Isolates were also observed for the production of lobed

hyphopodia, perithecia, and phialidic structures after a 10-week incubation period on PDA at 25°C. All plates used for cultural characterizations were sealed with Parafilm M before starting the incubation period. Hyphopodia, perithecia, asci, ascospores, and phialidic structures were observed using an Olympus CH30RF100 compound light microscope (Olympus Optical Co., Ltd., Tokyo, Japan), and images were acquired with an AM4023 Dino-Eye USB digital eyepiece (AnMo Electronics Corp., New Taipei, Taiwan). Mean phialospore and ascospore lengths were determined from a total of 10 spores obtained from each isolate using the measuring tool provided with DinoCapture 2.0 software (version 1.5.4; AnMo Electronics Corp.). Measurements were made under a ×40 objective lens, and spores were acquired either by flooding plates or from agar scrapings.

Significant difference of phialospore lengths between groups was determined with a one-way analysis of variance (ANOVA) using PROC GLM with SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA), and means were separated using Fisher's protected least significant difference (LSD) test at $P = 0.05$.

Determination of growth rate. Actively-growing mycelium was transferred from each isolate to PDA using a sterile 5-mm cork borer, and plates were allowed to incubate at 20, 25, 30, and 35 ± 1°C for three days. Radial growth was obtained by taking two perpendicular measurements of the colony diameter, calculating their average, and dividing the mean by two. Radial growth was measured every 24 h, and the mean growth rate (mm day^{-1}) was calculated by averaging radial growth acquired from

one and two days following the first day of incubation. There were three replicates per isolate.

Data from each incubation temperature were analyzed separately, and each experiment was arranged as a completely randomized design. At each temperature, significant difference of growth rates between isolates was determined with a one-way ANOVA using PROC GLM. Means between isolates by temperature were separated using Fisher's protected LSD test at $P = 0.05$.

Rice seedling pathogenicity assay. Rice cv. Presidio was used for root infection assays, which were similarly performed as previously described for *G. graminis* (Bowyer et al. 1995; Dufresne and Osbourn 2001). Inocula was prepared by recovering each isolate onto PDA from long-term storage for the production of actively-growing mycelium. Rice seeds were surface sterilized in several volumes of 1.2% NaClO for 10 minutes and rinsed 10 times with sterile distilled water. Seeds were pre-germinated in a petri dish containing two 7.5-cm filter papers moistened with 3 ml of sterile distilled water before sealing with Parafilm M and incubating at 29°C for approximately 24 h. Filter paper was added to the bottoms of 66-ml plastic cone-tainers (Stuewe & Sons, Inc., Corvallis, OR, USA), which were subsequently filled with sterile moistened vermiculite (Therm-O-Rock West, Inc., Chandler, AZ, USA) to leave approximately 3 cm of space at the top of each cone-tainer. Five plugs of actively-growing mycelium from each isolate were cut using a 5-mm cork borer and placed on the surface of vermiculite in each cone-tainer. Five sterile PDA plugs were used as a negative control. There were four replicates per treatment. Each tube was then filled with an additional

layer of vermiculite to leave about 1.5 cm of space. Two germinating rice seeds were added at the top of this layer, which were then covered with a final layer of vermiculite to fill to capacity. Prepared units were placed in a Model 2015 Diurnal Growth Chamber (VWR, West Chester, PA, USA) set at a constant temperature of 25°C with a photoperiod of 16 h and light intensity of 86.6 µmol m^{-2} s^{-1}. All cone-tainers were watered daily to saturation for the first week of incubation and once every four days thereafter. After the first week of incubation, each tube was fertilized with 5 ml of Miracle-Gro Water Soluble All Purpose Plant Food (Scott's Miracle-Gro Products, Inc., Port Washington, NY, USA) according to the label recommendations. Following an incubation period of 26 or 42 days post inoculation (dpi), roots were carefully washed free of vermiculite under running tap water, and shoot lengths, root lengths, and root lesions were measured to determine disease development.

A disease scale developed by Datnoff (Datnoff et al. 1997) was used to evaluate the severity of root lesions, where roots were scored based on the following: 1 = no discoloration of the root system; 2 = 1 to 25% of roots exhibiting black lesions or a general tan discoloration of the entire root system; 3 = 26 to 50% of roots with black coalescing lesions; 4 = 51 to 75% of roots with black coalescing lesions; 5 = 76 to 100% of roots with black lesions.

Leaf sheathes were observed for the development of hyphopodia and perithecia, and images were acquired with a USB digital eyepiece. Ten ascospores from each isolate were measured under a ×40 objective lens using the measuring tool provided with DinoCapture 2.0 software. Infected root tissue was surface sterilized with 0.57% NaClO,

rinsed once with sterile distilled water, and plated on the previously-described *Ggg* semi-selective medium for reisolation of *Ggg*.

The experiment was performed twice under similar conditions, with the exception of the incubation period duration, where the first and second experiments were terminated after 26 and 42 dpi, respectively. Experiments were analyzed separately as a completely randomized design, and significant differences of disease severity, shoot lengths, and root lengths between treatments and between groups were determined with a one-way ANOVA using PROC GLM. Means between treatments and between groups were separated using Fisher's protected LSD test at $P = 0.05$.

DNA extraction. Genomic DNA was isolated from *Ggg* according to modified procedures by Thomas et al. (Thomas and Kenerley 1989). Each isolate was grown on PDA at 25°C until petri dishes were entirely colonized. Fifty ml of glucose yeast extract broth (GYEB; 0.5 g of glucose, 0.25 g of yeast extract, and 50 ml of distilled water) was heavily inoculated with agar scrapings of mycelium using a sterile scalpel and was allowed to incubate for four days at 25°C while shaking at 100 rpm. Fungal tissue was harvested by filtering with sterile 7.5-cm filter paper and washing with several volumes of sterile distilled water. Harvested tissue was pressed dry with sterile paper towels, and ground to a fine powder in liquid nitrogen. Approximately 0.25 to 0.5 g of crushed fungal tissue was added to a 1.5-ml microcentrifuge tube and mixed with 500 µl of extraction buffer (50 mM Tris-HCl (pH 8.0), 50 mM sodium-EDTA (pH 8.0), and 2% sodium dodecyl sulfate), which was incubated for 30 min at 68°C. After incubation, a phenol-chloroform extraction method was performed to purify DNA according to

standard protocol (Sambrook et al. 1989). Purified DNA was treated with RNase and stored at -20°C until further use.

PCR, sequencing, and phylogenetic analysis. A PCR method with slight modifications was used for determining if amplification of each isolate could be achieved using a *Ggg*-specific primer set (Table 1) (Rachdawong et al. 2002). PCR amplification was performed with the GoTaq PCR Core System I kit (Promega, Madison, WI, USA) in 25-µl reactions containing 1.5 mM $MgCl_2$, 0.625 U of GoTaq DNA polymerase, 5 µl of 5× Green GoTaq Flexi Buffer, 200 µM dNTPs, 0.5 µM of each primer, and 0.2 µg of fungal DNA. PCR amplification was carried out in a 2720 thermal cycler (Applied Biosystems, Warrington, UK) under the following cycling conditions: initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 45 s, 59°C for 45 s, 72°C for 2 min, and a final extension step of 72°C for 10 min.

For each isolate, regions of three loci were amplified: (i) ITS, partial 18S and 28S rRNA genes, ITS regions, and intervening 5.8S rRNA gene, (ii) LSU, partial 28S rRNA gene, and (iii) RPB1, partial RNA polymerase II largest subunit gene. ITS, LSU, and RPB1 regions were amplified using ITS1/ITS4, LS1/LR5, and RPB1F/RPB1R primer sets, respectively (Table 1) (Klaubauf et al. 2014; Rehner and Samuels 1995; White et al. 1990). Non-degenerate primers were also developed for amplifying a partial RPB1 region, which were termed RPB1-NF and RPB1-NR for forward and reverse primers, respectively. RPB1-NF and RPB1-NR primers were designed to flank RPB1 sequences of published *Ggg*, *Gga*, and *Ggt* taxa retrieved from the National Center for Biotechnology Information (NCBI) GenBank database

Table 1. List of primers used and developed for amplification and sequencing of *Gaeumannomyces graminis* var. *graminis*

Locus[a]	Primer name	Orientation and sequence (5' - 3')	Reference
ALG	*Ggg*-specific	F-CAC CCC CGG TCC CTG CGT A	Rachdawong et al. 2002
	AV3	R-TGC TCA TGG TGG TTC CTG CG	Rachdawong et al. 2002
ITS	ITS1	F-TCC GTA GGT GAA CCT GCG G	White et al. 1990
	ITS4	R-TCC TCC GCT TAT TGA TAT GC	White et al. 1990
LSU	LS1	F-GTA CCC GCT GAA CTT AAG C	Rehner and Samuels 1995
	LR5	R-TCC TGA GGG AAA CTT CG	Rehner and Samuels 1995
RPB1	RPB1F	F-AGA CGA TYG AGG AGA TCC AGT T	Klaubauf et al. 2014
	RPB1R	R-ART CCA CAC GCT TAC CCA TC	Klaubauf et al. 2014
	RPB1-NF	F-AAG ATC CTG GAG ATG GTG TGC C	This study
	RPB1-NR	R-TGC CCT TGG ACG AGC G	This study

[a] ALG: partial avenacinase-like gene; ITS: internal transcribed spacer regions and intervening 5.8S rRNA gene; LSU: partial 28S rRNA gene; RPB1: partial RNA polymerase II largest subunit gene.

(http://www.ncbi.nlm.nih.gov/nucleotide) using Primer Premier software (Table 2) (version 5.0; Premier Biosoft International, Palo Alto, CA, USA).

PCR of ITS, LSU, and RPB1 regions were performed with the GoTaq PCR Core System I kit in 50-ul reactions containing 1.5 mM $MgCl_2$, 1.25 U of GoTaq DNA polymerase, 10 µl of 5× Green GoTaq Flexi Buffer, 300 µM dNTPs, 0.8 µM of each primer, and 0.2 µg of fungal DNA.

PCR amplification of the ITS region was performed under the following cycling conditions: initial denaturation step at 95° C for 3 min, followed by 40 cycles of 95°C for 45 s, 50°C for 45 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR amplification of the LSU region was performed under the following cycling conditions: initial denaturation step of 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 57°C for 1 min, 72°C for 70 sec, and a final extension step of 72°C for 10 min. PCR amplification of the RPB1 region using the RPB1F/RPB1R primer set was performed under the following cycling conditions: initial denaturation step at 95°C for 5 min, followed by 12 cycles of 95°C for 30 s, 57 to 51°C (decreasing for 0.5°C every cycle) for 20 s, 72°C for 70 s; 25 cycles of 95°C for 30 s, 51°C for 20 s, 72°C for 70 s; and a final extension step of 72°C for 5 min. PCR amplification using the RPB1-NF/RPB1-NR primer set was achieved under the following cycling conditions: initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 56°C for 1 min, 72° C for 1 min, and a final extension step of 72°C for 5 min. PCR amplification using the ITS1/ITS4, LS1/LR5, and RPB1-NF/RPB1-NR primer sets were carried out in a 2720 thermal cycler, and PCR amplification using the RPB1F/RPB1R primer set was

Table 2. Species names, isolate numbers, source locations, hosts, and GenBank accession numbers of fungi evaluated during this study

			GenBank accession numbers[b]		
Species and isolate numbers[a]	**Location**	**Host**	**ITS**	**LSU**	**RPB1**
Gaeumannomyces graminis var. *graminis*					
MZTX9-1	USA: Texas	*Stenotaphrum secundatum*	KR610476	KR610491	KR610506
MZTX13-1	USA: Texas	*Stenotaphrum secundatum*	KR610477	KR610492	KR610507
MZTX28-1	USA: Texas	*Stenotaphrum secundatum*	KR610478	KR610493	KR610508
MZTX29-1	USA: Texas	*Stenotaphrum secundatum*	KR610479	KR610494	KR610509
MZTX34-1	USA: Texas	*Stenotaphrum secundatum*	KR610480	KR610495	KR610510
MZTX35-1	USA: Texas	*Stenotaphrum secundatum*	KR610481	KR610496	KR610511
MZTX38-1	USA: Texas	*Stenotaphrum secundatum*	KR610482	KR610497	KR610512
MZTX40-1	USA: Texas	*Stenotaphrum secundatum*	KR610483	KR610498	KR610513
MZTX42-1	USA: Texas	*Stenotaphrum secundatum*	KR610484	KR610499	KR610514
MZTX44-2	USA: Texas	*Stenotaphrum secundatum*	KR610485	KR610500	KR610515
MZTX45-1	USA: Texas	*Stenotaphrum secundatum*	KR610486	KR610501	KR610516

Table 2. (Continued)

Species and isolate numbers[a]	Location	Host	GenBank accession numbers[b]		
			ITS	LSU	RPB1
MZTX50-1	USA: Texas	*Stenotaphrum secundatum*	KR610487	KR610502	KR610517
MZTX67-1	USA: Texas	*Stenotaphrum secundatum*	KR610488	KR610503	KR610518
MZTX70-1	USA: Texas	*Stenotaphrum secundatum*	KR610489	KR610504	KR610519
MZTX73-1	USA: Texas	*Stenotaphrum secundatum*	KR610490	KR610505	KR610520
M33	USA: Florida	*Stenotaphrum secundatum*	JF710374	JF414896	JF710442
M53	USA: Florida	unknown	JF414847	JF414897	JF710443
M54	USA: Florida	unknown	JF414848	JF414898	JF710444
M57	USA: Florida	*Stenotaphrum secundatum*	JF414849	JF414899	JF710446
CBS 235.32	USA: Arkansas	*Oryza sativa*	JX134669	JX134681	KM485049
CBS 352.93 = PD 93/290	Netherlands: near Barendrecht	*Ctenanthe* sp.	KM484834	DQ341496	KM485050
CBS 902.73	Australia: New South Wales	*Stenotaphrum secundatum*	KM484836	KM484953	KM485052
CBS 903.73	Australia: New South Wales	*Pennisetum clandestinum*	KM484837	KM484954	KM485053

Table 2. (Continued)

Species and isolate numbers[a]	Location	Host	GenBank accession numbers[b]		
			ITS	LSU	RPB1
Gaeumannomyces graminis var. *avenae*					
CBS 187.65	Netherlands: Flevoland	*Avena sativa*	JX134668	JX134680	JX134722
CBS 870.73	Australia: Western Australia	*Avena sativa*	KM484833	DQ341495	KM485048
Gaeumannomyces graminis var. *tritici*					
CBS 186.65	Netherlands: Flevoland	*Hordeum vulgare*	KM484838	KM484955	KM485054
CBS 905.73	Australia: Western Australia	*Triticum aestivum*	KM484841	KM484958	KM485057
Magnaporthe poae					
ATCC 64411	USA	*Triticum aestivum*	Genome	Genome	Genome

[a] ATCC: American Type Culture Collection, Manassas, Virginia, USA; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

[b] ITS: internal transcribed spacer regions and intervening 5.8S rRNA gene; LSU: partial 28S rRNA gene; RPB1: partial RNA polymerase II largest subunit gene; Genome sequences of *Magnaporthe poae* strain ATCC 64411 were retrieved from Broad Institute.

performed in a MasterCycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). PCR products were extracted and purified from a 1.8% agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol.

