ECOLOGY AND MANAGEMENT OF LARGE PATCH OF ZOYSIAGRASS, CAUSED BY *RHIZOCTONIA SOLANI* AG 2-2 LP

by

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B.S., Ahmadu Bello University, Nigeria, 2000
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Large patch, caused by the fungus *Rhizoctonia solani* anastomosis group (AG) 2-2 LP, is the most common and severe disease of zoysiagrass (*Zoysia* spp). Despite the importance of this disease, few studies have examined pathogen biology, cultivar susceptibility, cultural controls, and chemical controls. The objectives of this dissertation were: (1) Characterize large patch isolates based on anastomosis pairing, in-vitro mycelial growth rates, nuclear counts, virulence, PCR, and amplified fragment length polymorphism (AFLP); (2) Determine the effects of cultivation (aerification, verticutting, and sand topdressing) on disease severity; (3) Evaluate different fall and spring applications of the fungicides flutolanil, azoxystrobin, and triticonazole; (4) Evaluate the susceptibility of fifteen new zoysiagrass germplasm lines from parental crosses including *Z. japonica*, *Z. matrella*, and *Z. pacifica*. All the *R. solani* isolates from large patch-infected zoysiagrass from Kansas belonged to AG 2-2 LP. Variations were observed among the isolates in their average number of nuclei per cell, mycelial growth rates and virulence. There was also variation in the amplified fragment length polymorphism (AFLP) DNA fingerprints, suggesting possible underlying genetic differences of biological significance among members of AG 2-2 LP. Cultivation did not affect soil moisture or temperature. Cultivation also did not reduce patch sizes, nor influence turf recovery rate from large patch. From 2009 to 2011, spring and fall N fertility was consistently associated with lower percentages of diseased turf in both cultivated and non-cultivated plots at Manhattan and Haysville. In general, two fall applications of fungicide did not reduce disease compared to one fall application. Fungicides applied in the fall when thatch temperatures ranged from 17.8^{o}C to 23.2^{o}C reduced disease compared to untreated controls. Early spring applications reduced disease compared to later spring

applications. In germplasm screening studies, all progeny had similar disease levels compared to Meyer in the growth chamber, but only 6 consistently had disease levels as low as Meyer in the field. Growth chamber results did not correlate to field results.

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Approved by:

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Table of Contents

List of Figures ... xii

List of Tables ... xiv

Acknowledgements ... xvi

Dedication ... xviii

Chapter 1 - Introduction ... 1

References ... 9

Chapter 2 - Phenotypic and Genotypic Characterization of *Rhizoctonia solani* Isolates from Zoysiagrass in Kansas ... 13

Abstract ... 13

INTRODUCTION ... 13

MATERIALS AND METHODS ... 17

Pathogen isolation and storage ... 17

In vitro phenotypic characteristics, anastomosis grouping of isolates, and PCR identification ... 18

Average number of nuclei per hyphal cell ... 19

Mycelial growth rates and virulence ... 19

AFLP analysis of genomic DNA of isolates ... 21

Isolation of genomic DNA ... 21

Digestion and ligation ... 23

Pre-amplification ... 24

First and second selective amplifications ... 24

Preparation of DNA samples for analysis ... 25

Cluster analysis of isolates ... 25

Data analysis ... 25

RESULTS ... 26

In vitro phenotypic characteristics, anastomosis grouping of isolates, and PCR identification ... 26

Average number of nuclei per hyphal cell ... 26

Mycelial growth rates and virulence 26
Relationship between nuclear number and mycelial growth of isolates 27
AFLP analysis of genomic DNA of isolates 27
DISCUSSION 28
References 30
Chapter 3 - Effect of cultivation and timing of nitrogen fertilization on large patch disease of zoysiagrass 40
Abstract 40
INTRODUCTION 40
MATERIALS AND METHODS 44
Pathogen isolation, storage, and inoculation 44
Summer cultivation and fertility treatments 45
Disease assessment 46
Data analysis 47
Microclimate measurements 48
RESULTS 50
Effects of summer cultivation on soil and thatch temperatures and volumetric soil water content 50
Effects of summer cultivation and timing of nitrogen application on large patch 50
Manhattan – 2008 50
Manhattan – 2009 51
Manhattan – 2010 51
Manhattan – 2011 52
Haysville – 2009 52
Haysville – 2010 52
Haysville – 2011 53
Olathe – 2009 53
Olathe – 2010 53
Olathe – 2011 53
DISCUSSION 54
References 57

Chapter 4 - Evaluation of spring and fall fungicide applications for large patch management in zoysiagrass 75
Abstract 75
INTRODUCTION 75
MATERIALS AND METHODS 79
Inoculum preparation 79
Site description and inoculation of study plots 80
Fungicide timing studies 80
Fall applications, 2008 81
Spring applications, 2009 81
Fall applications, 2009 82
Spring applications, 2010 82
Soil temperature measurement 82
Data analysis 83
RESULTS 84
Efficacy of fall 2008 applications on large patch in spring 2009 84
Efficacy of spring 2009 applications 84
Fall applications, 2009 85
Spring applications, 2010 86
DISCUSSION 87
References 89
Chapter 5 - Evaluation freeze-tolerant zoysiagrass genotypes for susceptibility to large patch disease caused by *Rhizoctonia solani* AG 2-2 LP. 96
Abstract 96
INTRODUCTION 96
MATERIALS AND METHODS 99
Pathogen isolation and storage 99
Growth chamber studies 100
Plant inoculation and disease assessment 100
Field studies 101
Plot inoculation and disease assessment 101

Data analysis 102
RESULTS 104
Growth chamber inoculation and disease assessment 104
Field experiments 104
2009 104
2010 105
Correlation analysis 105
DISCUSSION 106
References 108
Chapter 6 - Conclusions 115

List of Figures

Figure 2.1 Light microscopy images displaying C3 (perfect fusion) and C2 (imperfect fusion) between two paired isolates of *R. solani* on a water agar-coated glass slide. 33

Figure 2.2 Agarose gel electrophoresis of the PCR products of 43 *R. solani* isolates using the AG 2-2 LP-specific primer, P22-LP.. 34

Figure 2.3 Comparison of the virulence of four *R. solani* AG 2-2 LP isolates on Meyer zoysiagrass. 35

Figure 2.4 Scatter plot showing the relationship between the colony diameter and the average numbers of nuclei per cell of *R. solani* AG 2-2 LP isolates from Kansas and Missouri. 36

Figure 2.5 Cluster dendrogram of 23 *R. solani* isolates belonging to AG 2-2 LP including one each of AG 1, 2-2 IIIB, 2-1A, and 3. 37

Figure 3.1 Experimental plots at Manhattan in the spring of 2009 following inoculation of plots during the fall of 2008, showing large patch symptoms of approximate uniform size in the inoculated plots and symptoms from pre-existing natural infections. 60

Figure 3.2 Effect of core-aerification, verticutting and sand topdressing on soil and thatch temperatures at Manhattan in 2009 and 2010. 61

Figure 3.3 Effect of core-aerification, verticutting and sand topdressing on volumetric soil water content, measured with dual probes installed at 13 cm below the thatch layer, in cultivated and non-cultivated plots at Manhattan in 2009 and 2010.. 62

Figure 3.4 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2008 at Manhattan, KS. 63

Figure 3.5 Analysis of digital image of plot with large patch symptoms showing image before (left) and after (right) analysis with the SigmaScan Pro 5 (SPSS 5) image analysis software. 64

Figure 3.6 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2009 at Manhattan, KS. 65

Figure 3.7 Effect of summer cultivation and timing of nitrogen application on large patch disease symptoms during the spring of 2010 at Manhattan, KS. 66

Figure 3.8 Effect of summer cultivation and timing of nitrogen application on large patch during spring 2009 at Haysville, KS. 67
Figure 3.9 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2009 at Olathe, KS.. 68

List of Tables

Table 2.1 Anastomosis group of *Rhizoctonia solani* isolates from zoysiagrass, average number of nuclei per cell, and colony diameter after incubation at 25°C for 72 h on PDA. 38

Table 2.2 Colony diameter of 34 *Rhizoctonia solani* AG 2-2 LP isolates from Kansas and Missouri, averaged by location, after incubation at different temperatures for 72 h. 39

Table 3.1 Experimental locations with schedule of cultural practices. 69

Table 3.2 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by relative patch size during spring 2008 at Manhattan, KS. 70

Table 3.3 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by relative patch size during spring and early summer of 2009 at Manhattan, KS. 71

Table 3.4 Effect of summer cultivation and timing of nitrogen application on large patch disease symptoms as measured by digital image analysis during spring 2010, fall 2010, and spring 2011 at Manhattan, KS. 72

Table 3.5 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by patch size (2009) and digital image analysis (26 June 2009, 2010 and 2011) at Haysville, KS. 73

Table 3.6 Effect of summer cultivation and timing of nitrogen application on large patch as measured by patch size (2009) and digital image analysis (2010 and 2011) at Olathe, KS. 74

Table 4.1 Application-timing schedule for flutolanil, azoxystrobin, and triticonazole for the management of large patch on 'Meyer' zoysiagrass. 91

Table 4.2 Effect of single and sequential fall 2008 applications of flutolanil on large patch in the spring of 2009. 92

Table 4.3 Effect of single and sequential spring 2009 applications of flutolanil, azoxystrobin, and trinity on large patch. 93

Table 4.4 Effect of single fall 2009 applications of flutolanil, azoxystrobin, and triticonazole on large patch in the spring of 2010. 94

Table 4.5 Effect of single spring 2010 applications of flutolanil, azoxystrobin, and triticonazole on large patch in the spring of 2010. 95

Table 5.1 Backgrounds of the zoysiagrass genotypes 110
Table 5.2 Large patch (caused by *R. solani* AG 2-2 LP) disease incidence of new zoysiagrass lines and 'Meyer' under growth chamber conditions[z] in 2009. 111
Table 5.3 Large patch (caused by *R. solani* AG 2-2 LP) disease incidence of new zoysiagrass lines and 'Meyer' under growth chamber conditions[z] in 2010. 112
Table 5.4 Large patch (caused by *R. solani* AG 2-2 LP) diameter and percentage of diseased turf of new zoysiagrass lines and 'Meyer' under field conditions at Manhattan, KS in 2009. . 113
Table 5.5 Percentage of large patch (caused by *R. solani* AG 2-2 LP) diseased turf of new zoysiagrass lines and 'Meyer' under field conditions at Manhattan, KS in 2010. 114

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Dedication

In loving memory of my parents, Michael and Bose Obasa, for their faith in me, and their support for every step of my academic aspiration.

Chapter 1 - Introduction

Rhizoctonia species are ubiquitous soil-borne basidiomycete (sub-division: basidiomycotina) fungi that are ecologically diversified as plant pathogens, soil saprophytes, and mycorrhizae of several orchids (Ogoshi, 1987). *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris* (A. B. Frank) Donk) is a species complex comprising many related but genetically isolated subspecific groups (Carling *et al*, 2002, Ogoshi, 1987, Anderson, 1982). Identification of groups and subspecific groups of *R. solani* has traditionally been based on hyphal anastomosis (fusion) reactions and are called anastomosis groups (AG's) (Carling, 1996). There are currently 14 AG's described in the literature (AG-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, and AG-BI) (Carling *et al*., 2002). Anastomosis groups 1, 2, 3, 4, 6, 8, and 9, have further been divided into subgroups (Carling *et al*., 2002, Ogoshi, 1987). Currently, AG 2 represents the most heterogeneous AG with seven subsets described: AG-2-1, AG-2-2 IIIB, AG-2-2 IV, AG-2-2 LP, AG-2-3, AG-2-4, and AG 2 BI (Carling *et al*., 2002, Hyakumachi *et al*., 1998). Members of AG 2 are also known to form bridging anastomosis reactions with members of AG 3 and AG 8 (Carling *et al*., 2002; Carling, 1996). Much work has been conducted to distinguish among the subgroups of *R. solani* using anastomosis pairings, pathogenicity, DNA sequencing, and other methods. (Jiangfeng *et al*., 2005; Liu and Sinclair, 1992; Salazar *et al*., 2000; Carling *et al*., 2002; Johnk and Jones, 1993; Toda *et al*., 2004; Aoyagi *et al*., 1998; Green *et al*., 1993). Variation within subgroups has been less explored.

Rhizoctonia species affect both warm-season (C4 photosynthetic pathways) and cool-season (C3 photosynthesizing) turfgrasses and incite leaf and sheath diseases such as brown patch, large patch, yellow patch, leaf and sheath spot in different hosts (Smiley *et al*., 2005).

Four anastomosis groups of *R. solani*, AG 1- IA, AG-2, AG-4, and AG-5, have so far been identified to infect turfgrasses (Burpee and Martin 1992, Aoyagi *et al*., 1998). And more *Rhizoctonia* diseases in turfgrasses are being reported. For instance, a newly described binucleate *Rhizoctonia* in anastomosis group AG-D was recently reported as causing a sheath blight and patch symptoms in the C4 turfgrass *Zoysia japonica* (Hayakawa *et al*, 2006). Another new *Rhizoctonia* pathogen tentatively named only for the teleomorph *Waitea circinata* var. *circinata* has been reported in several C3 turfgrasses (Toda *et al.*, 2005, de la Cerda *et al*., 2007). Examples of other plant diseases caused by different AG's of *R. solani* include rice sheath blight, stem canker of potato, black scurf of potato, damping off of cotton and tobacco, bare patch and crater disease of wheat, root and crown rot of sugar beet, and brown patch of cool-season turfgrasses (Lee and Rush, 1983; Banville *et al.*, 1996; Frank and Leach, 1980; Bacharis *et al*., 2010; Neate and Warcup, 1985, Carling *et al*., 1996; Herr, 1996; Burpee and Martin, 1993). Although members of an AG are often associated with infection within a range of host species and given a subAG classification, the exact mechanisms of that specificity are not known. Evidence from studies of the infection process suggests that extracellular enzymes including pectinolytic and cellulolytic enzymes aid in host penetration by members of some AG (Bertagnolli *et al*., 1996; Liu and Sinclair 1991, Hofman and Jongebloed 1988; Marcus *et al*., 1986; Brookhouser and Weinhold, 1979). In contrast, in isolates of some other AG's such as AG 1, host penetration may also be by mechanical means with the aid penetration pegs (Matsuura, 1986). During the colonization of host tissues after penetration, *R. solani* has been reported to secrete DNAse, RNAse, alpha-amylase, chitinase, beta-glucanase, xylanase, protease, and urease (Bertagnolli *et al*., 1996), but their involvement in host colonization is not well understood. In other studies, *R. solani* has been shown to produce several toxins, some of which may be

involved in pathogenesis. For instance, a host specific non-protein containing glucose, mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine capable of reproducing symptoms of rice sheath blight, caused by AG 1A has been identified (Vidhyasekaran *et al*., 1997). It was further shown that a phytotoxin from *R. solani* correlates with sheath blight susceptibility in rice (Brooks, 2007). Other phytotoxins identified from *R. solani* have included phenylacetic acid (PAA) and one or more of its hydroxyl derivatives (Mandava *et al*., 1980), and fumaric acid (Hyakumachi *et al*., 1980).

Zoysiagrass (*Zoysia japonica* Steud. and *Z. matrella* (L.) Merr.) is a warm-season (C4), perennial turfgrass that is widely used on golf courses as well as on lawns in the "transition-zone" of the United States, a region that includes Kansas and states eastward to Virginia and North Carolina. Most zoysiagrass cultivars, with the exception of a few such as Zenith, are vegetatively propagated from sprigs or sods. As a warm-season turfgrass, optimum root and shoot growth of zoysiagrass occurs during the high temperature conditions of the summer months in the transition zone. Compared to certain cool-season (C3) turfgrass species such as creeping bentgrass (*Agrostis stolonifera* L.), which experiences optimum root and shoot growth during the lower temperature conditions characteristic of spring and autumn months in the transition zone, zoysiagrass has lower water, fertilizer, and pesticide requirements for maintenance while maintaining a high-quality surface (Fry *et al*., 2008), making it a potentially more sustainable species. However, an important consideration in the adoption and widespread use of zoysiagrass is large patch disease, caused by *R. solani* AG 2-2 LP. Large patch is a serious problem for turfgrass managers, particularly along the northern range of zoysiagrass adaptation in North America (Green *et al*., 1993), but also represents a major problem everywhere

zoysiagrass is utilized. Large patch also occurs in St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze) and bermudagrass (*Cynodon dactylon* (L.) Pers.).

In the transition zone of the U.S., symptoms of large patch appear during spring (April-May) and autumn (September-October) as roughly circular light-brown to straw-colored patches with slightly matted areas of bright-orange discoloration that eventually fade to a tan or dull brown color with bright orange margins (Smiley *et al*., 2005; Tisserat *et al*., 1994; Green *et al*., 1993). Patches can range in size up to 6 meters or more in diameter (Tisserat *et al*., 1994; Green *et al*., 1993) with healthy turf sometimes scattered within the patches. Symptoms on individual plants occur as reddish-brown to black lesions on the basal leaf sheaths. Infection of the leaf sheath results in the girdling of shoots, which cuts off water and nutrient supplies to the upper parts of the plant and culminates in the bright orange discoloration characteristic of the disease (Tisserat *et al*., 1994). During summer conditions, regrowth within patches often results in full turf recovery though weeds can encroach while the turfgrass is thinned.

Large patch symptom development in zoysiagrass is favored by relatively cool and humid weather. Thatch temperatures of 15 to 25°C, compacted and poorly drained soils, and excessive and prolonged wetness near the leaf surface are optimal conditions for the development of large patch symptoms (Green *et al*, 1993). However, large patch symptoms are suppressed during summer, supposedly by thatch and soil temperatures above 30°C (Green *et al*, 1993). In addition to environmental conditions, several cultural practices such as mowing height (Green *et al*., 1994) and water management (Green *at al*., 1994) can also influence large patch development.

Currently, management of large patch is primarily by fungicide application during spring and/or fall, because cultural management practices do not provide an acceptable level of disease control and there have been few studies of the effects of cultural practices. Green *et al*. (1994)

studied the effects of mowing height, nitrogen (N) source, and N application rates on large patch development and severity on zoysiagrass. They found that lower mowing heights resulted in more severe disease. In addition, large patch was not affected by N source (urea, urea formaldehyde, poultry litter, sewage sludge, and bovine waste) or the two different application rates of 74 kg and 148 kg of N per hectare per year. They did not however study the effect of different fertilization timings. In an effort to promote faster emergence from winter dormancy some turfgrass managers practice early spring fertilization. Similarly, N fertility is applied by some during late fall to extend the duration of retention of green color by zoysia, thereby delaying the onset of dormancy (Fry *et al*., 2008). The effects of the practices, if any, remains to be elucidated.