Automated sequencing reactions were performed using the ABI Big Dye (Perkin-Elmer, Norwalk, CT, USA) reaction mix, where 6 µl of each extracted template and 2 µl of either ITS, LSU, or RPB1 forward or reverse primers (5 µM) were added to 2 µl of ABI Big Dye. PCR reactions were performed using a 2720 thermal cycler under the following conditions: initial denaturation step at 96°C for 2 min, followed by 40 cycles of 96°C for 30 s, 42°C for 15 s, and 60°C for 4 min. Following PCR reactions, 10 µl of nuclease free water was added to each reaction, and Spin-50 mini-columns with Sephadex-G50 (BioMax, Odenton, MD, USA) were used to purify PCR products according to the manufacturer's protocol. Cleaned reactions were vacuum-dried for 30 min and submitted to the Texas A&M University Gene Technology Laboratory for sequencing of targeted genes using an ABI 3100 Automated Sequencer (Applied Biosystems).

ITS, LSU, and RPB1 sequences determined during this study were aligned with published *Ggg*, *Ggt*, and *Gga* sequences retrieved from the NCBI GenBank database and genomic sequences of *Magnaporthe poae* strain ATCC 64411 retrieved from Broad Institute (Table 2) (www.broadinstitute.org). Sequences were aligned using DNAMAN software (version 8.0; Lynnon Biosoft, Quebec, Canada). Partial ITS regions and entire intervening 5.8S rDNA, partial LSU, and partial RPB1 sequences were selected from alignments and combined for a phylogenetic analysis using the maximum likelihood

(ML) statistical method (best fit model TN93 + G) with 1,000 bootstrap replicates using MEGA6 software (Tamura et al. 2013).

Nucleotide sequence accession numbers. ITS, LSU, and RPB1 sequences determined in this study and utilized for the multilocus phylogeny reconstruction can be found under the accession numbers listed in Table 2.

Results

Morphological characterization. Evaluation of colony morphologies after one and two weeks of growth on PDA revealed three distinct phenotypic groups: (i) highly melanized with a round colony formation (termed M group), (ii) non to slightly melanized with a round colony formation (termed L group), and (iii) highly melanized with an irregular colony formation (termed H group; Fig. 1). Each isolate could be placed in either of the M, L, or H groups, which composed 10.5, 80.3, and 9.2% of the entire *Ggg* collection, respectively.

After 10 weeks of growth on PDA, high quantities of lobed hyphopodia were observed underneath cultures and on the sides of petri dishes for all M and H group isolates (Table 3). Low quantities of lobed hyphopodia were observed on petri dishes containing MZTX44-2 and 9-1, and it appeared that no hyphopodia were developed by the remaining L group isolates. Flask-shaped perithecia were readily produced by all H group isolates and one isolate (MZTX73-1) from the M group (Table 3). Perithecia were embedded or produced near the surface of the culture medium and were dark brown to black (Fig. 1). Ascospores from PDA were hyaline with multiple septations, filiform,

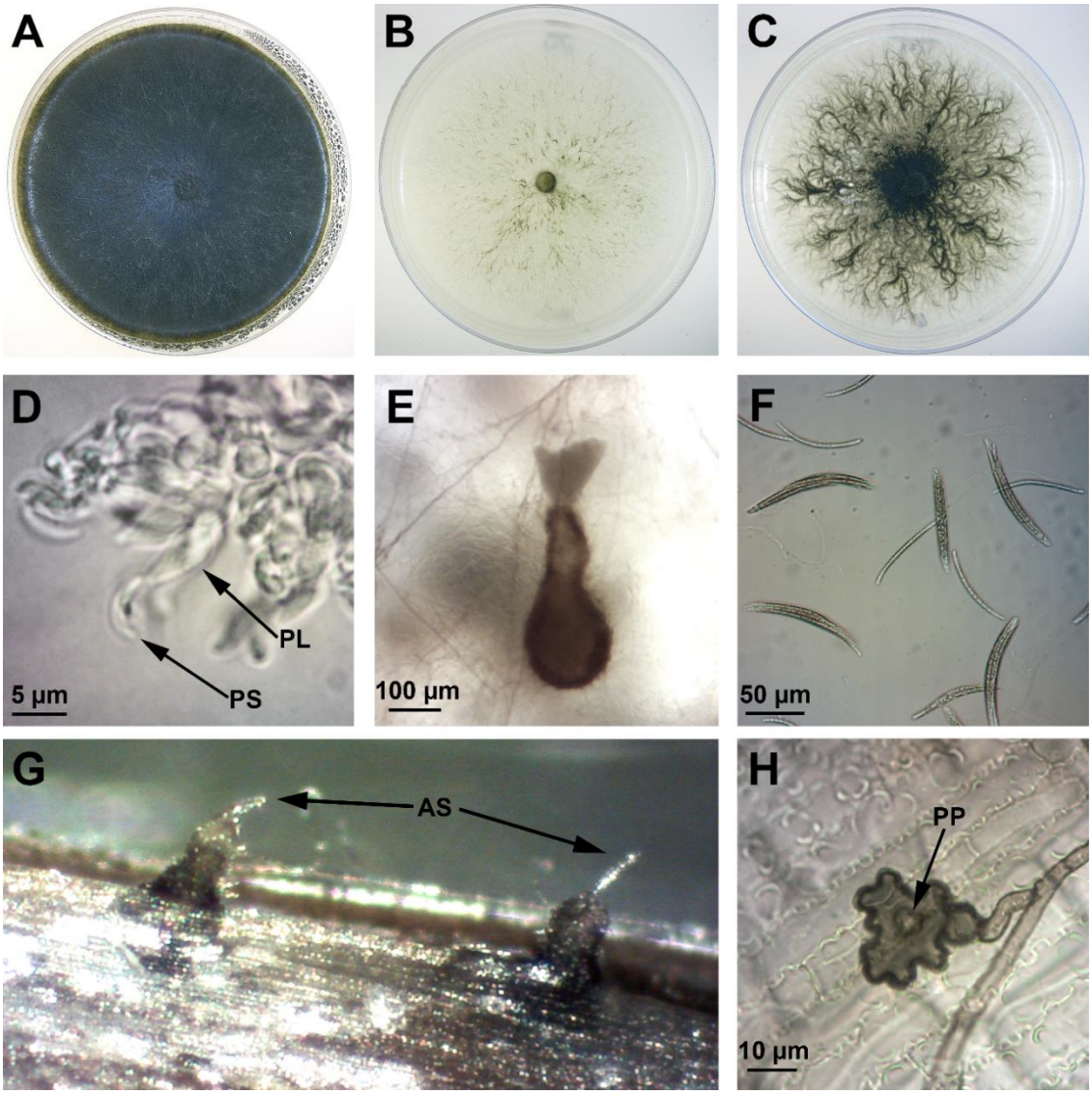

Fig. 1. Morphological features of *Gaeumannomyces graminis* var. *graminis* (*Ggg*) observed during this study. **A**, **B**, and **C**, cultures of *Ggg* after one week of growth on potato dextrose agar (PDA) representing M, L, and H groups, respectively; **D**, phialides and phialospores produced on PDA (arrows labeled PL and PS indicate phialide and phialospore, respectively); **E**, perithecium and liberating asci and ascospores produced on PDA; **F**, asci and ascospores produced on PDA; **G**, perithecia protruding through leaf sheath of rice seedling (arrows labeled AS indicate liberating asci and ascospores); **H**, lobed hyphopodium branching from ectotrophic runner hypha produced upon leaf sheath of rice seedling (arrow labeled PP indicates penetration peg).

Table 3. Morphological characteristics and PCR production developed by *Gaeumannomyces graminis* var. *graminis*

		PDA[a]			Rice sheath[b]		
Groups	**Isolates**	**Lobed hyphopodia[c]**	**Mean ascospore length**	**Mean phialospore length**	**Lobed hyphopodia**	**Mean ascospore length**	**PCR[f]**
M	MZTX13-1	++	ND[d]	7.8 ± 1.1	+	ND	0
	MZTX34-1	++	ND	7.5 ± 1.0	+	81.5 ± 5.1	0
	MZTX38-1	++	ND	7.4 ± 0.9	+	84.2 ± 4.7	0
	MZTX50-1	++	ND	9.1 ± 2.5	+	86.5 ± 4.8	0
	MZTX73-1	++	90.8 ± 5.2[e]	8.0 ± 1.0	+	82.8 ± 4.0	0
L	MZTX28-1	0	ND	6.3 ± 0.6	+	ND	+
	MZTX40-1	0	ND	6.9 ± 0.6	+	ND	+
	MZTX44-2	+	ND	8.6 ± 1.2	+	ND	+
	MZTX45-1	0	ND	6.8 ± 0.9	+	ND	+
	MZTX9-1	+	ND	7.8 ± 1.3	+	ND	+
H	MZTX29-1	++	83.0 ± 5.4	7.8 ± 1.2	+	80.8 ± 8.1	0
	MZTX35-1	++	83.1 ± 4.2	8.0 ± 0.8	+	89.1 ± 3.9	0
	MZTX42-1	++	91.2 ± 5.5	8.5 ± 1.6	+	ND	0
	MZTX67-1	++	84.9 ± 5.8	9.0 ± 1.6	+	82.6 ± 4.6	0
	MZTX70-1	++	86.3 ± 9.4	8.3 ± 1.2	+	86.7 ± 5.9	0

[a] *Gaeumannomyces graminis* var. *graminis* (*Ggg*) cultures on potato dextrose agar observed after 10 weeks at 25°C.
[b] Rice cv. Presidio seedlings were observed 42 days post inoculation with *Ggg*.
[c] Lobed hyphopodia produced on petri dishes were categorized based on the following: 0 = hyphopodia not developed; + = low quantities of hyphopodia production; ++ = high quantities of hyphopodia.
[d] ND = not determined due to failure of perithecial development.
[e] Means followed by ± standard deviation.
[f] PCR amplification using a *Ggg*-specific primer set targeting the avenacinase-like gene.

slightly curved, and ranged from 83.0 to 91.2 µm (Fig. 1; Table 3). Hyaline and lunate phialospores were produced by all isolates tested (Fig. 1; Table 3). The phialospore length produced by the L group was shorter than M and H groups, and phialospore lengths among M and H groups were similar (Fig. 2). Mean phialospore lengths for M, L, and H groups were 7.9, 7.3, and 8.3 µm and ranged from 7.4 to 9.1, 6.3 to 8.6, and 7.8 to 9.0 µm, respectively.

Lobed hyphopodia and perithecia were formed on rice sheathes and culms, based on observations after 42 dpi (Fig. 1). Lobed hyphopodia were developed by all isolates tested; however, perithecia were not produced by all L group isolates, one isolate from the M group (MZTX13-1), and one isolate from the H group (MZTX42-1; Table 3). Perithecia were embedded under black lesions of rice sheathes and protruded for the liberation of ascospores (Fig. 1). Perithecia and ascospore morphologies were similar to those observed from PDA cultures. Mean ascospore lengths ranged from 80.8 to 89.1 µm (Table 3).

Determination of growth rate. Five isolates belonging to each of the M, L, and H groups were evaluated for growth rate at 20, 25, 30, and 35 ± 1°C. All M group isolates, with the exception of MZTX13-1, optimally grew at 30°C, and all L and H group isolates, with the exception of MZTX70-1, optimally grew at 25°C (Table 4). Optimal growth rates of MZTX13-1 and 70-1 were 25 and 30°C, respectively.

A one-way ANOVA determined a significant difference ($P < 0.0001$) of growth rates between isolates at each temperature. M and L group isolates grew faster than H group isolates based on growth rates at 20°C (Table 4). The mean growth rates of M and

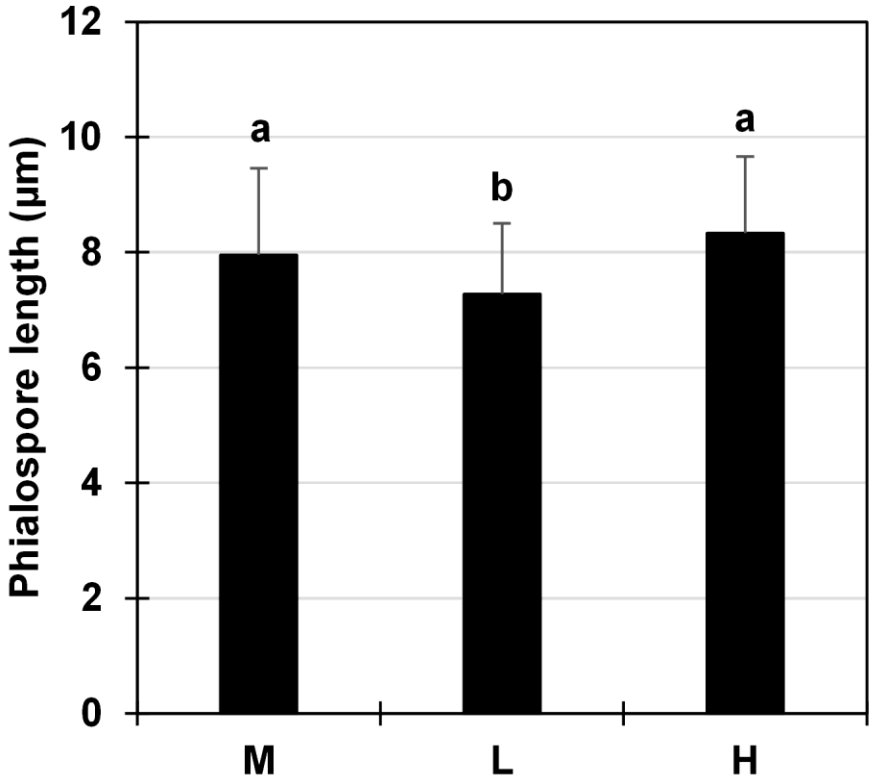

Fig. 2. Mean phialospore lengths produced by M, L, and H groups of *Gaeumannomyces graminis* var. *graminis*. Means followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$. Error bars indicate the standard deviation of the mean.

Table 4. Radial growth rates of *Gaeumannomyces graminis* var. *graminis* on potato dextrose agar at 20, 25, 30, and 35°C

		Mean radial growth rate (mm day^{-1})[y]			
Groups	**Isolates**	**20°C**	**25°C**	**30°C**	**35°C**
M	MZTX13-1	6.2 a[z]	9.0 bc	8.2 b	0.9 e
	MZTX34-1	5.7 ab	9.0 bc	10.5 a	6.3 a
	MZTX38-1	5.2 b	9.5 ab	10.3 a	6.8 a
	MZTX50-1	3.3 d	6.1 h	6.7 cd	5.3 b
	MZTX73-1	6.2 a	9.7 a	10.6 a	5.4 b
L	MZTX28-1	4.3 c	7.3 fg	5.7 ef	0.7 ef
	MZTX40-1	5.3 b	8.1 de	7.2 c	0.4 ef
	MZTX44-2	4.5 c	7.6 ef	6.5 d	0.8 ef
	MZTX45-1	5.5 ab	8.1 de	3.6 h	1.0 e
	MZTX9-1	6.0 a	8.6 cd	7.3 c	0.2 f
H	MZTX29-1	2.3 e	6.7 g	5.3 fg	3.7 c
	MZTX35-1	1.3 f	5.8 h	4.0 h	3.5 c
	MZTX42-1	2.4 e	5.3 i	4.9 g	2.5 d
	MZTX67-1	2.6 e	6.7 g	6.1 de	1.8 d
	MZTX70-1	1.4 f	4.4 j	6.1 de	2.1 d

[y] Radial growth was measured every 24 h for three days, and the mean growth rate was calculated by averaging radial growth acquired from one and two days following the first day of incubation.

[z] Means followed by the same letter in each column are not significantly different based on Fisher's protected least significant difference test at $P = 0.05$.

L groups were 5.3 and 5.1 mm day^{-1} and ranged from 3.3 to 6.2 and 4.3 to 6.0 mm day^{-1}, respectively. The H group grew at an average rate of 2.0 mm day^{-1}, which ranged from 1.3 to 2.6 mm day^{-1}.

At 25°C, all M group isolates, with the exception of MZTX50-1, grew faster than all H group isolates (Table 4). MZTX50-1 grew slower than MZTX29-1 and 67-1 and was similar to MZTX35-1. MZTX50-1 grew at an average rate of 6.1 mm day^{-1}, while the remaining M group isolates grew ≥9.0 mm day^{-1}. MZTX38-1 and 73-1 grew faster than all L group isolates, and growth rates of MZTX13-1 and 34-1 were similar to MZTX9-1. MZTX50-1 grew slower than all L group isolates. At 30°C, all M group isolates, with the exception of MZTX50-1, also grew at greater rates than isolates belonging to the L and H groups (Table 4). MZTX50-1 grew faster than two L group isolates (MZTX28-1 and 45-1) and three H group isolates (MZTX29-1, 35-1, and 42-1); however, MZTX50-1 grew at a similar rate to three L group isolates (MZTX40-1, 44-2, 9-1) and two H group isolates (MZTX67-1 and 70-1).

Finally, M, L, and H group isolates, with the exception of MZTX13-1, differed based on growth rates at 35°C (Table 4). MZTX13-1 grew at an average rate of 0.9 mm day^{-1} and was similar to most L group isolates. The L group grew at an average rate of 0.6 mm day^{-1} with a range from 0.2 to 1.0 mm day^{-1}. The M and H groups grew at an average rate of 4.9 and 2.7 mm day^{-1} with a range from 0.9 to 6.8 and 1.8 to 3.7 mm day^{-1}, respectively.

Rice seedling pathogenicity assay. All isolates were capable of producing distinct root lesions on rice cv. Presidio seedlings after 26 and 42 dpi, and a significant

difference ($P < 0.0001$) of disease severity ratings between treatments was observed in both experiments (Fig. 3). After 26 dpi, three of the isolates (MZTX40-1, 45-1, and 67-1) produced disease severities that were similar to the control (Table 5). Three isolates from the M group (MZTX13-1, 34-1, and 73-1) produced disease severity ratings that were similar to one isolate from the L group (MZTX9-1), three isolates from the H group (MZTX29-1, 35-1, and 70-1), and were more aggressive than the remaining L and H group isolates tested. MZTX38-1 and 50-1 were only more aggressive than MZTX40-1, 45-1, and 67-1.

Disease severity ratings were approximately two-fold higher after 42 dpi, and disease severities produced by all isolates were greater than the control (Table 5). All M group isolates, with the exception of MZTX50-1, were more aggressive than all L group isolates. MZTX50-1 produced a mean disease severity rating of 3.6, which was not different from any of the means produced by all L and H group isolates. Four of the M group isolates (MZTX13-1, 34-1, 38-1, and 73-1) were more aggressive than four of the H group isolates (MZTX35-1, 42-1, 67-1, and 70-1), while only MZTX38-1 was more aggressive than MZTX29-1. Most L and H group isolates produced similar disease severities, and MZTX29-1 was more aggressive than MZTX9-1, 28-1, and 45-1.

After 26 and 42 dpi, a significant difference ($P < 0.0001$) of disease severities between M, L, and H groups was observed. In each experiment, the M group was more aggressive than both L and H groups, and L and H groups were not different from each other (Fig. 4A). After 26 dpi, mean disease severity ratings for the M, L, and H groups

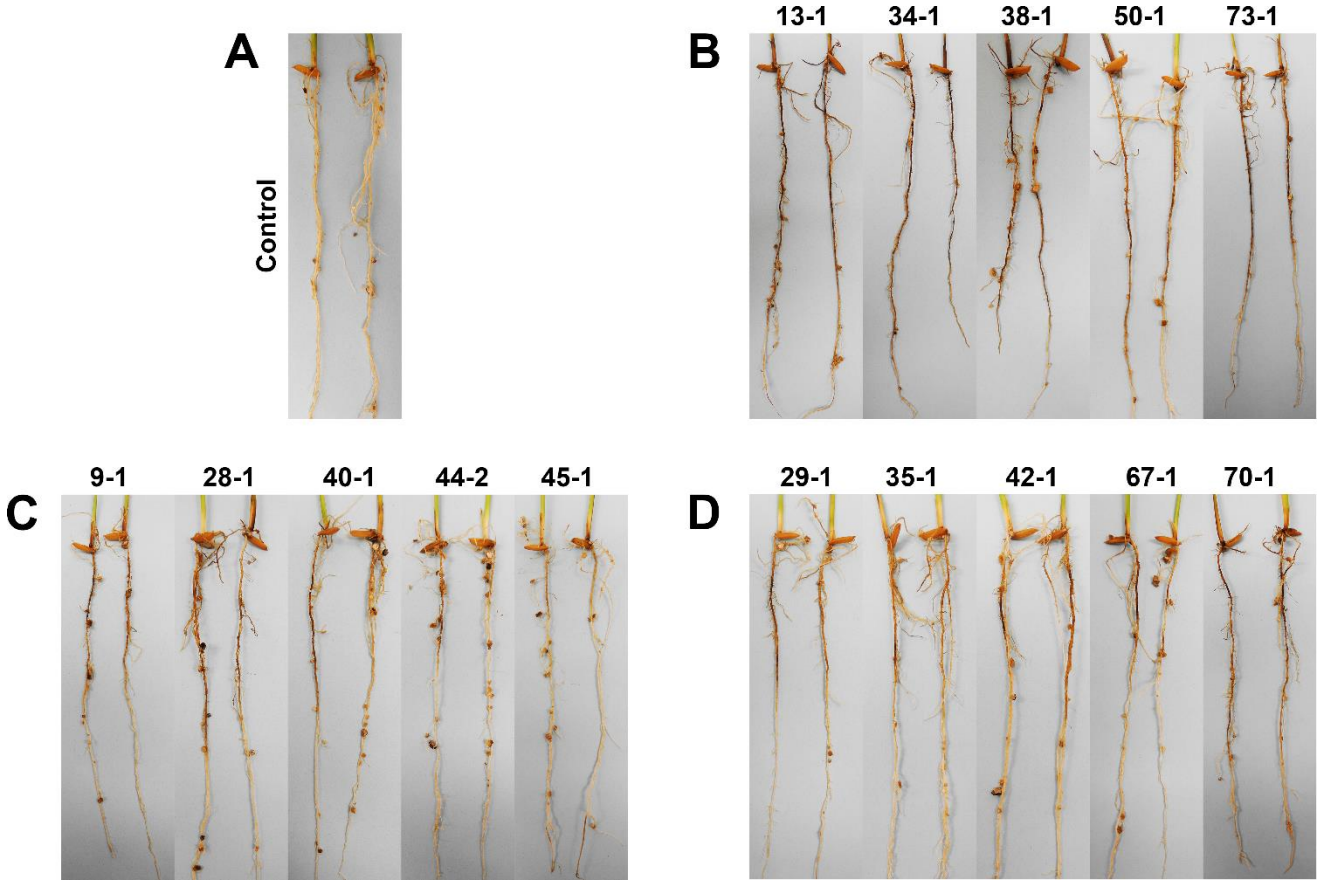

Fig. 3. Root lesions of rice cv. Presidio seedlings produced by *Gaeumannomyces graminis* var. *graminis* M, L, and H group isolates 26 days post inoculation. **A**, rice seedlings treated with five plugs of potato dextrose agar; **B**, **C**, and **D**, rice seedlings treated with five plugs of actively-growing mycelium belonging to M, L, and H group isolates, respectively. Numbers indicate isolate numbers.

Table 5. Comparison of root disease severity ratings and shoot lengths of rice cv. Presidio seedlings 26 and 42 days post inoculation (dpi) with *Gaeumannomyces graminis* var. *graminis*

		Mean disease severity[y]		Mean shoot lengths (cm)	
Groups	**Isolates**	**26 dpi**	**42 dpi**	**26 dpi**	**42 dpi**
M	MZTX13-1	2.8 ab[z]	4.6 ab	14.0 abcd	13.8 cde
	MZTX34-1	3.3 a	4.6 ab	10.6 d	14.0 cde
	MZTX38-1	2.3 bcd	4.8 a	11.1 d	12.1 e
	MZTX50-1	2.4 bcd	3.6 cd	13.8 abcd	12.5 de
	MZTX73-1	2.6 ab	4.5 ab	16.6 abc	14.9 bcde
L	MZTX9-1	2.5 bc	3.0 d	12.3 cd	18.6 ab
	MZTX28-1	1.9 cdef	3.0 d	10.1 d	17.4 abc
	MZTX40-1	1.5 efg	3.4 cd	16.5 abc	13.8 cde
	MZTX44-2	1.8 def	3.4 cd	17.8 a	15.3 bcde
	MZTX45-1	1.4 fg	3.0 d	12.8 bcd	14.5 bcde
H	MZTX29-1	2.4 bcd	3.9 bc	11.9 cd	12.9 cde
	MZTX35-1	2.1 bcde	3.1 cd	17.1 ab	13.1 cde
	MZTX42-1	1.8 cdef	3.1 cd	14.0 abcd	16.9 abcd
	MZTX67-1	1.4 fg	3.3 cd	14.0 abcd	14.0 cde
	MZTX70-1	2.3 bcd	3.2 cd	12.6 bcd	13.7 cde
Control	PDA	1.0 g	1.1 e	14.5 abcd	19.9 a

[y] Disease severity ratings were based on a 1 to 5 scale: 1 = no discoloration of the root system; 2 = 1 to 25% of roots exhibiting black lesions or a general tan discoloration of the entire root system; 3 = 26 to 50% of roots with black coalescing lesions; 4 = 51 to 75% of roots with black coalescing lesions; 5 = 76 to 100% of roots with black lesions.