In the turfgrass industry, cultivation refers to aerification (punching solid or hollow cores in the soil to create holes) and verticutting (slicing into the turf canopy and roots with vertical blades). These practices lead to improved soil moisture and oxygen conditions, resulting in improved root growth as well as increased microbial activity that is essential to the biodegradation of thatch (Christians, 2004). On zoysiagrass turf, aerating and verticutting fairways when large patch is active has been anecdotally reported to result in the development of new satellite infections on healthy areas of turf by infected cores (Spurlock, 2009), but the effects of cultivation in summer is not known. In addition, the effect of timing of nitrogen (N) fertilization and cultivation on large patch development and severity in zoysiagrass is not known, although turfgrass managers have associated severe large patch outbreaks to excessive N fertilization (Green *et al*., 1994). Similarly, high N applications were also associated with increased susceptibility of cool-season turfgrasses to *Rhizoctonia* brown patch (Smiley *et al*.,

1992; Cook *et al*., 1964). Furthermore, the influence of the interplay between cultivation and timing of fertilization, if any, on large patch remains to be elucidated.

The chemical control of large patch disease employs several classes of fungicides including the sterol biosynthesis demethylation inhibitors (DMI), quinone outside inhibitors (QoI, respiration inhibitors), polyoxins (chitin inhibitors), carboxamides (respiration inhibitors), and aromatic hydrocarbons (lipid and membrane synthesis inhibitors) that are labeled for large patch. However, while fungicide applications can be useful for suppressing large patch, optimum timing of application remains uncertain. Preventative fungicide applications made before the development of large patch symptoms have been demonstrated to provide better disease control than applications made after the onset of disease symptoms (Tisserat *et al*., 1993). Preventative applications made during fall not only inhibit fall symptoms, but also suppress or delay disease development during the following spring (Tisserat *et al*., 1994). As a general rule, the recommendation for the timing of the first fungicide application is when thatch temperatures drop below 21°C. However, neither the optimum thatch or soil temperatures for fungicide application nor the number of applications required for optimum control is known. It is not uncommon for turf managers to plan applications based on calendar dates. However, changing environmental conditions may influence the efficacy of such applications.

In addition to large patch disease, the relative lack of an acceptable level of winter hardiness and long period of winter dormancy are other limiting factors in the widespread use of zoysiagrass cultivars in the transition zone. The level of winter injury suffered varies widely among zoysiagrass genotypes (Patton and Reicher, 2007). ‘Meyer’ Zoysiagrass (*Zoysia japonica* Steud.), a vegetatively propagated zoysiagrass cultivar, is the most widely used cultivar used on golf courses in the transition zone since 1952 (Fry *et al*., 2008). Meyer and ‘Zenith’ zoysiagrass

(*Z. japonica*), which is seed-propagated, have better freeze-tolerance than cultivars such as Zorro, Diamond, and Royal, which are *Z. matrella* (Patton *et al*., 2007). However, Meyer is slow to establish and recover, and it is coarser in texture than *Z. matrella* cultivars (Patton and Reicher, 2007; Fry and Dernoeden, 1987). Since 2004, turfgrass researchers at Kansas State University have evaluated over 600 new zoysiagrass progeny for winter survival and quality (Zhang and Fry, 2006; Okeyo *et al*., 2011). These progeny were the result of genotypic crosses made at Texas A&M-Dallas, most of which involved one parent from *Z. japonica* and one from a *Z. matrella* cultivar or Emerald (*Z. japonica* × *Z. pacifica*). The crosses were made in an effort to develop one or more cultivars with freezing tolerance as good as or better than Meyer's, as well as having good density, finer leaf texture, and quality. In a recent study, Okeyo *et al*. (2011) found among the zoysiagrass progeny that some of those associated with reciprocal crosses involving *Z. matrella* (L.) Merr. × *Z. japonica* or 'Emerald' × Meyer, 'Cavalier' (*Z. matrella*), and DALZ 0102 (*Z. japonica*) showed freezing tolerance comparable with Meyer. Furthermore, some also were superior to Meyer in autumn green color retention, but not spring green color retention (Okeyo *et al*., 2011).

Despite previous research on the biology and management of large patch, gaps remain in the understanding of the pathogen, the influence of cultural practices on disease development, optimal use of fungicides, and differences in cultivar susceptibility. In chapter two of my dissertation, I will characterize 36 *Rhizoctonia* isolates from zoysiagrass exhibiting large patch symptoms from different golf courses in Kansas based on: AG group, mycelia growth rates, nuclear counts, virulence, PCR, and amplified fragment length polymorphism (AFLP). In chapter three, I will evaluate the effect of cultivation on soil moisture, soil and thatch temperatures, and large patch development; as well as evaluate the effect of timing of

fertilization on large patch development. In chapter four, I will evaluate large patch control efficacy with several spring and fall fungicide application timings for azoxystrobin (QoI), flutolanil (carboxamide), and triticonazole (DMI). In chapter five, I will evaluate the susceptibility to large patch of fourteen new freeze-tolerant zoysiagrass progenies, and Meyer, under growth chamber and field conditions.

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Chapter 2 - Phenotypic and Genotypic Characterization of *Rhizoctonia solani* Isolates from Zoysiagrass in Kansas

Abstract

Large patch, the most common and severe disease of zoysiagrass (*Zoysia* spp) is caused by *Rhizoctonia solani* Kuhn anastomosis group (AG) 2-2 LP, a subgroup of AG 2. Thirty-six fungal isolates from zoysiagrass in Kansas and Missouri were characterized based on anastomosis pairing, in-vitro mycelial growth rates, nuclear counts, virulence, PCR, and amplified fragment length polymorphism (AFLP). All the *R. solani* isolates belonged to AG 2-2 LP. Variations were observed among the isolates in the average number of nuclei per cell, mycelia growth rates and virulence. A significant negative correlation existed between average number of nuclei per cell and mycelia growth rate. There was also variation in the isolates' amplified fragment length polymorphism (AFLP) DNA fingerprint, suggesting possible underlying genetic differences of biological significance among members of AG 2-2 LP.

INTRODUCTION

Rhizoctonia species are ecologically diversified as plant pathogens, soil saprophytes, and mycorrhizae of several orchids (Ogoshi, 1987). *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris* (A. B. Frank) Donk) is a species complex comprising many related but genetically isolated subspecific groups (Carling *et al*, 2002, Ogoshi, 1987, Anderson, 1982). Identification of groups and subspecific groups of *R. solani* has traditionally been based on hyphal anastomosis (hyphal fusion) reactions and are called anastomosis groups (AG's) (Carling, 1996). There are

currently 14 AG's described in the literature (AG 1-13, and AG-BI) (Carling *et al*., 2002). Anastomosis groups 1, 2, 3, 4, 6, 8, and 9, have further been divided into subgroups (Carling *et al*., 2002, Ogoshi, 1987). Currently, AG 2 represents the most heterogeneous AG with seven subsets currently described: AG-2-1, AG-2-2 IIIB, AG-2-2 IV, AG-2-2 LP, AG-2-3, AG-2-4, and AG 2 BI (Carling *et al*., 2002, Hyakumachi *et al*., 1998). Members of AG 2 are also known to form bridging anastomosis reactions with members of AG 3 and AG 8 (Carling *et al*., 2002; Carling, 1996).

Rhizoctonia species affect both warm-season (C4 photosynthetic pathway) and cool-season (C3 photosynthetic pathway) turfgrasses and incite different symptoms in different hosts. Binucleate *Rhizoctonias* are associated with the teleomorph *Ceratobasidium*. *Rhizoctonia cerealis* is a binucleate species that causes yellow patch in cool-season turf. A newly described binucleate *Rhizoctonia* in anastomosis group AG-D was recently reported as causing a sheath blight and patch symptoms in the C4 turfgrass *Zoysia japonica* (Hayakawa *et al*, 2006). *Rhizoctonia solani* Kühn, a multinucleate *Rhizoctonia,* was identified as the first *Rhizoctonia* pathogen of turfgrass on creeping bentgrass (*Agrostis palustris* Hudson) in the United States over 90 years ago (Piper and Coe, 1919, Aoyagi *et al*, 1998) and has been reported on several other C3 turfgrasses. *R. solani* has also been reported on C4 turfgrass species including bermudagrass (*Cynodon dactylon* (L.) Pers.), St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kuntze), and Zoysiagrass (*Zoysia* Willd. spp.). *R. solani* AG 2-2 IIIB is the primary pathogen of C3 turfgrasses, though multinucleate *R. zeae* and *R. oryzae* (teleomorph *Waitea circinata*) also cause disease in C3 turfgrasses. In addition, a newly-described pathogen tentatively named only for the teleomorph *Waitea circinata* var. *circinata* has been reported in several C3 turfgrasses (Toda *et al.*, 2005, de la Cerda *et al*., 2007).

Large patch, the most common and severe disease of zoysiagrass, is caused by *R. solani* AG 2-2 LP (Hyakumachi *et al*., 1998, Aoyagi *et al*., 1998). Large patch also occurs in St. Augustinegrass and bermudagrass. Large patch is particularly serious along the northern range (ex: Kansas, central Illinois, northern Virginia) of zoysiagrass adaptation and use in North America (Couch, 1973; Burpee and Martins, 1992). Symptoms of large patch on zoysiagrass commonly appear during spring and autumn. In laboratory tests, a temperature range of 15 to 25°C was found to be optimum for infection of zoysiagrass by the fungus (Green *et al*., 1993). It has been suggested that the lack of symptoms during summer months is because thatch and soil temperatures often exceed 30°C, potentially hindering growth of the fungus while at the same time favoring zoysiagrass root and shoot growth (Green *et al*., 1993). Large patch symptoms may, however, develop in shaded areas during unusually cool, moist summers (Green *et al*., 1993). Patches can range in size, up to 6 meters or more in diameter (Tisserat *et al*., 1994; Green *et al*., 1993) under favorable conditions of prolonged leaf wetness, humidity greater than 95%, thatch temperatures of 20-25°C and night temperatures above 20°C (Smiley *et al*., 2005, Green *et al*., 1993). Affected turf has fewer living tillers and a reduced rate of leaf growth. Lower leaf sheaths of affected grasses appear water-soaked, with reddish brown or black lesions. Affected tillers subsequently turn orange to orange-yellow.

Much work has been conducted to distinguish the subgroups of *R. solani* using anastomosis pairings, pathogenicity, DNA sequencing, and other method. (Jiangfeng *et al*., 2005; Liu and Sinclair, 1992; Salazar *et al*., 2000; Carling *et al*., 2002; Johnk and Jones, 1993; Toda *et al*., 2004; Aoyagi *et al*., 1998; Green *et al*., 1993). Variation within subgroups has been less explored. Furthermore, recent research in C3 and C4 turfgrasses has revealed newly-described species and subgroups of *Rhizoctonia*, highlighting the need for re-examination of causal

organisms. Our objective was to characterize 36 *Rhizoctonia* isolates from zoysiagrass exhibiting large patch symptoms from different golf courses in Kansas based on: AG, mycelia growth rates, nuclear counts, virulence, PCR, and amplified fragment length polymorphisms (AFLP).

MATERIALS AND METHODS

Pathogen isolation and storage

Large patch-infected zoysiagrass samples were obtained from fairways of a total of seven golf courses in Kansas and one from Missouri close to the Kansas border. Turf plugs 5 to 10 cm in diameter were removed from the edge of discrete patch areas. For each golf course sample, only one *Rhizoctonia* isolate per patch was saved for analysis. In all, thirty-six representative isolates were recovered from large patch-infected zoysiagrass samples from the 7 sites in Kansas and one from the Missouri site (Table 1). Leaf-sheath sections measuring 1 to 2 cm with blight symptoms were removed from infected plants, separately surface-sterilized with 0.5% NaOCl for about 2 min, blotted dry, and placed on 9-cm-diameter plates of one-fourth strength potato-dextrose agar (Difco Laboratories, Maryland) amended with tetracycline (10 mg/L) and streptomycin (10 mg/L) (designated as "1/4 PDA^{++}") (Biotech Research Grade, Fisher Scientific Inc., New Jersey). Cultures were maintained at 23°C in the dark. Initial identification of *Rhizoctonia solani* AG 2-2 LP isolates was based on cultural characteristics including absence of sclerotia and distinct zonation, and mycelial color of 2-week-old cultures maintained on PDA^{++} at 23°C in the dark (Hyakumachi *et al*., 1998; Aoyagi *et al*., 1998). Hyphal-tip cultures of isolates were subsequently obtained and maintained on 1/4 PDA^{++} at 23°C in the dark. The nuclear counts of the *Rhizoctonia* isolates were then determined using the 4', 6'-diamidino-2-phenylindole (DAPI) staining techniques of Martin (1987) described below with slight modifications. Two known tester isolates, LP 17Li and Rh 146, representing AG 2-2 LP and AG 2-2 IIIB respectively were similarly stored.

***In vitro* phenotypic characteristics, anastomosis grouping of isolates, and PCR identification**

Isolates were identified on the basis of hyphal anastomosis with the AG 2-2LP reference isolate. Five-mm diameter mycelial plugs from hyphal tip cultures of each isolate were paired with plugs of the reference isolate 4 cm apart on a clean glass slide coated with a thin layer of water agar and placed in a Petri dish with moistened filter paper (Martin and Lucas, 1984). The Petri dishes were then incubated at 23°C for 24 to 48 h in the dark. A cover slip was subsequently placed over the region of hyphal contact and the resultant overlapping hyphae were viewed under a compound microscope at 400× magnification. Hyphal fusions between paired isolates were observed for positive anastomosis. Perfect fusion, which includes fusion of cell walls and cytoplasm with continuous living cytoplasm in the fusion site, indicates that the paired isolates belong to the same AG and vegetative compatibility population (VCP) (Yokoyama *et al*., 1985). Imperfect fusion, which involves cell wall fusion accompanied by plasmolysis of the fused cells indicate that the paired isolates belong to the same AG but different VCP (Yokoyama and Ogoshi, 1986). Percent fusion frequency (%FF), a measure of the incidence of hyphal fusion between two isolates (Carling *et al*., 1988), was estimated using the formula %FF = A(100)/B where A is the sum of fusion points in 15 microscopic fields, and B is the sum of contact points in the 15 microscopic fields (Sneh *et al*., 1998). A fusion frequency of fifty percent between paired isolates is considered high and indicates close relatedness of the paired isolates (Sneh *et al*, 1998). All pairings were tested twice.

Polymerase chain reaction (PCR) amplification, using AG 2 subgroup specific primers (Carling *et al*., 2002), was used to identify the AG 2 subgroup to which each isolate belonged. Genomic DNA was extracted as described below using a modified method of Jiangfeng *et al*. (2005). PCR conditions consisted of 1.88 mM $MgCl_2$, 5.0 pmoles of the forward and reverse

primers each, and 100 μg of gDNA. PCR thermocycling protocols (MJ Research PTC-100 Peltier thermal cycler) were: 94°C for 2 min, followed by 31 cycles at 94°C for 40 s; 55°C (for primers P24 and P22), 60°C (for P23, P21), or 62°C (for P22-LP, P22-IV, P22-IIIB) for 1 min; 72°C for 10 s; then 72°C for 7 min, and finally held at 4°C. DNA from isolates belonging to AG's other than AG 2-2 LP were included in the PCR run as negative controls. The PCR products were visualized with ethidium bromide (1 μl per 100 ml of Agarose) in 1% Agarose gel with ultraviolet light.

Average number of nuclei per hyphal cell

To determine the average number of nuclei per cell of each isolate, a 5-mm-diameter mycelial plug of each was incubated on water agar-coated slides (Martin and Lucas, 1984). After 48 h, the mycelia plug was removed using a sterile scalpel and 500 μl of diluted fluorescent DNA-binding probe 4', 6'-diamidino-2-phenylindole (DAPI) (2 μl DAPI + 498 μl McIlvaine buffer) was added to the hyphal growth on the water agar and incubated for 2 minutes at room temperature. The slide was then washed with 3 ml of McIlvaine buffer to remove the staining solution. The slides were subsequently mounted with one drop of the fluorescent enhancer, Vectarshield (Vector Cat. No. H-1000), covered with a thin glass cover slip (24 mm × 30 mm Fisher Co.) and viewed under a Zeiss fluorescent microscope (Axioplan2 imaging v. 4.6/ x-cite series 120). The number of the fluorescently-labeled nuclei per cell was counted from twenty randomly selected hyphal cells per isolate.

Mycelial growth rates and virulence

Mycelial plugs (5-mm diameter) from the margins of 3-day-old cultures of each of the thirty-six AG 2-2 LP isolates were removed and placed on 10 ml PDA^{++} contained in 9-cm-diameter Petri dishes. The two reference isolates were also included. Three replicated plates of

each isolate were incubated in the dark at 5, 10, 15, 20, 23, 25, 28, 30, and 35°C. Colony diameters were measured and expressed as the average diameter along two pre-drawn perpendicular lines on the underside of each Petri dish at 24 h-intervals until at least one isolate completely filled the plate. The areas under the mycelia growth curve (AUMGC) for the colony diameter of each isolate after 72 h of incubation at the nine different temperatures was calculated as $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where y_i is the colony diameter at time t_i, and t_i is the time of the ith rating (Madden *et al*., 2007).

An assay was subsequently conducted to determine the relationship between in vitro mycelia growth rate and virulence. Stolons of the zoysiagrass cultivar 'Meyer' were collected from the edges of an established field plot at the Rocky Ford Turfgrass Research Station in Manhattan, KS. The stolons were rinsed under tap water to remove soil debris, surface sterilized with 0.5% NaOCl for 3 min, and rinsed in two changes of tap water. Prepared stolons were then propagated in potting media (Metro Mix 510, SUN GRO., Washington) contained in 5 × 5 cm plastic pots and kept under an intermittent mist system in the greenhouse at 25°C for 8 weeks. Pots with stolon sections containing 3 to 10 shoots were removed from the mist chamber and maintained at 28°C and a 16 h-photoperiod for an additional 12 weeks. Pots were then inoculated with eight to ten oat kernels infested with isolate ACC1, CGC F18(D), MCC3, or LP2, four isolates with varying in vitro growth rates, or with non-infested sterile oat kernels placed on the soil surface in each pot. The infested oat kernels of the four isolates were prepared following the method described by Tisserat *et al*. (1989). Oat kernels were autoclaved twice for 20 min at 121°C. When cool, 6-8 plugs of PDA^{++} cultures of each isolate were added. The cultures were maintained for two weeks at 23°C with intermittent shaking of the jars to mix the mycelial

growth with the oat kernels. The infested oat kernels were used for the inoculation procedure without drying.