[z] Means followed by the same letter in each column are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

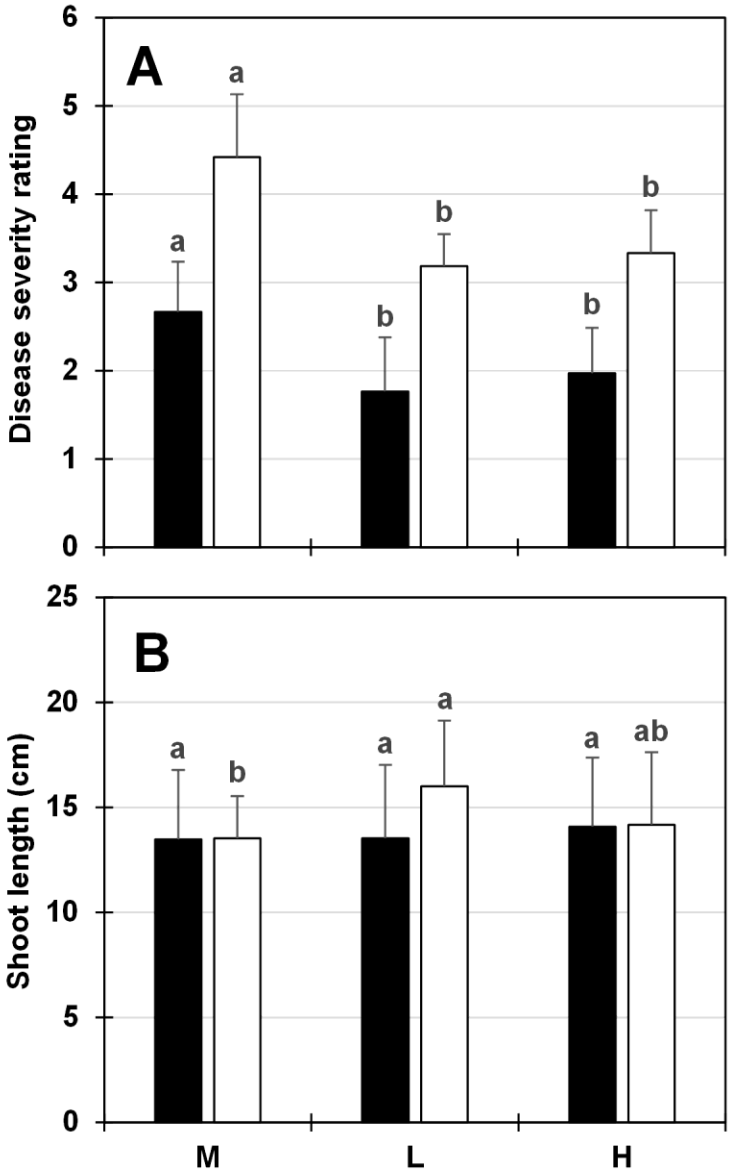

Fig. 4. Effects of *Gaeumannomyces graminis* var. *graminis* M, L, and H groups on **A**, root disease severity ratings and **B**, shoot lengths of rice cv. Presidio seedlings. Disease severity ratings were based on a 1 to 5 scale: 1 = no discoloration of the root system; 2 = 1 to 25% of roots exhibiting black lesions or a general tan discoloration of the entire root system; 3 = 26 to 50% of roots with black coalescing lesions; 4 = 51 to 75% of roots with black coalescing lesions; 5 = 76 to 100% of roots with black lesions. Solid and empty bars represent means obtained after 26 and 42 days post inoculation, respectively. Means followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$. Error bars represent the standard deviation of the mean.

were 2.7, 2.0, and 1.8, respectively. After 42 dpi, mean disease severity ratings for the M, L, and H groups were 4.4, 3.2, and 3.3, respectively.

A comparison of shoot lengths among treatments determined differences after 26 ($P = 0.0359$) and 42 ($P = 0.0149$) dpi. After 26 dpi, none of the isolates tested suppressed shoot lengths as compared to the PDA control (Table 5). After 42 dpi, shoot lengths were suppressed by all isolates except MZTX9-1, 28-1, and 42-1. A comparison of mean shoot lengths among M, L, and H groups determined no difference after 26 dpi ($P = 0.838$) but a difference after 42 dpi ($P = 0.0448$). After 42 dpi, the mean shoot length produced by the M group was less than the L group (Fig. 4B). Mean shoot lengths between L and H groups and M and H groups were similar. After 26 and 42 dpi, mean root lengths among treatments and between groups were also similar ($P \geq 0.0949$).

PCR, sequencing, and phylogenetic analysis. Amplification of a 1,086 bp region was achieved for all L group isolates using the *Ggg*-specific primer set (Fig. 5; Table 3). All M and H group isolates failed to amplify. The non-degenerate RPB1-NF/RPB1-NR primer set successfully amplified a partial RPB1 region of approximately 800 bp that could be aligned with entire RPB1 sequences retrieved from the NCBI GenBank database (Table 1; Table 2). Multiple sequence alignments provided the selection of 445, 752, and 614 nucleotide characters with gaps from regions of ITS, LSU, and RPB1 loci, respectively.

A total of 1,811 nucleotide characters from combined sequences were used to determine the Tamura-Nei model with gamma distribution as the best fit nucleotide substitution model for the ML analysis. The best fit model analysis and multilocus

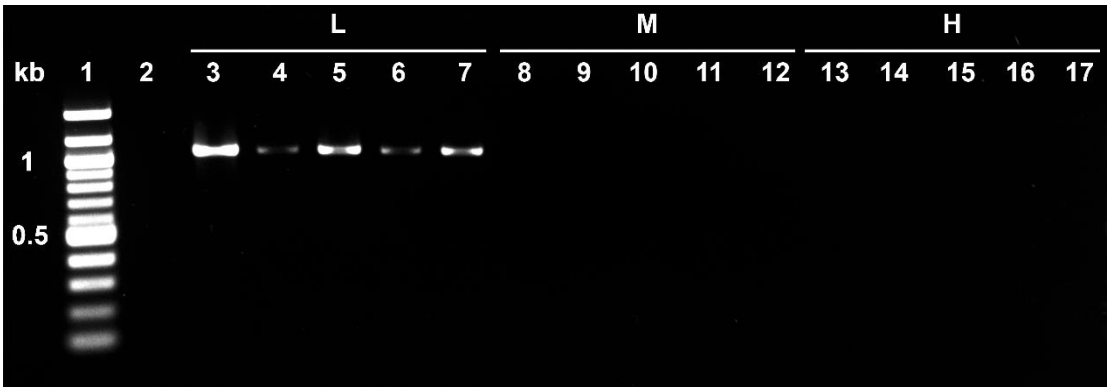

Fig. 5. Amplification of *Gaeumannomyces graminis* var. *graminis* (*Ggg*) genomic DNA using *Ggg*-specific primers designed to amplify a 1,086 bp region from the avenacinase-like gene. Lane 1 contains a 100-bp DNA ladder. Lane 2 contains no DNA. Lanes 3 to 7, 8 to 12, and 13 to 17 contain five L group isolates (MZTX9-1, 28-1, 44-2, 40-1, and 45-1), M group isolates (MZTX38-1, 13-1, 50-1, 73-1, and 34-1), and H group isolates (MZTX70-1, 35-1, 42-1, 67-1, and 29-1), respectively. Ladder and polymerase chain reaction products were stained with ethidium bromide and fluorescently visualized with ultraviolet light.

phylogeny reconstruction were determined with combined sequences after complete deletion of gaps. The multilocus phylogeny reconstruction produced an ML tree consisting of two major clades (Fig. 6). Five *Ggg* taxa (CBS 235.32, CBS 352.93, M33, M53, and M54) and all M and H group isolates, with the exception of MZTX13-1, formed a major clade with a bootstrap support of 83%. Within this clade, M and H group isolates were further resolved into two subclades supported by bootstrap values of 98 and 99%, respectively. Two *Ggg* taxa (CBS 352.93 and M53) were placed in the same subclade as M group isolates, and all H group isolates formed a clade of their own. MZTX13-1 and all L group isolates, along with three *Ggg* taxa (CBS 902.73, CBS 903.73, and M57), were grouped with *Gga* and *Ggt* in the second major clade. Within this clade, MZTX13-1 was grouped with M57 with a bootstrap support of 72%. All L group isolates, along with CBS 902.73 and CBS 903.73, were further separated from *Gga* and *Ggt* taxa and formed a subclade with a bootstrap support of 78%. The group containing *Gga* and *Ggt* taxa was supported by a bootstrap value of 99% and each variety was further separated into their respective clades.

Discussion

An assessment of 76 *Ggg* isolates obtained from St. Augustinegrass throughout Texas revealed three distinct morphological groups (M, L, and H) based on cultural characterizations of melanization and colony formation. All M, L, and H group isolates were capable of producing lobed hyphopodia and exhibited a *Phialophora*-like anamorph, which is valuable evidence to support that the isolates tested represent *Ggg*

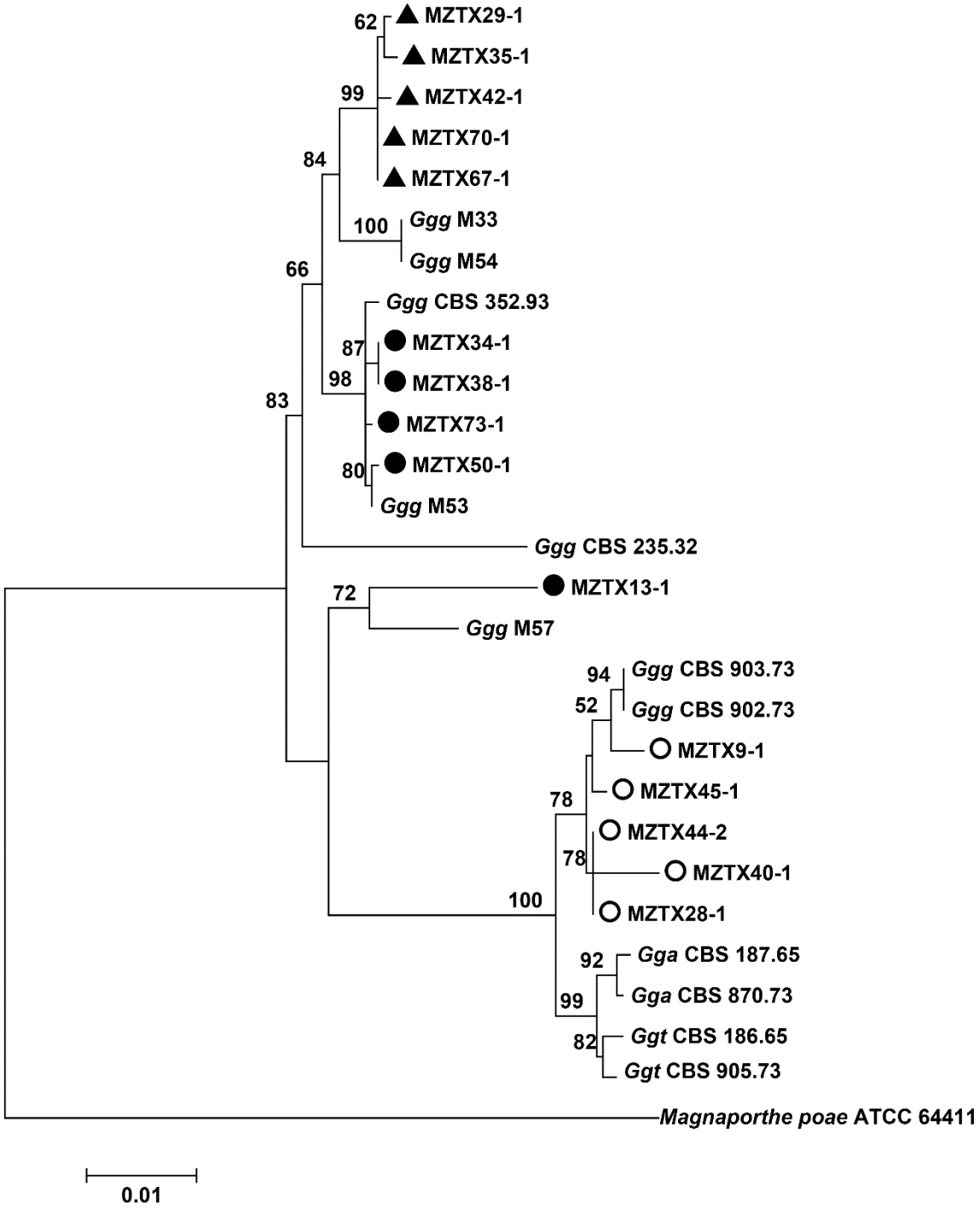

Fig. 6. Maximum likelihood tree based on the combination of partial ITS, LSU, and RPB1 sequences showing relationships between groups of *Gaeumannomyces graminis* var. *graminis* and *G. graminis* varieties *avenae* and *tritici*. Solid circles, empty circles, and solid triangles represent M, L, and H group isolates, respectively. Bootstrap values >50% are labeled above nodes. *Magnaporthe poae* ATCC 64411 was used as outgroup.