The inoculated pots of each isolate were then arranged in plastic trays filled with water to a depth of 2.5 cm. Each tray was covered with clear plastic to maintain a high relative humidity and maintained at 25°C and a 13 h-photoperiod in a Conviron ATC60 growth chamber (Conviron, Pembina, ND).

After five days, and at 5-day intervals thereafter, three replicate pots were removed from each tray and assessed by determining the percentage of individual shoots in each pot with distinct, water-soaked brown lesions on the leaf sheath (Green *et al*. 1993). To verify the causal pathogen of observed lesions, representative samples of infected tissues from each pot were surface-sterilized and incubated on 1/4 PDA^{++} at 25°C for re-isolation of *R. solani*. After 25 days, the last three pots in each tray were rated for disease incidence. The area under the disease progress curve (AUDPC) was calculated using the formula $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where y_i is the amount of disease incidence, and t_i is the time of the *i*th rating (Madden *et al*., 2007).

AFLP analysis of genomic DNA of isolates

Isolation of genomic DNA

Genomic DNA was isolated from 23 *R. solani* AG 2-2 LP isolates using a modified method of Jiangfeng *et al*. (2005). Isolates were grown from 5 mm-diameter potato dextrose agar-mycelial discs at 25°C for 5 days in the dark in complete media (modified from Correll *et al*. (1987) and containing per liter of distilled H_2O: sucrose, 30 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $NaNO_3$, 2 g; N-Z amine A (casein), 2.5 g; yeast extract (Difco), 1.0 g; 10 ml vitamin solution (contained per liter of 50% ethanol: thiamine HCl, 100 mg; riboflavin, 30 mg; pyridoxine HCl, 75 mg; D-pantothenate Ca, 200 mg; *p*-aminobenzoic acid, 5 mg; nicotinamide,

75 mg; choline Cl, 200 mg; folic acid, 5 mg; D-biotin, 5 mg; and *myo*-inositol, 4 g); 0.2 ml trace element solution. The trace element solution contained (per 95 ml of distilled H_2O) – citric acid, 5 g; $ZnSO_4$. $7H_2O$, 5 g; $FeNH_4)(SO_4)_2$. $6H_2O$, 1 g; $CuSO_4$. $5H_2O$, 0.25 g; $MnSO_4$. H_2O, 50 mg; H_3BO_4, 50 mg; and $NaMoO_4$. $2H_2O$, 50 mg. After incubation, mycelia were collected by filtration in a 16.5 cm diameter filter paper (KenAG Non Gauze milk filter) and ground into fine powder in pre-chilled mortars and pestles with liquid nitrogen.

The powder was transferred into a 1.5 ml micro-centrifuge tube and filled to the 0.5 ml mark. Next, 700 μl of 65°C 2% cetyltrimethylammonium (CTAB) solution and 7 μl of 2-mercaptoethanol were added to each tube. The tubes were vortexed briefly (2-3 sec) to disperse any clumps of mycelia, placed in a 65°C incubator for 10 min, briefly (2-3 sec) vortexed again to further homogenize the mixtures in the tubes, and returned back into the 65°C incubator for an additional 20 min. At the end of the incubation periods, 350 μl of chloroform:iso-amyl alcohol (24:1 v/v) was added to the tubes and vortexed briefly to thoroughly mix the aqueous and organic phases that formed in the tubes. The tubes were mixed gently by hand for an additional 5 minutes then centrifuged at 13,500 × g for 6 min to separate the organic and aqueous phases. 600 μl of the aqueous (upper) phase was transferred into a fresh, sterile 1.5 ml micro-centrifuge tube without disturbing the middle layer of cellular debris that had formed between the two layers. 600 μl of cold iso-propanol (2-propanol) was next added to the recovered aqueous phase in the new tubes and the mixture inverted several times to thoroughly mix and subsequently set aside for approximately 5 min at room temperature to allow nucleic acid precipitation in each tube. The tubes were then centrifuged for 5 min at 10,300 × g to pellet the nucleic acids. The aqueous/alcoholic mixture in each tube was thereafter decanted from the pellets and allowed to

air-dry for about 5 min. 600 µl of TE buffer was added to the crude pellets in each tube to begin to resuspend them overnight at 4°C.

Once the samples were completely resuspended, 300 µl of phenol:chloroform:iso-amyl-alcohol (25:24:1 v/v/v) was added to each tube, the mixture was vortexed briefly (2-3 sec), and then gently by hand for an additional 3 min to thoroughly mix the aqueous and organic phases that formed in the tubes. The tubes were again centrifuged for 5 min at 13,500 × g to separate the organic and aqueous phases. 500 µl of the aqueous (upper) phase was transferred into a fresh, sterile 1.5 ml micro-centrifuge tube without disturbing the layer of denatured protein that formed between the two layers. 1.0 µl of RNAse A was added into each tube, vortexed briefly to fully disperse the RNAse, and incubated at 37°C for 30 min. 500 µl of cold iso-propanol (2-propanol) was added to each tube and the mixture thoroughly mixed by inverting the tubes several times and then set aside for about 5 min to again allow nucleic acid precipitation in each tube. The tubes were centrifuged for 5 min at 10,300 × g to pellet the DNA, the supernatant was removed and the pellets were allowed to air-dry for 3 min. DNA was washed by adding 1 ml of cold 70% (v/v) ethanol into each tube, decanting the ethanol, and allowing the tubes to air-dry for 3 min. After a second wash, the tubes (with lids opened) were placed in a 65°C incubator for 5 min to evaporate the remaining ethanol. After drying, 50 µl of TE buffer was added to the pellets in each tube and resuspended overnight.

Digestion and ligation

Approximately 200 ng from the extracted DNA of each *Rhizoctonia* isolate was digested overnight at room temperature with the restriction enzymes *Eco*RI and *Mse*I, and ligated to 5 µM *Eco*RI and 50 µM *Mse*I adapters with T4 DNA ligase (6 units/µl, New England Biolabs) in a combined reaction step.

Pre-amplification

Following incubation, the digestion/ligation reaction product was diluted 1:9 with double distilled water (ddH_2O). Pre-amplification of the diluted DNA was carried out in a total reaction volume of 20 µl that included; 10x AFLP buffer, 25mM dNTP, Taq DNA polymerase, 50 ng/ µl *Eco*RI pre-amplification primer (5'-CTC GTA GAC TGC GTA CCA ATT C-3'), and *Mse*I pre-amplification primer (5'-GAC GAT GAG TCC TGA GTA A-3'). The pre-amplification reaction was programmed to run at 94°C for 60 s, followed by 20 cycles of 56°C for 30s, 56°C for 60 s, and 72°C for 60 s. The reaction products were stored at -20°C.

First and second selective amplifications

The first selective amplification step involved the use of the non-labeled *Eco*RI selective primer *E*-CC and a non-labeled *Mse*I primer, *M*-CC. The second selective amplification used the 5'-/56-FAM-labeled *Eco*RI selective primer with a single selective-nucleotide extension *E*-C and the non-labeled *Mse*I primer, *M*-CC. These primer pairs were based on preliminary studies using 8 isolates to optimize the number and reproducibility of generated alleles.

The first selective amplification consisted of 4 µl of diluted (1:19) pre-amplification product, 2.0 µl 10x AFLP buffer, 0.5 µl dNTP, 0.25 µl Taq polymerase, 1.0 µl each of non-labeled *Eco*RI and *Mse*I selective primers, and 11.25 µl of ddH_2O to make a final volume of 20.0 µl. The second selective amplification consisted of 2.0 µl of diluted (1:10,000) first selective amplification product, 2.0 µl 10x AFLP buffer, 0.5 µl dNTP, 0.25 µl Taq polymerase, 2.0 µl each of labeled *Eco*RI and non-labeled *Mse*I primers, and 11.25 µl ddH_2O to make a final reaction volume of 20.0 µl. Both the first and second selective amplification reactions were programmed on an MJ Research PTC-100 Peltier DNA thermal cycler to run at 94°C for 30 sec, 13 touchdown cycles (-0.7°C/cycle) at 65°C for 30 sec, 72°C for 1 min, 94°C for 30 sec, and a final 30 sec at

94°C followed by 23 cycles at 56°C for 30 sec, 72°C for 1 min, and 94°C for 30, then a single cycle at 56°C of 30 sec, and 72°C for 5 min.

Preparation of DNA samples for analysis

The products of the second selective amplification step were diluted (2:18) with distilled water, vortexed, and centrifuged. From each sample, 2 µl was taken and transferred into PCR plate reaction wells. Ten microliters of labeled size-standard (GeneScan 500 LIZ Applied Biosystems, #4322682) containing 0.15 µl of LIZ and 9.85 µl Hi-Di Formamide (Applied Biosystems) was added into each reaction well. The sample mixtures were then denatured at 95°C for 5 min, placed on ice for another 5 min, and then centrifuged. Analysis of the samples was performed on an ABI 3730 sequencer (Applied Biosystems). The AFLP study was repeated three times.