(Walker 1981). However, *Phialophora* spp. such as *P. radicicola* Cain and *P. graminicola* (Deacon) Walker are also capable of producing lobed hyphopodia (Walker 1981); therefore, isolates cannot be confirmed as *Ggg* based solely on hyphopodia shape and the observation of a *Phialophora*-like anamorph. Many of the isolates tested also produced flask-shaped perithecia either on PDA or on the culms of rice seedlings, and mean ascospore lengths were consistent with previous descriptions of *Ggg* (Freeman and Ward 2004); however, perithecia were never produced by MZTX13-1 and all L group isolates. The failure of perithecial development restates that identification of *G. graminis* varieties based solely on morphological features can be inconclusive and that molecular techniques may provide a more informative approach (Bateman et al. 1992; Bryan et al. 1995; Holden and Hornby 1981).

Rachdawong et al. (Rachdawong et al. 2002) previously designed *Ggg*-specific primers for amplifying a 1,086 bp region from the avenacinase-like gene, and primer specificity was confirmed based on the failure to amplify closely-related fungi such as *Gga*, *Ggt*, and *Phialophora* spp. such as *P. radicicola* and *P. graminicola*. In our study, amplification of a 1,086 bp region was achieved with L group isolates using the *Ggg*-specific primers, supporting the identification of these isolates as *Ggg*. Interestingly, isolates belonging to the M and H groups failed to amplify, indicating that DNA polymorphisms within the avenacinase-like gene likely exist within *Ggg*. DNA polymorphisms within the avenacinase-like gene may provide a suitable target for future phylogenetic analyses. Based on the combination of morphological characteristics and a PCR identification method, M, L and H group isolates, with the exception of

MZTX13-1, could be confirmed as *Ggg*; however, a multilocus phylogenetic analysis indicated that MZTX13-1 formed a well-supported group with a published *Ggg* taxon (M57), providing support for the identification of this isolate as *Ggg*.

M, L, and H group isolates were also differentiated based on growth rate. In fact, radial growth rates at 35°C provided statistical separation of isolates in accordance with their morphological groups; however, MZTX13-1 did not differ from most L group isolates. With few exceptions, it also appeared that M group isolates were adapted to warmer temperatures as compared to those in the L and H groups. This is of particular interest, since host species of *Ggg* are cultivated throughout subtropical and tropical regions (Elliott 1991; Elliott et al. 1993; McCarty and Lucas 1989; Walker 1981; Wilkinson 1994; Wilkinson and Kane 1993).

Rice seedling pathogenicity assays were performed to characterize variations of aggressiveness. Disease severity ratings based on root lesions consistently supported that the M group was more aggressive than L and H groups, while L and H groups were similar. Rice shoot lengths also supported similar variations of aggressiveness between groups; however, mean shoot lengths between M and H groups were similar. Previous reports have identified groups of *Ggt* according to pathogenicity (Hollins and Scott 1990; Lebreton et al. 2004). Hollins et al. (Hollins and Scott 1990) first described N- and R-isolates of *Ggt*, associated with the inability or ability to infect rye, respectively, and subsequent reports supported that these groups were genetically distinct (Bryan et al. 1995; Bryan et al. 1999; O'Dell et al. 1992). Lebreton et al. (Lebreton et al. 2004) also

reported two genetically distinct groups of *Ggt*, G_1/G_2, where the G_2 group was significantly more aggressive than the G_1 group.

Datnoff (Datnoff et al. 1997) demonstrated that *Ggg* isolates obtained from St. Augustinegrass, bermudagrass, and rice were not host-specific; however, it also appeared that *Ggg* isolates were most aggressive on the host from which they were obtained. Pathogenicity assays using St. Augustinegrass and bermudagrass can be cumbersome and time-consuming, particularly since most cultivars are sterile and require the production of aerial stolons to provide disease-free plant material (Datnoff et al. 1997; Elliott 1995). Rice seedlings provided a clean, efficient, and less-cumbersome method for evaluations of pathogenicity. However, future studies should expand pathogenicity assays to encompass turfgrass hosts, particularly St. Augustinegrass, in order to confirm variations of aggressiveness.

A multilocus phylogeny reconstruction based on partial sequences of ITS, LSU, and RPB1 loci strongly supported the differentiation of *G. graminis* varieties and M, L, and H groups based on morphological and physiological variations. M, L, and H group isolates were clearly separated into well-supported clades; however, MZTX13-1 formed a separate clade with M57. The ML tree also sheds light on the evolutionary relationships between *Ggg*, *Gga*, and *Ggt*. It appeared that M and H groups, with the exception of MZTX13-1, were more closely related as compared to the L group, while the L group was more closely related to *Gga* and *Ggt*. The close phylogenetic relationship between M and H groups was consistent with their similarities in melanization, hyphopodia production, mean phialospore length, and the ability to

sexually reproduce on the culms of rice seedlings; however, M and H groups were differentiated based on colony morphology, growth rate, and aggressiveness, which is supported by their separation into respective subclades. The L group, along with MZTX13-1 and *Ggg*, *Gga*, and *Ggt* taxa, appeared to diverge and evolve separately from M and H groups and other *Ggg* taxa. This is also consistent with the similar growth rate observed between MZTX13-1 and most of the L group isolates. Furthermore, the distant phylogenetic relationship between the L group and M and H groups is consistent with their clear differentiation of melanization, phialospore length, hyphopodia production, and perithecial development observed during this study.

Previous reports have also described closely and distantly related groups of *Ggg* to *Gga* and *Ggt*. Fouly (Fouly et al. 1997) separated *Ggg* into four groups based on length variation of an amplified portion of 18S rDNA, where one of these groups produced a similar product size to *Gga* and *Ggt*. A phylogenetic analysis of the ITS region also separated *Ggg* isolates into two groups, one of which showed a closer relationship to *Gga* and *Ggt* (Fouly 2002). Two sets of sequence insertions within the SSU rDNA were also discovered by Fouly (Fouly et al. 2011), where *Gga* and *Ggt* isolates were unique to set I, and *Ggg* isolates were arranged in both sets. The polyphyletic nature of *Ggg* in this study is also consistent with a previous report by Ward (Ward and Bateman 1999), where *Ggg* was described as polyphyletic based on a phylogenetic analysis of the ITS region. Variability of *Ggg* has also been described in other studies (Elliott et al. 1993; Fouly 2002; Fouly et al. 2011; Fouly et al. 1997; Fouly et al. 1996; Ward and Akrofi 1994; Ward and Bateman 1999).

The phylogenetic analysis also supported that *Gga* and *Ggt* were more closely related to each other than to *Ggg*. This is consistent with morphological characteristics and host ranges previously described for varieties of *G. graminis* (*Gga* and *Ggt* produce simple hyphopodia and are pathogenic to wheat and other cereals, while *Ggg* produces lobed hyphopodia and is pathogenic to rice and various warm-season turfgrasses (Elliott 1991; Elliott et al. 1993; McCarty and Lucas 1989; Walker 1981; Wilkinson 1994; Wilkinson and Kane 1993)). Other genetic approaches also supported the close relationship between *Gga* and *Ggt* based on restriction analyses (Fouly et al. 1997; Ward and Akrofi 1994) and phylogenetic analyses (Bryan et al. 1995; Fouly 2002; Fouly et al. 2011; Ward and Bateman 1999) of rDNA, RAPD analyses (Bryan et al. 1999; Fouly et al. 1996), and differences in codon usage (Bryan 1995).

The resolution of intravarietal complexities associated with *Ggg* is important for the development of proper identification and diagnostic procedures, as well as the development disease management programs for TARR. The identification of highly aggressive groups of *Ggg*, as well as those better adapted to regions where St. Augustinegrass is cultivated, is important from biological and applied perspectives. Proper management strategies, whether chemical, biological, or cultural, revolve around a thorough understanding of the targeted pathogen. Currently, the biological aspects of TARR are not well understood. Future studies should continue to resolve the variability within *Ggg* and further investigate associations between specific groups and disease incidences and severities.

CHAPTER III

FUNGICIDE SENSITIVITY OF *Gaeumannomyces graminis* var. *graminis* ISOLATED FROM ST. AUGUSTINEGRASS IN TEXAS

Introduction

Gaeumannomyces graminis (Sacc.) von Arx & D. Olivier *var. graminis* (*Ggg*) is a soil-borne, ectotrophic root infecting (ERI) fungus that is associated with take-all root rot (TARR) of St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) (Elliott et al. 1993), bermudagrass (*Cynodon dactylon* (L.) Pers. × *transvaalensis* Burtt-Davy) decline (Elliott 1991), and spring dead spot (McCarty and Lucas 1989) of bermudagrass. *Ggg* has also been reported to cause root rot of centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) (Wilkinson 1994) and zoysiagrass (*Zoysia japonica* Steud.) (Wilkinson and Kane 1993). Collectively, turfgrass diseases caused by *Ggg* may be described as root decline of warm-season turfgrasses (Smiley et al. 2005). Symptoms of root decline are initially characterized by chlorotic patches resulting from necrosis of the infected root system. As the disease progresses, infected areas will begin to thin and eventually turn bare, where restoration can be difficult to achieve.

Penetrant fungicides, such as methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs), have been widely used for turfgrass disease management since the 1970s, 1980s, and 1990s, respectively (Martin 2003). To date, several reports have indicated increased resistance of fungal turfgrass pathogens to the MBCs, DMIs, and QoIs. Golembiewski

(Golembiewski et al. 1995) reported the first case of DMI resistance in a turfgrass pathogen population, *Sclerotinia homoeocarpa* F.T. Bennett, where acceptable control of dollar spot was no longer achieved with triadimefon, fenarimol, and propiconazole. Reduced sensitivity to propiconazole and complete resistance to thiophanate-methyl were also observed in *S. homoeocarpa* isolates obtained from golf courses in Ohio (Jo et al. 2006). The first case of resistance of *Pyricularia grisea* (Cooke) Sacc. (teleomorph *Magnaporthe grisea* (Hebert) Barr) to QoIs was reported in 2002, where azoxystrobin could no longer provide desired control of gray leaf spot caused by the resistant fungal population (Vincelli and Dixon 2002). QoI resistance of *Colletotrichum graminicola* (Ces.) G.W. Wils., a fungal pathogen causing anthracnose of annual bluegrass (*Poa annua*) and bentgrasses (*Agrostis spp*.), has also been reported (Avila-Adame et al. 2003).

Fungicide sensitivities are typically represented by EC_{50} values, the effective concentration of a fungicide that inhibits growth by 50% (Brent 1988; Jo et al. 2006; Köller and Scheinpflug 1987). Traditional methods of EC_{50} determination require exposing individual isolates to serial concentrations of a particular fungicide, which can be time-consuming and laborious. Alternatively, fungicide sensitivities may be predicted by determining relative growth at a single discriminatory dose, a more efficient method that has been previously used for resistance screening of *S. homoeocarpa* to DMIs (Golembiewski et al. 1995; Jo et al. 2006; Miller et al. 2002) and *Colletotrichum cereale* sensu lato Crouch, Clarke, and Hillman to azoxystrobin (Wong et al. 2007).

QoIs bind specifically to an outer quinol-oxidizing site of the cytochrome bc_1 enzyme complex, inhibiting respiration via interruption of electron transfer between cytochrome b and cytochrome c_1 (Bartlett et al. 2002). Some fungi possess the ability of bypassing the effects of QoIs by induction of alternative respiration (Avila-Adame and Köller 2002; Joseph-Horne et al. 2001; Köller et al. 2002). Thus, for proper *in vitro* assessment of QoI sensitivities, growth media is often amended with salicylhydroxamic acid (SHAM) to inhibit alternative respiration (Avila-Adame et al. 2003; Kim et al. 2003; Olaya and Köller 1999; Vincelli and Dixon 2002; Wong et al. 2007). The ability of *Ggg* to utilize alternative respiration is currently unknown, and determining whether this pathway is induced is a requisite for the development of proper screening assays.

MBC, DMI, and QoI fungicides for the control of TARR and root-decline of warm-season turfgrasses are currently labeled for residential and commercial use; however, reports of fungicide sensitivities for *Ggg* are limiting and fungicide sensitivity distributions are not understood. The objectives of this study were to (i) determine if *Ggg* can utilize alternative respiration for circumventing respiration inhibition by azoxystrobin, (ii) determine single discriminatory concentrations of azoxystrobin and tebuconazole for rapid screening and prediction of EC_{50} values, and (iii) determine azoxystrobin, tebuconazole, and thiophanate-methyl sensitivity distributions of *Ggg* isolated from St. Augustinegrass throughout Texas.

Materials and Methods

Determination of alternative respiration in *Ggg*. A total of 76 *Ggg* isolates were previously obtained from St. Augustinegrass throughout Texas, from which nine isolates were randomly selected. Actively-growing mycelium (5 mm in diameter) from each isolate was transferred to three replicates of PDA amended with azoxystrobin (Heritage TL; Syngenta Crop Protection, Inc., Greensboro, NC, USA) at 0.001, 0.003, 0.01, 0.03, 0.1, 1, 10, and 50 µg a.i. ml^{-1} with 100 µg ml^{-1} of SHAM (Alfa Aesar, Ward Hill, MA, USA). A stock solution of SHAM was prepared by dissolving in methanol to a final concentration of 100 mg ml^{-1}. Actively-growing mycelium was also transferred to three replicates of PDA amended with azoxystrobin at 0.01, 0.1, 1, 10, and 50 µg a.i. ml^{-1} in the absence of SHAM. Radial mycelial growth was measured after five to seven days of incubation in the dark at 25°C. The relative mycelial growth at each fungicide concentration in the presence or absence of SHAM was determined by dividing radial growth on fungicide-amended PDA by radial growth on PDA and multiplying by 100. Regression analyses using PROC REG with SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA) were used to estimate EC_{50} values from linear models by regressing relative growth against log transformed concentrations of azoxystrobin.

The experiment was repeated with three replicates per isolate and was arranged as a completely randomized design. Data from both experiments were subjected to a two-tailed *F* test for equality of variances of log transformed EC_{50} values to determine if experiments could be combined. Data were further subjected to a two-way factorial analysis of variance (ANOVA) for evaluation of experiment and isolate as the main

effects using PROC GLM. The lack of significant ($P \leq 0.05$) experiment or isolate-experiment interactions were used to finally determine if the experiments could be combined. If significant experiment or isolate-experiment interactions were not observed, experiment was dropped from the model, and a two-way factorial ANOVA was calculated using isolate and SHAM as the main effects. Least-square means *t* tests using PDIFF were used to compare log transformed EC_{50} values of individual *Ggg* isolates on PDA containing SHAM with those on PDA in the absence of SHAM.

Determination of azoxystrobin and tebuconazole discriminatory concentrations. Azoxystrobin EC_{50} values were determined for the aforementioned nine isolates and an additional 11 isolates selected from the existing *Ggg* collection. Tebuconazole EC_{50} values were determined for 15 isolates selected from the *Ggg* collection. For determination of azoxystrobin EC_{50} values, actively-growing mycelium from each isolate was transferred to three replicates of PDA amended with 100 mg ml^{-1} of SHAM and azoxystrobin at the following concentrations: 0.001, 0.003, 0.01, 0.03, 0.1, 1, 10, and 50 µg a.i. ml^{-1}. For tebuconazole, actively-growing mycelium was transferred to three replicates of PDA amended with tebuconazole (Torque; Cleary Chemical Corporation, Dayton, NJ, USA) at the following concentrations: 0.01, 0.05, 0.1, and 1 µg a.i. ml^{-1}. Radial mycelial growth was measured after incubation for five to seven days at 25°C in the dark. Relative growth at each fungicide concentration was calculated as previously described. Linear analyses were performed using PROC REG to regress relative growth against log transformed azoxystrobin or square root transformed tebuconazole concentrations to estimate EC_{50} values.

The experiment was repeated for azoxystrobin and tebuconazole analyses, and a two-tailed *F* test for equality of variances was used to determine if experiments could be combined. If experiment or isolate-experiment interactions were not significant ($P \leq 0.05$) in the combined analysis, experiment was dropped from the model, and the mean EC_{50} values were determined using data from both experiments.

Linear models were created using PROC REG to regress log transformed azoxystrobin and tebuconazole EC_{50} values against the relative growth of associated isolates on PDA amended with a given fungicide concentration: azoxystrobin at 0.001, 0.003, 0.01, 0.03, 0.1, 1, 10, or 50 µg a.i. ml^{-1} or tebuconazole at 0.01, 0.05, 0.1, or 1 µg a.i. ml^{-1}. Discriminatory concentrations for azoxystrobin and tebuconazole were determined based on a comparison of significance and coefficients of determination (R^2) from models representing each fungicide concentration. Azoxystrobin and tebuconazole EC_{50} values estimated by relative growth at the discriminatory dose ($EC_{50(D)}$) were then determined for each isolate. Regression analyses between azoxystrobin and tebuconazole EC_{50} and $EC_{50(D)}$ values were finally performed using PROC REG.

Determination of fungicide sensitivity distributions of *Ggg*. Azoxystrobin and tebuconazole sensitivities of remaining *Ggg* isolates were determined based on mycelial growth on PDA amended with predetermined discriminatory concentrations. Actively-growing mycelium from each isolate was transferred to three replicates of unamended PDA or PDA amended with the discriminatory concentration of azoxystrobin or tebuconazole using a sterile 5-mm cork borer. PDA with azoxystrobin was also amended with SHAM to a final concentration of 100 µg ml^{-1}. Radial mycelial growth was

measured after incubation for five to seven days at 25°C in the dark. The relative growth was determined for each isolate, and the regression equation associated with the azoxystrobin or tebuconazole discriminatory linear model was used to predict $EC_{50(D)}$ values.

Each experiment was repeated, and a two-tailed *F* test for equality of $EC_{50(D)}$ variances was used to determine if experiments could be combined. Frequency distributions, means, medians, ranges, and skewness were determined using EC_{50} and $EC_{50(D)}$ values obtained for all isolates, and a Shapiro-Wilk test for normality was performed using PROC UNIVARIATE NORMAL. Because fungal sensitivity to thiophanate-methyl and other MBCs follows single-step resistance or disruptive selection for fungicide resistance (Köller and Scheinpflug 1987), PDA amended with thiophanate-methyl (3336F; Cleary Chemical Corporation, Dayton, NJ, USA) at 500 µg a.i. ml^{-1} was used to screen *Ggg* isolates for resistance to thiophanate-methyl. Each isolate was qualitatively assessed for growth or no growth on PDA amended with thiophanate-methyl after an incubation period of five to seven days at 25°C in the dark.

Results

Determination of alternative respiration in *Ggg*. All linear models used for determining EC_{50} values for each isolate were significant ($P \leq 0.05$); therefore, EC_{50} values could be reliably estimated. Variances of log transformed EC_{50} values between two separate experiments were not different ($P = 0.055$). Analysis of combined data from both experiments determined no difference between experiment ($P = 0.959$) and

the isolate-experiment interaction ($P = 0.695$). Therefore, the combined data were used for further analyses. The two-way factorial ANOVA of log transformed EC_{50} values determined a significant difference for the main effects of isolate and SHAM, and their interaction was significant ($P < 0.0001$). EC_{50} values of all isolates were significantly lower when SHAM was included in the media (Table 6).

Determination of azoxystrobin and tebuconazole discriminatory concentrations. According to a two-tailed *F* test, variances of azoxystrobin EC_{50} values from 20 *Ggg* isolates between experiments were not different ($P = 0.402$), and the isolate-experiment interaction in the combined analysis was not significant ($P = 0.232$); therefore, data from both experiments were combined and used for further analyses. All isolates were able to grow at concentrations ranging from 0.001 to 0.1 µg a.i. ml^{-1}; however, mycelial growth of five isolates (MZTX10-2, 12-1, 18-2, 33-1, and 8-1) was completely inhibited at 1 µg a.i. ml^{-1}, all but four isolates (MZTX13-1, 34-1, 35-1, and 38-1) were completely inhibited by 10 µg a.i. ml^{-1}, and only MZTX13-1 was able to grow at 50 µg a.i. ml^{-1} (Table 7). The highest R^2 value (0.952) was observed in the regression model using relative growth at 10 µg a.i. ml^{-1}; however, mycelial growth of all but four isolates was completely inhibited at this concentration. Therefore, a discriminatory concentration of 0.1 µg a.i. ml^{-1} was deemed more suitable for prediction of $EC_{50(D)}$ values for wide-ranged sensitivities ($R^2 = 0.839$; Fig. 7A; Table 7). The accuracy of $EC_{50(D)}$ prediction was supported by the high correlation ($R^2 = 0.808$) between azoxystrobin EC_{50} and $EC_{50(D)}$ values (Fig. 8A).

Table 6. Comparison of azoxystrobin EC_{50} values between isolates of *Gaeumannomyces graminis* var. *graminis* in the presence or absence of salicylhydroxamic acid (SHAM)

	EC_{50} (µg a.i. ml^{-1})[a]		
Isolate	**+SHAM**	**-SHAM**	***P* value[b]**
MZTX18-2	0.0155	0.2729	<0.0001
MZTX12-1	0.0173	0.3289	<0.0001
MZTX10-2	0.0272	0.6049	<0.0001
MZTX24-1	0.0274	0.2333	<0.0001
MZTX50-1	0.0297	0.0727	0.0003
MZTX22-1	0.0534	0.5457	<0.0001
MZTX8-1	0.0581	0.4993	<0.0001
MZTX38-1	0.0762	0.1632	0.0027
MZTX13-1	0.3945	0.6279	0.0512

[a] Mean EC_{50} values obtained from combined results of two experiments when individual isolates were either exposed (+SHAM) or not exposed (-SHAM) to salicylhydroxamic acid. EC_{50} values were obtained by plating each isolate on serial concentrations of azoxystrobin and estimating the concentration that could inhibit growth by 50%.

[b] *P* values for individual isolates were determined from least-square means *t* tests between the presence or absence of SHAM using log transformed EC_{50} values.

Table 7. Comparison of linear regression models between different concentrations of azoxystrobin and tebuconazole for determining discriminatory concentrations

Fungicide and concentration (µg a.i. ml^{-1})	**Slope (*m*)**	**Intercept (*b*)**	**Coefficient of Determination (R^2)**	***P* value**	***n*[a]**
Azoxystrobin					
0.001	0.0233	-3.63142	0.08	0.2268	20
0.003	0.05159	-6.24823	0.3638	0.0049	20
0.01	0.02072	-2.84398	0.2539	0.0235	20
0.03	0.02713	-2.66184	0.6302	<0.0001	20
0.1	0.02141	-1.97555	0.8394	<0.0001	20
1	0.01926	-1.57079	0.5434	0.0017	15
10	0.04089	-1.35566	0.952	0.0243	4
50	-	-	-	-	1
Tebuconazole					
0.01	0.0205	-2.98115	0.732	<0.0001	15
0.05	0.01529	-1.9462	0.9369	<0.0001	15
0.1	0.01731	-1.7858	0.9443	<0.0001	15
1	-	-	-	-	1

[a] Number of isolates able to grow at associated fungicide concentration.

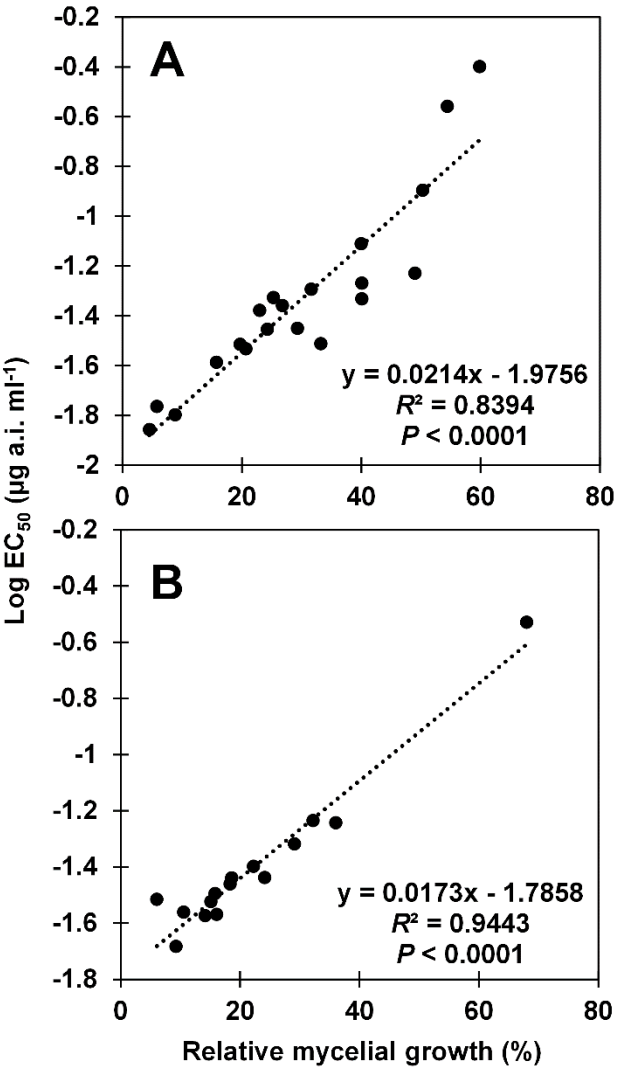

Fig. 7. Linear models regressing log EC_{50} values against relative mycelial growth of 20 and 15 *Gaeumannomyces graminis* var. *graminis* isolates on potato dextrose agar with 0.1 µg a.i. ml^{-1} of **A**, azoxystrobin and **B**, tebuconazole, respectively. EC_{50} values were determined by estimating the concentration that could inhibit radial growth by 50% after plating on serial fungicide concentrations. Relative mycelial growth is represented by the percent growth on fungicide-amended media as compared to an unamended control.