Cluster analysis of isolates

Cluster dendrogram of isolates based on the AFLP-generated alleles were constructed in "R" statistical program using the hierarchical clustering (function hclust) method available in standard R (R Development Core Team, 2011). Class designation of isolates within the dendrogram was done using the "Vegan" function "cascadeKM".

Data analysis

Statistical analysis of data was performed with Minitab version 16 (Minitab Inc., Pennsylvania) statistical software. Mean comparisons were performed using Tukey's family error rate at *P*-value of 0.05. Assessment of the relationships between the average number of nuclei per cell and colony diameter were carried out using Pearson's product moment correlation coefficient and the regression analysis function in Minitab 16.

RESULTS

In vitro phenotypic characteristics, anastomosis grouping of isolates, and PCR identification

Phenotypic characteristics of the isolates in culture on PDA^{++} included brown-colored mycelia with dark-brown main runner hyphae, as well as a lack of zonation and absence of distinct sclerotia, consistent with the descriptions of isolates from zoysiagrass reported by Hyakumachi *et al*. (1998). Pairings with the AG 2-2 LP reference isolate LP 17Li revealed that all the isolates obtained from large patch-infected zoysiagrass obtained from Kansas and Missouri belonged to AG 2 (Table 1, Figure 1), with an average fusion frequency of 65%. Agarose gel analysis of the PCR products from the use of the AG 2-2 LP-specific primer, P22-LP, showed each zoysiagrass isolate's DNA yielded the expected product size of around 400 base pairs (bp) (Figure 2) from the use of the AG 2-2 LP-specific primer P22-LP. DNA from tester isolates belonging to groups other than AG 2-2 LP yielded no fragments.

Average number of nuclei per hyphal cell

All the *R. solani* isolates were multinucleate. Significant variations in the average number of nuclei per cell were observed among individual isolates, and ranged from 6.0 in isolate RF4 to 13 in isolate LP2 (Table 1).

Mycelial growth rates and virulence

Optimum in vitro radial growth of most of the zoysiagrass isolates occurred at 25°C with 52.3% of all isolates having a minimum growth of 5 cm after 72 h of incubation, compared with 40.9% and 50% at 23°C and 28°C respectively (data not shown). For most isolates, there was greatly reduced growth at 5 or 35 °C (Table 2).

Zoysiagrass inoculated with either of the two isolates (CGC F18(D) and ACC 1) with higher in vitro growth rates developed symptoms more quickly than zoysiagrass inoculated with either of the two isolates (MCC3 and LP2) with lower in vitro growth rates (Figure 3). In addition, the overall disease severity as measured by AUDPC was higher for the two isolates with faster in vitro growth (Figure 3).

Relationship between nuclear number and mycelial growth of isolates

There was a significant negative correlation ($P < 0.05$, $r = -0.53$) between average number of nuclei per cell and in vitro mycelia growth rate (Figure 4).

AFLP analysis of genomic DNA of isolates

Digestion of genomic DNA from 23 isolates of *R. solani* yielded 37 to 95 alleles. The result of the hierarchical clustering of the AFLP-generated alleles of representative isolates from the different locations in Kansas, as well as of the reference isolates of *R. solani* belonging to AG's 1, 2-1A, 2-2 IIIB, and 3 is shown in Figure 5. Isolates grouped into six classes consistent with the known AG designation (Table 1) of each isolate. However, five large patch isolates – Rh 146, TPK F9, RF6, ACC2, and ACC3 formed two separate classes distinct from the other AG 2-2 LP isolates (Figure 5).

DISCUSSION

All the *R. solani*-large patch isolates of zoysiagrass evaluated in the current study by the methods of anastomosis, cultural characteristics, and by PCR with specific primers were identified as belonging to AG 2-2 LP. This finding is consistent with the designation by Hyakumachi *et al.* (1998) of *R. solani* AG 2-2 isolates of warm-season turfgrasses. Furthermore, mycelial growth of the isolates was optimal at 25°C, consistent with studies of isolates from zoysiagrass by Green *et al.* (1993) and Hyakumachi *et al.* (1998).

Significant variations were observed in the average number of nuclei per cell of the isolates, including among isolates from within the same location, indicating heterogeneity within sites. We also found that the average number of nuclei per cell was negatively correlated with in vitro mycelia growth rates. Additionally, mycelia growth rate correlated positively with virulence in the four representative isolates evaluated. The biological significance of this is unknown. Further study of more isolates may explain the relationship between nuclear number, mycelial growth rate, and virulence in the field.

The AG 2-2 LP isolates formed a distinct class separate from those of the four other AG's evaluated. The LP 17Li isolate, which represented a separate class among the AG 2-2 LP isolates in the cluster dendrogram, is an AG 2-2 LP-reference isolate originally from St. Augustinegrass from South Carolina. Isolates from the same site did not always cluster together, indicating that there may be different clones within the same golf course. Different mycelial growth rates were also measured in isolates from the same location. In the field, some patches expand or recover more quickly than others which may be due to microclimate but perhaps also inherent isolate differences.

In the current study, genetic variation among our AG 2-2 LP isolates was evaluated by the method of AFLP because it is considered effective for investigating closely related plant pathogen species and subspecies, and has several advantages over RAPD and RFLP (Majer *et al*., 1996). We generated reliable AFLP protocols for fingerprinting and comparison of our isolates which suggest the existence of subtle genetic differences among our AG 2-2 LP isolates. These protocols can be utilized for larger scale population genetic studies to investigate questions about diversity within and between sites, mechanisms of disease spread (within and between sites), diversity among hosts (e.g., zoysiagrass vs bermudagrass isolates), and other ecological and epidemiological questions.

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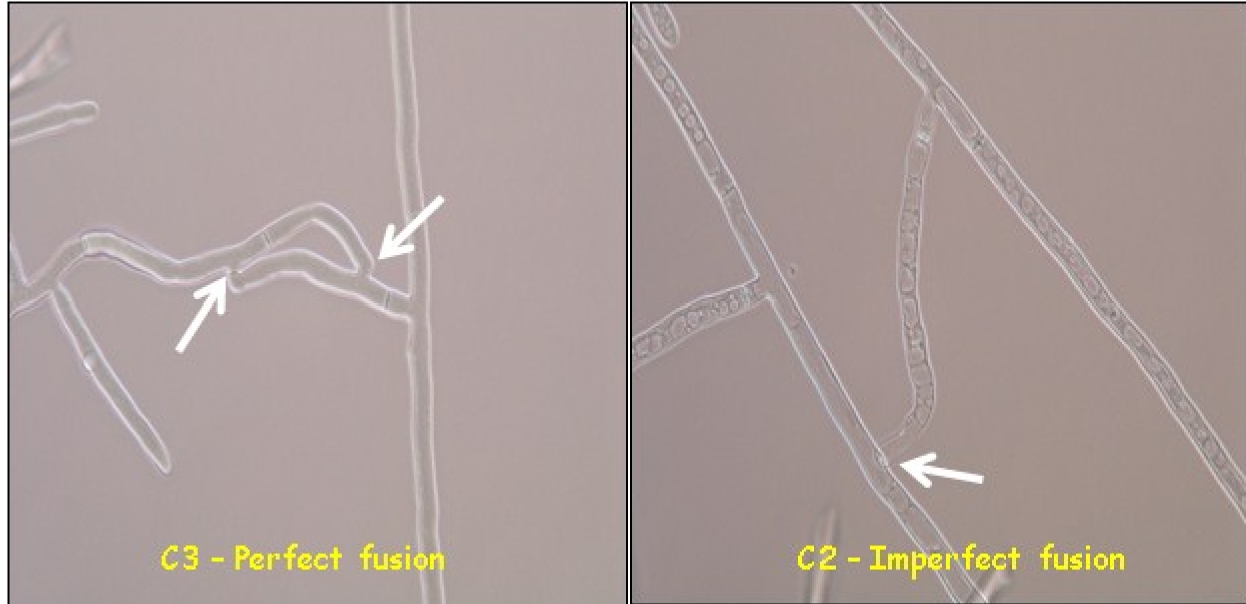

Figure 2.1 Light microscopy images displaying C3 (perfect fusion) and C2 (imperfect fusion) between two paired isolates of *R. solani* on a water agar-coated glass slide. The perfect fusion reaction (arrows) involved fusion of cell wall and cytoplasm of the paired isolates, while an imperfect fusion (arrow) involved cell wall fusion followed by death of the anastomosing cells and their adjacent cells. Microscopic examinations were made at 400x.