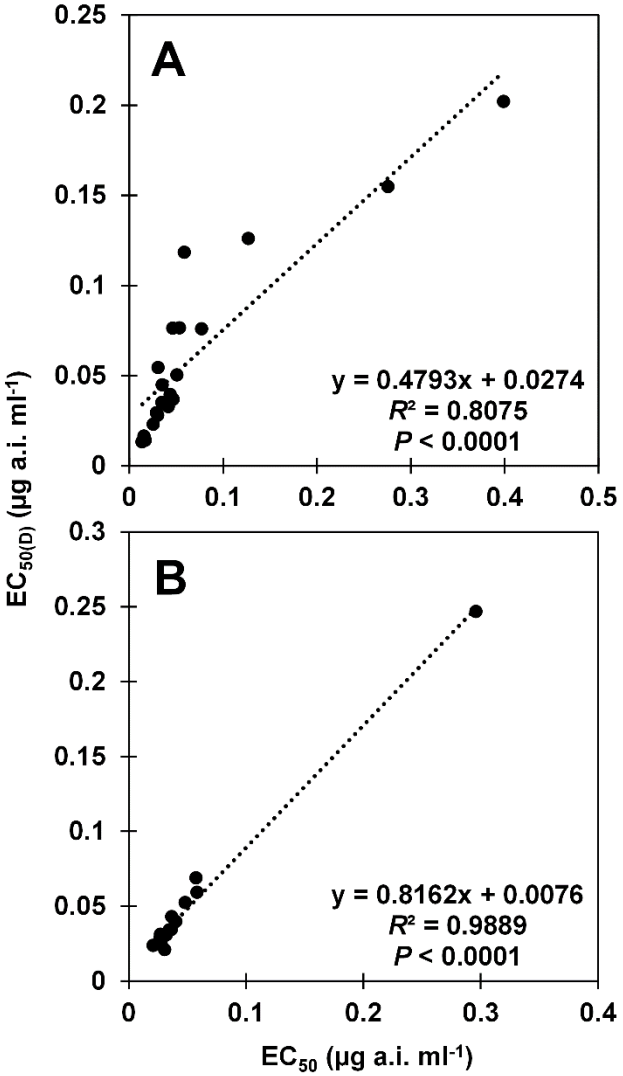

Fig. 8. Relationship between EC_{50} and $EC_{50(D)}$ values of **A**, azoxystrobin and **B**, tebuconazole for 20 and 15 *Gaeumannomyces graminis* var. *graminis* isolates, respectively. EC_{50} values represent the fungicide concentration that could inhibit radial growth by 50% after plating on potato dextrose agar amended with serial fungicide concentrations. $EC_{50(D)}$ values were determined by estimating the fungicide concentration that could inhibit radial growth by 50% after plating on potato dextrose agar amended with a single discriminatory fungicide concentration of 0.1 µg a.i. ml^{-1}.

Data from both experiments assessing tebuconazole EC_{50} values of 15 *Ggg* isolates were also combined based on equality of variances ($P = 0.778$) and no difference among experiments ($P = 0.422$) and the isolate-experiment interaction ($P = 0.274$). All isolates grew <50% when exposed to tebuconazole at 0.1 µg a.i. ml^{-1} with the exception of MZTX74-1; therefore, an additional concentration of tebuconazole at 1 µg a.i. ml^{-1} was used to estimate an EC_{50} value for this isolate. The highest R^2 value (0.944) was observed in the regression model using relative growth at 0.1 µg a.i. ml^{-1}, which was determined as the discriminatory dose of tebuconazole (Fig. 7B; Table 7). Furthermore, a high correlation ($R^2 = 0.989$) between tebuconazole EC_{50} and $EC_{50(D)}$ values was observed (Fig. 8B).

Determination of fungicide sensitivity distributions of *Ggg*. Tebuconazole $EC_{50(D)}$ values produced variances that were not significantly different between experiments ($P = 0.761$); therefore, data were combined, and mean $EC_{50(D)}$ values were obtained for individual isolates. Tebuconazole EC_{50} values ($N = 76$) ranged from 0.021 to 0.296 with a mean of 0.049 and median of 0.042. The frequency distribution had a skewness of 5.54 and was not normal according to a Shapiro-Wilk test ($P < 0.0001$; Fig. 9).

Variances of azoxystrobin $EC_{50(D)}$ values between each experiment were not significantly different ($P = 0.798$); therefore, data were combined. Azoxystrobin EC_{50} values ranged from <0.014 to 0.399 ($N = 75$). Twenty-eight isolates were completely inhibited by the azoxystrobin discriminatory dose (0.1 µg a.i. ml^{-1}) at the point that control plates were completely colonized; therefore, their $EC_{50(D)}$ values were predicted

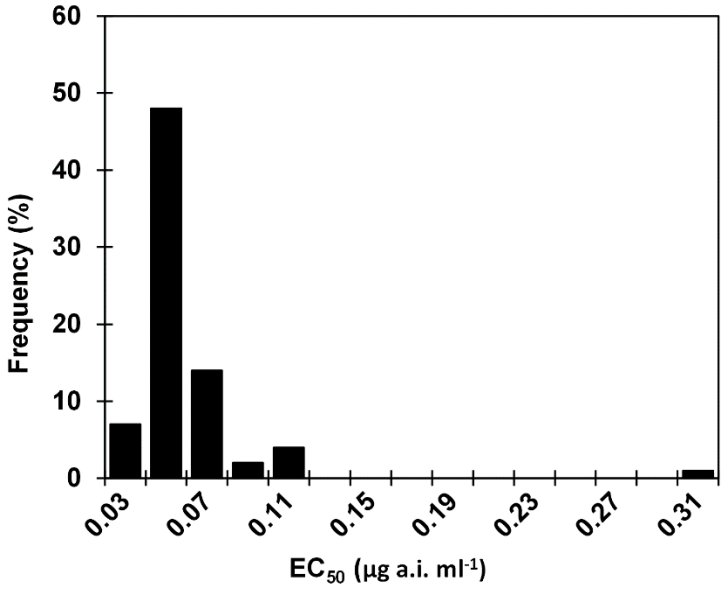

Fig. 9. Frequency distribution of tebuconazole EC_{50} values belonging to 76 *Gaeumannomyces graminis* var. *graminis* isolates from St. Augustinegrass in Texas. EC_{50} values were represented by the predicted concentration that could inhibit growth by 50% based on serial concentrations of tebuconazole or relative growth at the discriminatory concentration of 0.1 µg a.i. ml^{-1}. Values on the x-axis represent midpoints of each histogram class.

to be <0.014 based on the relative growth and EC_{50} value for the most sensitive isolate (MZTX55-1) able to grow at 0.1 µg a.i. ml^{-1}. A frequency distribution of azoxystrobin sensitivities was not determined, since $EC_{50(D)}$ values of 28 tested isolates could not be estimated using the predetermined discriminatory concentration. One isolate (MZTX71-1) lost viability during long-term storage and was only used in tebuconazole and thiophanate-methyl sensitivity assays. All isolates used in this study were completely inhibited by the thiophanate-methyl discriminatory dose of 500 µg a.i. ml^{-1}.

Discussion

Development of *in vitro* fungicide screening assays based on single discriminatory concentrations allowed the elucidation of the distribution of fungicide sensitivities of *Ggg* in the collection of isolates infecting St. Augustinegrass throughout Texas. Knowledge of fungicide sensitivities in *Ggg* populations is critical for designing proper fungicide programs for controlling TARR, a chronic disease of St. Augustinegrass throughout the Southern United States. The *Ggg* population assessed in this study was found to be sensitive to three major fungicide groups, MBCs, DMIs, and QoIs, all of which are speculated to be applied for TARR management. Furthermore, the sensitivities presented here may provide a baseline when determining fungicide resistance development during future studies.

QoIs bind specifically to an outer, quinone oxidizing site of the cytochrome bc_1 enzyme complex (complex III) and inhibit respiration along the electron transport chain (Bartlett et al. 2002). The determination of an alternative respiration pathway in *Ggg* was

necessary for proper measurement of azoxystrobin sensitivities since fungi have the ability to overcome the mode of action of QoIs via alternative respiration (Vincelli and Dixon 2002; Wise et al. 2008). Increased sensitivities of *Ggg* to azoxystrobin in the presence of SHAM indicated that *Ggg* is capable of utilizing alternative respiration for circumventing the mode of action of QoIs. In previous reports, SHAM has been used to inhibit effects of alternative oxidase (Avila-Adame et al. 2003; Kim et al. 2003; Vincelli and Dixon 2002; Wong et al. 2007), and others have reported that sensitivities to QoIs increase in the presence of SHAM (Bradley and Pedersen 2011; Olaya and Köller 1999; Olaya et al. 1998; Wise et al. 2008).

Point mutations within the cytochrome b gene, such as F129L and G143A amino acid substitutions, have been reported to confer resistance in other fungi (Gisi et al. 2002). In fact, these mutations have been described in isolates of the turfgrass pathogen *P. grisea*, where EC_{50} values were reported as 33.53 and 3.48 for isolates containing the G143A and F129L substitutions, respectively (Kim et al. 2003). Vincelli (Vincelli and Dixon 2002) reported an azoxystrobin baseline sensitivity of *P. grisea*, in which the mean EC_{50} value was determined as 0.029 µg a.i. ml^{-1} with a range of 0.015 to 0.064 µg a.i. ml^{-1}, and isolates suspected as resistant to azoxystrobin exhibited a mean EC_{50} value of 20 µg a.i. ml^{-1} with a range of 2.39 to 44.8 µg a.i. ml^{-1}. Wong (Wong et al. 2007) also reported a baseline sensitivity of *C. cereale*, where the mean EC_{50} value of a population unexposed to azoxystrobin was 0.037 µg a.i. ml^{-1} with a range of 0.006 to 0.089 µg a.i. ml^{-1}. True azoxystrobin baseline sensitivities of *Ggg* could not be determined because previous exposure to QoIs was unknown for the collected isolates. However, in

comparison to previous reports, the results presented here may still reflect baseline sensitivities of *Ggg*. The EC_{50} values presented here ($\leq$0.399 µg a.i. ml^{-1}) were not high when compared to those previously reported for resistant fungi from intensively managed turfgrass and were similar to the aforementioned baseline sensitivities. St. Augustinegrass is commonly used for low-maintenance residential turf and home lawns; therefore, the low EC_{50} values of *Ggg* presented here may be related to the fact that control of TARR is not heavily dependent upon fungicide applications. Still, the availability of QoIs for controlling TARR justifies the importance for continual monitoring of sensitivity distributions and for better understanding potential risks of QoI resistance.

A single discriminatory concentration was previously employed for rapid fungicide sensitivity screening of azoxystrobin for a turfgrass pathogen, *C. cereale*, in which a concentration of 0.031 µg a.i. ml^{-1} was used based on the relationship (R^2 = 0.80) between log transformed EC_{50} values and relative mycelial growth at this concentration (Wong et al. 2007). In this study, the relationship between relative growth of *Ggg* at 0.03 µg a.i. ml^{-1} and log transformed EC_{50} values of azoxystrobin was not as strong (R^2 = 0.63) as the discriminatory dose of 0.1 µg a.i. ml^{-1} (R^2 = 0.839). However, relative growth at 0.03 µg a.i. ml^{-1} may be alternatively used for predicting $EC_{50(D)}$ values, since all *Ggg* isolates in this study were able to grow at this concentration. Azoxystrobin EC_{50} values for *Ggg* were $\leq$0.399, and 37% of isolates tested were completely inhibited when exposed to the discriminatory dose of 0.1 µg a.i. ml^{-1}. Although this discriminatory concentration was not suitable for predicting $EC_{50(D)}$ values

for highly sensitive *Ggg* isolates, it did provide an effective tool for resistance screening, given that isolates exhibiting no growth at 0.1 µg a.i. ml^{-1} are likely to represent a high sensitivity with EC_{50} values of <0.014.

DMIs prevent biosynthesis of ergosterol by inhibiting demethylation at the 14-α carbon of 24-methylene dihydrolanosterol or lanosterol by binding to cytochrome P450-dependent 14-α demethylase (Gisi et al. 2000). Various mechanisms that confer resistance to DMIs have been described. Hulvey (Hulvey et al. 2012) reported the overexpression of *ShCYP51B*, a gene encoding cytochrome P450-dependent 14-α demethylase, and *ShatrD*, a gene encoding an efflux transporter of DMI fungicides, in *S. homoeocarpa* isolates displaying reduced DMI sensitivities. Several target site mutations in *CYP51* are also known to confer resistance to DMIs, where combined mutations confer higher resistance factors (calculated by dividing the EC_{50} value of the most resistant isolate by the EC_{50} value of the population mean) than single mutations (Gisi et al. 2000). Due to the polygenic nature of DMI-resistance, reduced sensitivities move in a gradual manner; therefore, it is important to establish a baseline sensitivity using isolates not previously exposed to DMIs in order to detect significant shifts to increased resistance (Russell 2004). During *in vitro* tebuconazole sensitivity assays of *C. cereale*, Wong (Wong and Midland 2007) reported a baseline EC_{50} mean of 0.082 µg a.i. ml^{-1} with a range of 38-fold; furthermore, a mean of 0.35 µg a.i. ml^{-1} and range of 3.6-fold was determined for a *C. cereale* population previously exposed to DMIs. Other reports of baseline EC_{50} values of tebuconazole for fungal pathogens include means from 0.007 to 0.124 µg a.i. ml^{-1} with ranges from 6- to 100-fold and resistance factors ranging from

1.99 to 6 (Franke et al. 1998; Ma et al. 2002; Thomas et al. 2012; Wilcox and Burr 1994). In this work, we determined the mean EC_{50} value of *Ggg* to be 0.049 µg a.i. ml^{-1} with a range of 14-fold and resistance factor of 6.02. Previous exposure to DMIs for the isolates obtained for this study is unknown; therefore, the DMI sensitivities reported here may not represent a true baseline for *Ggg*. However, since most *Ggg* isolates tested produced a frequency distribution at high sensitivities, it is still possible that this *Ggg* population represents a sensitive population, and the isolates presented here may be used as a reference baseline for tebuconazole. In order to confirm these isolates as baseline representatives, fungicide efficacy data may need to be acquired, or additional sampling from St. Augustinegrass confirmed as not previously treated with DMIs should be performed (Russell 2004).