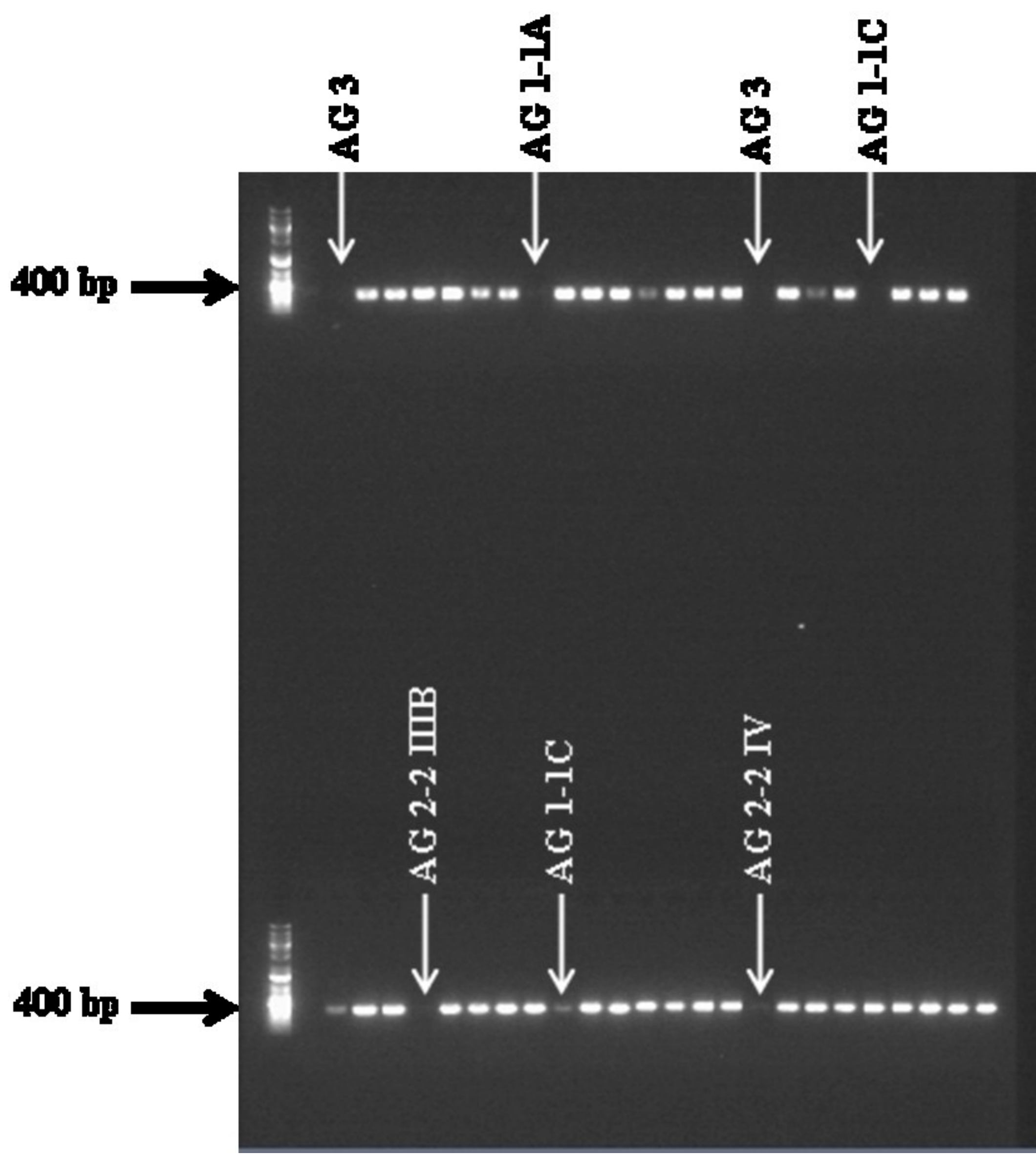

Figure 2.2 Agarose gel electrophoresis of the PCR products of 43 *R. solani* isolates using the AG 2-2 LP-specific primer, P22-LP. All 36 isolates from infected zoysiagrass in Kansas and Missouri used in this study had the expected band with a size of approximately 400 bp. Seven known tester isolates belonging to other AG's and AG 2-2 subgroups did not have the expected band.

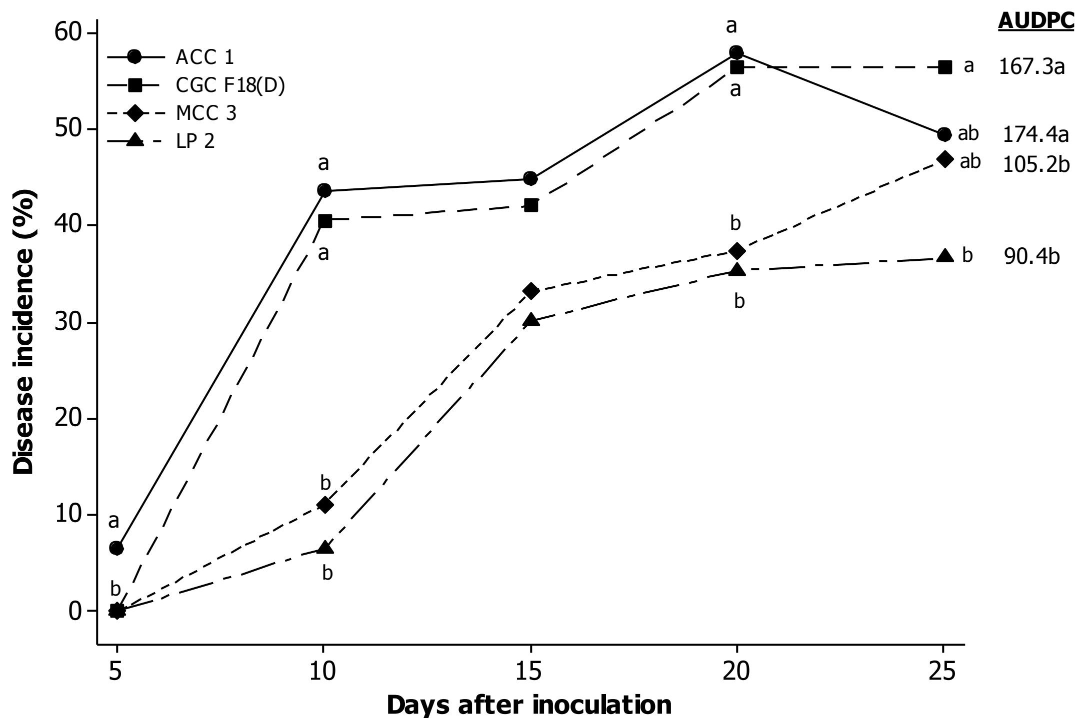

Figure 2.3 Comparison of the virulence of four *R. solani* AG 2-2 LP isolates on Meyer zoysiagrass. Isolates CGC F18(D) (circles) and ACC1 (triangles) had higher in vitro growth rates than isolates LP 2 (diamonds) and MCC 3 (squares). Three pots per isolate were inoculated with colonized oat kernels. Inoculated plants were maintained at 25°C, a 16 h-photoperiod, and high relative humidity under growth chamber conditions. Disease incidence was rated every 5 days and expressed as a percentage of individual shoots showing water-soaked sheath blighting symptom averaged across three replicated pots destructively sampled at each time point. Area under disease progress curve (AUDPC) was calculated as $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where y_i is the amount of disease (incidence), and t_i is the time of the *i*th rating. Points followed by the same letters are not statistically different (*P*=0.05). Values represent the area under the disease progress curve (AUDPC).

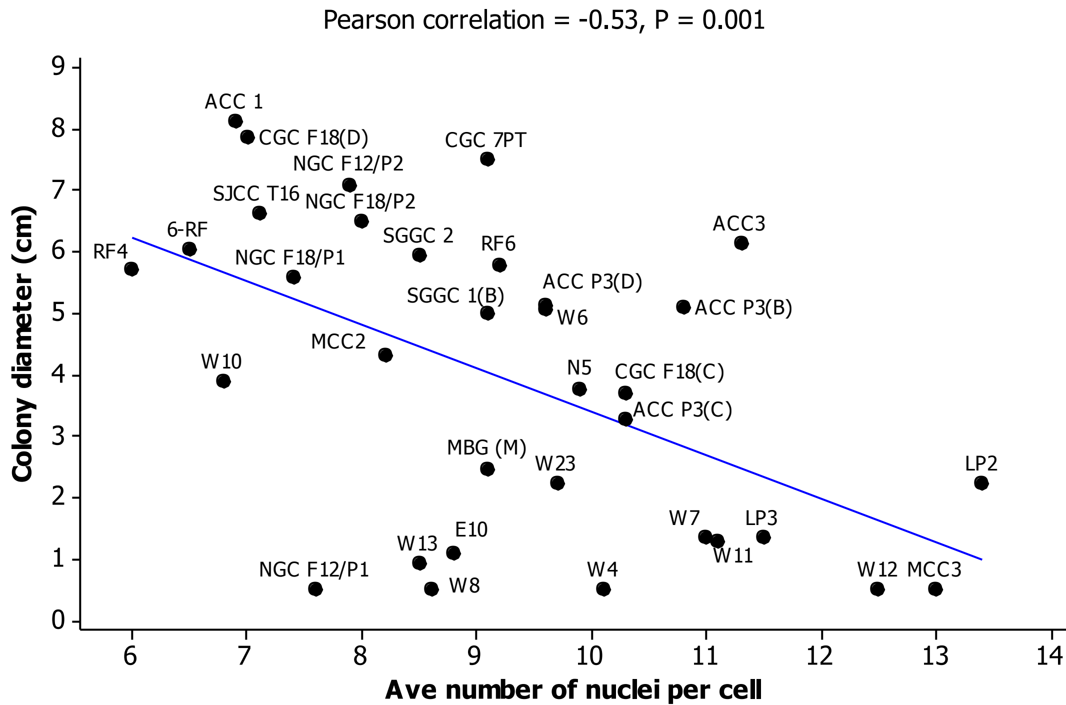

Figure 2.4 Scatter plot showing the relationship between the colony diameter and the average numbers of nuclei per cell of *R. solani* AG 2-2 LP isolates from Kansas and Missouri. Each point represents the average of three replicates per isolate. The colony diameter is the average mycelia growth after 72 h incubation at 25°C. The average number of nuclei is for twenty randomly selected hyphal cells per isolate.