Disruptive selection of resistance occurs following sequential applications of MBCs, since this class of fungicides specifically inhibits microtubule assembly via binding to beta-tubulin (Davidse 1986), and a single mutation of the beta-tubulin gene may confer complete resistance (Köller and Scheinpflug 1987; Orbach et al. 1986). In this study, all isolates were completely inhibited by thiophanate-methyl at 500 µg a.i. ml^{-1}. Jo et al. (Jo et al. 2006) determined a concentration of thiophanate-methyl at 1,000 µg a.i. ml^{-1} as an effective dose for screening for resistant isolates of *S. homoeocarpa*, which either inhibited or did not inhibit fungal growth. Complete inhibition of *Ggg* by thiophanate-methyl at 500 µg a.i. ml^{-1} suggests that none of the isolates obtained for this study have developed resistance to the MBC group of fungicides.

Fungicide sensitivities provided by this work allowed the development of proper fungicide screening assays of *Ggg* for three major fungicide groups: MBCs, DMIs, and QoIs. Based on the *Ggg* isolates obtained for this study, fungicide resistance of azoxystrobin, tebuconazole, and thiophanate-methyl is not prevalent, and the potential of future resistance development may not be as high as compared to other heavily managed turfgrass pathogens. However, resistance screening should be expanded to accommodate additional isolates of *Ggg*, such as those collected from sites previously treated with these fungicides, especially from heavily managed areas such as sod farms or other commercial landscapes. Here, rapid screening assays based on a single discriminatory concentration of azoxystrobin, tebuconazole, and thiophanate-methyl will provide a less cumbersome method for predicting EC_{50} values that can be used for future screening assays of *Ggg*.

CHAPTER IV

CONCLUSIONS

Morphological characteristics, along with PCR identification and a multilocus phylogenetic analysis, confirmed that all isolates evaluated were *Ggg* (Rachdawong et al. 2002; Walker 1981). Morphological, physiological, and genetic differences also revealed three groups (M, L, and H) of *Ggg* infecting St. Augustinegrass throughout Texas. On PDA, Group M produced highly melanized and round colony morphologies, followed by high quantities of lobed hyphopodia and a mean phialospore length similar to the H group but significantly longer than the L group. Compared to L and H groups, group M was more adapted to higher temperatures and was significantly more aggressive based on rice seedling pathogenicity assays. Group L produced non to slightly melanized and round colony morphologies with few to no lobed hyphopodia. Group H produced highly melanized colonies with an irregular formation, followed by high quantities of lobed hyphopodia. Optimal growth temperature and aggressiveness were similar between most L and H group isolates. Finally, most M and H group isolates were capable of perithecial development on the culms of rice seedlings, while perithecia were not developed by L group isolates. The failure of perithecial production restates that identification of *G. graminis* varieties solely based on morphological features can be inconclusive and that molecular approaches may be more definitive (Bateman et al. 1992; Bryan et al. 1995; Holden and Hornby 1981).

Datnoff (Datnoff et al. 1997) demonstrated that *Ggg* isolates were cross pathogenic on St. Augustinegrass, bermudagrass, and rice; however, it also appeared that isolates were most aggressive on the host from which they were obtained. Rice seedlings provided a clean, efficient, and less-cumbersome method for evaluating pathogenicity, particularly since most turfgrass cultivars are sterile and require the production of aerial stolons in an attempt to obtain disease-free plant material (Datnoff et al. 1997; Elliott 1995). However, future studies should expand pathogenicity assays to turfgrass hosts, particularly St. Augustinegrass, in order to confirm consistent variations of aggressiveness.

A multilocus phylogeny reconstruction based on partial sequences of ITS, LSU, and RPB1 loci strongly supported the separation of *G. graminis* varieties and M, L, and H groups based on morphological and physiological differences. The maximum likelihood (ML) tree produced during this study also sheds light on the evolutionary relationships between *Ggg*, *Gga*, and *Ggt*. M, L, and H group isolates, with the exception of MZTX13-1, were separated into their respective clades, where it appeared that M and H groups were more closely related to each other as compared to the L group. The close phylogenetic relationship between M and H groups supports their similarities of melanization, hyphopodia production, mean phialospore length, and the ability to sexually reproduce on the culms of rice seedlings. It also appeared that the L group, along with MZTX13-1 and *Ggg*, *Gga*, and *Ggt* taxa, diverged and evolved separately from M and H groups and other *Ggg* taxa. The observation of closely and distantly related groups of *Ggg* to *Gga* and *Ggt* is consistent with previous reports

(Fouly 2002; Fouly et al. 2011; Fouly et al. 1997). Furthermore, the phylogenetic analysis indicated that *Ggg* was polyphyletic, while *Gga* and *Ggt* each formed monophyletic groupings. This is consistent with previous work by Ward (Ward and Bateman 1999), where the polyphyletic nature of *Ggg* was also described. Other studies also report variability of *Ggg* (Elliott et al. 1993; Fouly 2002; Fouly et al. 2011; Fouly et al. 1997; Fouly et al. 1996; Ward and Akrofi 1994; Ward and Bateman 1999).

The phylogenetic analysis also supported that *Gga* and *Ggt* were more closely related to each other as compared to *Ggg*, which is consistent with morphological characteristics and host ranges previously described for varieties of *G. graminis* (Elliott 1991; Elliott et al. 1993; McCarty and Lucas 1989; Walker 1981; Wilkinson 1994; Wilkinson and Kane 1993)). Several molecular approaches also support the close relationship between *Gga* and *Ggt* (Bryan 1995; Bryan et al. 1995; Bryan et al. 1999; Fouly 2002; Fouly et al. 2011; Fouly et al. 1997; Fouly et al. 1996; Ward and Akrofi 1994; Ward and Bateman 1999).

The resolution of intravarietal complexities associated with *Ggg* is important considering proper identification and diagnostic procedures, as well as the development of disease management programs. Evaluations of morphological characteristics, pathogenicity, and genetic backgrounds can be implemented for distinguishing members of the G-P complex; however, specific groups of *Ggg* have not been well-described. To our knowledge, this is the first report to identify specific groups of *Ggg* based on phenotypic and genetic data. Group M isolates are of particular interest due to their high aggressiveness and adaptation to higher temperatures, which are common to regions

where host species are cultivated (Elliott 1991; Elliott et al. 1993; McCarty and Lucas 1989; Walker 1981; Wilkinson 1994; Wilkinson and Kane 1993). However, future studies should investigate pathogenicity of St. Augustinegrass and other turfgrass hosts to confirm consistent variations of aggressiveness between groups. Future studies should also continue resolving intravarietal complexities and investigate associations between specific groups of *Ggg* and disease incidences and severities of TARR.

An evaluation of nine *Ggg* isolates determined that sensitivities to azoxystrobin significantly increased in the presence of salicylhydroxamic acid (SHAM), indicating that *Ggg* can utilize alternative oxidase for circumventing the mode of action of QoIs. SHAM has been used to inhibit effects of alternative oxidase in other fungi (Avila-Adame et al. 2003; Kim et al. 2003; Vincelli and Dixon 2002; Wong et al. 2007), and increased sensitivities in the presence of SHAM have been previously reported (Bradley and Pedersen 2011; Olaya and Köller 1999; Olaya et al. 1998; Wise et al. 2008). The determination of alternative respiration in *Ggg* also indicated that SHAM is a critical component for proper *in vitro* QoI sensitivity assays of this pathogen.

A single discriminatory concentration of azoxystrobin and tebuconazole was determined as 0.1 µg a.i. ml^{-1}, which provided an accurate and rapid tool for *in vitro* resistance screening and EC_{50} prediction. Azoxystrobin EC_{50} values ranged from <0.014 to 0.399, while 28 isolates were completely inhibited by the discriminatory dose. Tebuconazole EC_{50} values ranged from 0.021 to 0.296 with a mean of 0.049. All isolates were completely inhibited by the discriminatory dose of thiophanate-methyl (500 µg a.i. ml^{-1}). In comparison to previous reports of resistance and baseline sensitivities of

azoxystrobin (Kim et al. 2003; Vincelli and Dixon 2002; Wong et al. 2007) and tebuconazole (Wong and Midland 2007) (Franke et al. 1998; Ma et al. 2002; Thomas et al. 2012; Wilcox and Burr 1994), the EC_{50} values presented here may reflect baseline sensitivities for *Ggg*. However, previous exposure of the isolates to QoIs and DMIs was unknown; therefore, true baseline sensitivities could not be confirmed. In order to confirm these isolates as true baseline representatives, azoxystrobin and tebuconazole efficacy should be determined (Russell 2004).

Knowledge of fungicide sensitivities in *Ggg* populations is critical to designing pragmatic fungicide programs for controlling TARR. The majority of isolates were obtained from low-maintenance residential St. Augustinegrass, where the control of TARR is not heavily dependent on fungicide applications. This might reflect the low EC_{50} values determined for azoxystrobin, tebuconazole, and complete inhibition of growth by thiophanate-methyl. Still, the availability of QoIs, DMIs, and MBCs for controlling TARR justifies the importance for continual monitoring of sensitivity distributions and for better understanding potential risks for fungicide resistance. Future studies should determine true baseline sensitivities of *Ggg* to the fungicides tested and expand resistance screening to accommodate isolates from heavily managed landscapes.

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APPENDIX

GENOMIC DNA EXTRACTION

A phenol-chloroform method was used for genomic DNA extraction (Sambrook et al. 1989). Approximately 0.25 to 0.5 g of fungal tissue was mixed with 500 µl of extraction buffer (50 mM Tris-HCl (pH 8.0), 50 mM sodium-EDTA (pH 8.0), and 2% sodium dodecyl sulfate), followed by an incubation period of 30 min at 68°C. Five hundred µl of phenol:chloroform:IAA (25:24:1) was then added and was incubated in ice for 5 min, followed by light vortexing and centrifugation at 14,000 rpm for 4 min at room temperature. The aqueous phase was transferred to a new 1.5-ml microcentrifuge tube, which was again treated with phenol:chloroform:IAA (25:24:1) as previously described. For complete removal of cellular debris, the aqueous phase was transferred to a new 1.5-ml microcentrifuge tube and was subsequently treated with 400 µl of chloroform:IAA followed by light vortexing and a 14,000 rpm centrifugation at room temperature. For precipitation of DNA, the aqueous phase was transferred to a new 1.5-ml microcentrifuge tube, and a 1/10-volume of 3M sodium acetate (pH 5.2) and 2.5-volume of 95% ethanol was added. After gentle inversion and incubation in ice for 5 min, centrifugation at 14,000 rpm for 3 min at room temperature was performed to pellet DNA. The supernatant was removed and pelleted DNA was washed twice with 200 µl of 70% ethanol. Finally, washed DNA was air dried and resuspended in 100 µl of nuclease free water, followed by a 2-µl treatment of RNase at 37°C for 30 min. All extracted genomic DNA was stored at -20°C.

Table 8. Texas counties and isolates of *Gaeumannomyces graminis* var. *graminis* from St. Augustinegrass used for population diversity and fungicide sensitivity analyses

County	Isolates
Brazoria	MZTX57-1
Brazos	MZTX25-2, MZTX85-1[c], MZTX80-1, MZTX10-2[bcd], MZTX11-1[b], MZTX47-1, MZTX8-1[bcd], MZTX9-1[b]
Collin	MZTX67-1, MZTX36-1
Comanche	MZTX88-1
Conroe	MZTX12-1[cd]
Dallas	MZTX75-1, MZTX76-1, MZTX66-1, MZTX33-1[d], MZTX51-1, MZTX6-2, MZTX7-1, MZTX24-1[cd], MZTX70-1, MZTX68-1, MZTX30-2[d], MZTX39-1, MZTX38-1[cd], MZTX45-1, MZTX72-1, MZTX18-2[cd], MZTX52-1[d], MZTX74-1[c], MZTX87-1
Denton	MZTX61-1[d], MZTX50-1[cd]
Gillespie	MZTX73-1
Harris	MZTX34-1[d], MZTX43-1
Henderson	MZTX14-1, MZTX35-1[d], MZTX37-1
Jefferson	MZTX1-2[a], MZTX2-2[a]
Kaufman	MZTX53-2
McLennan	MZTX49-1, MZTX59-1[d]
Montgomery	MZTX63-1, MZTX32-2
Nacogdoches	MZTX28-1
Nueces	MZTX19-2, MZTX40-1
Parker	MZTX86-1, MZTX31-1
Smith	MZTX64-1
Tarrant	MZTX91-1, MZTX13-1[cd], MZTX22-1[cd], MZTX41-1, MZTX5-2[c], MZTX65-1[d], MZTX93-1, MZTX44-2, MZTX15-2[cd], MZTX23-1[c], MZTX42-1, MZTX56-1, MZTX71-1, MZTX16-1, MZTX29-1, MZTX55-1[d], MZTX90-1
Travis	MZTX20-1[cd], MZTX89-1, MZTX83-1
Williamson	MZTX62-2, MZTX69-2, MZTX21-1

[a] Isolates collected from the Texas A&M AgriLife Research Center in Beaumont, Texas.
[b] Isolates collected from the Texas A&M University in College Station, Texas; all other isolates, with the exception of MZTX80-1 and MZTX12-1, were provided by the Texas Plant Disease Diagnostic Laboratory.
[c] Isolates used for determining a discriminatory concentration of tebuconazole.
[d] Isolates used for determining a discriminatory concentration of azoxystrobin.