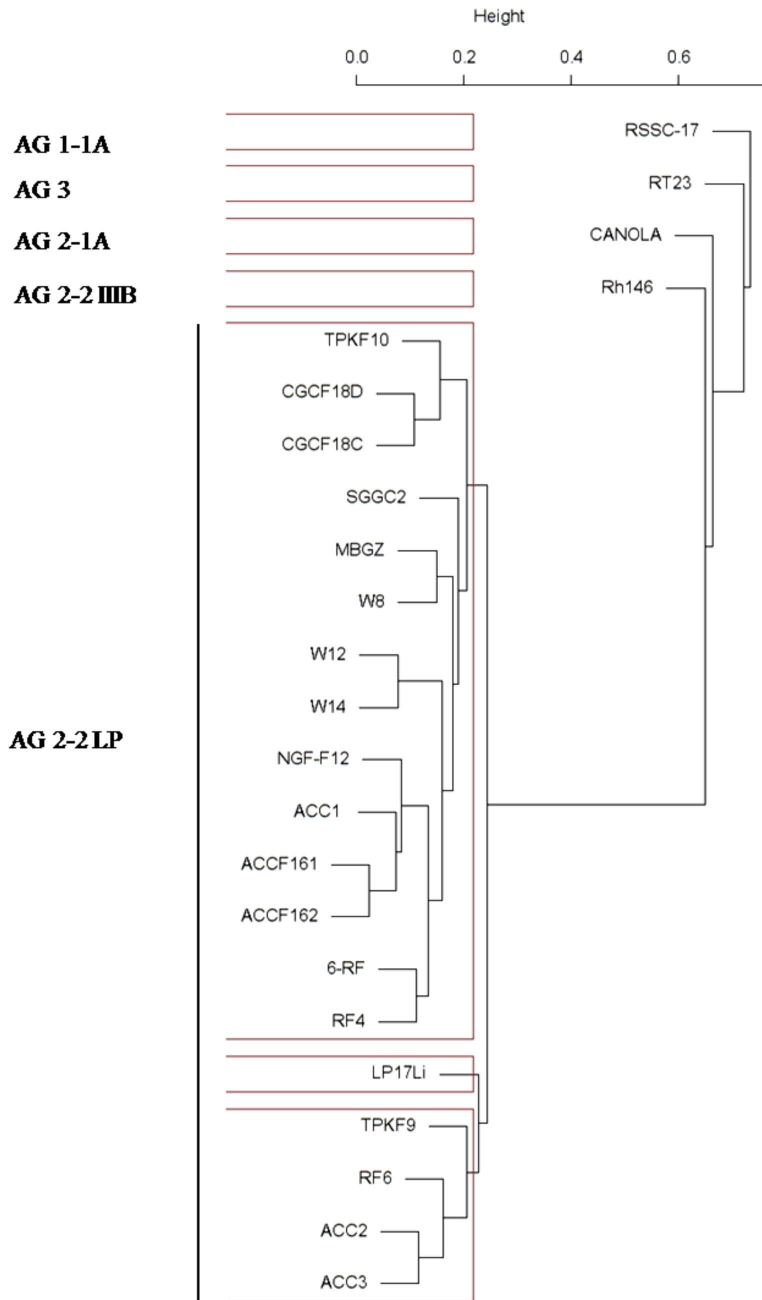

Figure 2.5 Cluster dendrogram of 23 *R. solani* isolates belonging to AG 2-2 LP including one each of AG 1, 2-2 IIIB, 2-1A, and 3. Clustering was based on reproducible AFLP-ECC/MCC primer markers generated per isolate, from three replicated experiments, at the 300 rfu cut-off using GeneMapper. Cluster dendrogram was constructed in "R" statistical program using the hierarchical clustering function "hclust", and class designation was done using the "Vegan" function "cascadeKM".

Table 2.1 Anastomosis group of *Rhizoctonia solani* isolates from zoysiagrass, average number of nuclei per cell, and colony diameter after incubation at 25°C for 72 h on PDA.

Isolate[z]	Original host	Site[y]	AG	Sub-group	Average[x] nuclei/cell	Colony[w] diameter (cm)
TPK F9	Zoysia	TPK	2	2-2 LP	-	-
TPK F10	Zoysia	TPK	2	2-2 LP	-	-
MCC3	Zoysia	MCC	2	2-2 LP	13.0ab	0.50p
W4	Zoysia	MCC	2	2-2 LP	10.1d-i	0.50p
W8	Zoysia	MCC	2	2-2 LP	8.6i-n	0.50p
W12	Zoysia	MCC	2	2-2 LP	12.5abc	0.50p
NGC F12/P1	Zoysia	NGC	2	2-2 LP	7.6l-r	0.50p
W13	Zoysia	MCC	2	2-2 LP	8.5i-o	0.93op
E10	Zoysia	MCC	2	2-2 LP	8.8h-m	1.10o
W11	Zoysia	MCC	2	2-2 LP	11.1c-f	1.28o
W7	Zoysia	MCC	2	2-2 LP	11.0c-f	1.33o
LP3	Zoysia	-	2	2-2 LP	11.5bcd	1.35o
LP2	Zoysia	-	2	2-2 LP	13.4a	2.23n
W23	Zoysia	MCC	2	2-2 LP	9.7e-j	2.23n
MBG(M)	Zoysia	MBG	2	2-2 LP	9.1h-l	2.45n
ACC P3(C)	Zoysia	ACC	2	2-2 LP	10.3d-h	3.25m
CGC F18(C)	Zoysia	ACC	2	2-2 LP	10.3d-h	3.70lm
N5	Zoysia	MCC	2	2-2 LP	9.9d-i	3.77l
W10	Zoysia	MCC	2	2-2 LP	6.8pqr	3.88kl
MCC2	Zoysia	MCC	2	2-2 LP	8.2j-p	4.32k
SGGC 1(B)	Zoysia	SGGC	2	2-2 LP	9.1h-l	4.98j
W6	Zoysia	MCC	2	2-2 LP	9.6f-k	5.05j
ACC P3(B)	Zoysia	ACC	2	2-2 LP	10.8d-g	5.08j
ACC P3(D)	Zoysia	ACC	2	2-2 LP	9.6f-k	5.13ij
NGC F18/P1	Zoysia	NGC	2	2-2 LP	7.4m-r	5.58hi
RF4	Zoysia	RF	2	2-2 LP	6.0r	5.72gh
RF6	Zoysia	RF	2	2-2 LP	9.2g-l	5.78fgh
LP 17Li	St. Augustinegrass	-	2	2-2 LP	-	5.80fgh
SGGC 2	Zoysia	SGGC	2	2-2 LP	8.5i-o	5.92fgh
6-RF	Zoysia	RF	2	2-2 LP	6.5qr	6.03efgh
ACC3	Zoysia	ACC	2	2-2 LP	11.3cde	6.12efg
Rh 146	Bentgrass	-	2	2-2 IIIB	-	6.20def
NGC F18/P2	Zoysia	NGC	2	2-2 LP	8.0k-q	6.48de
SJCC T16	Zoysia	SJCC	2	2-2 LP	7.1n-r	6.60d
NGC F12/P2	Zoysia	NGC	2	2-2 LP	7.9l-q	7.08c
CGC 7PT	Zoysia	ACC	2	2-2 LP	9.1h-l	7.50bc
CGC F18(D)	Zoysia	ACC	2	2-2 LP	7.0n-r	7.87ab
ACC1	Zoysia	ACC	2	2-2 LP	6.9o-r	8.10a

[z]Isolates LP 17Li and Rh 146 are standard tester isolates

[y]MCC-Manhattan country club, ACC-Alvamar country club, RF- Rocky ford, SGGC-Shadow Glen Golf Club, MBG-Meadowbrook Golf Club, NGC-Nicklaus golf club, SJCC- St. Joseph country club, TPK- Topeka.

[x]Values represent the average number of nuclei in twenty randomly selected hyphal cells per isolate. Values followed by the same letters are not statistically different (P=0.05).

[w]Values represent the average colony diameter of three replicate plates. Values followed by the same letters are not statistically different (P=0.05).

Table 2.2 Colony diameter of 34 *Rhizoctonia solani* AG 2-2 LP isolates from Kansas and Missouri, averaged by location, after incubation at different temperatures for 72 h.

	Colony diameter* (cm)									
	Temperature (C)									
Isolate	5	10	15	20	23	25	28	30	35	AUMGC**
W12	0.53(0.04)	0.76(0.11)	0.66(0.01)	0.54(0.07)	0.57(0.01)	0.50(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	3.29z
W8	0.50(0.00)	0.68(0.06)	0.64(0.04)	0.50(0.00)	0.50(0.00)	0.50(0.00)	0.55(0.09)	0.53(0.04)	0.50(0.00)	4.40y
W13	0.00(0.00)	0.00(0.00)	0.00(0.00)	1.03(0.15)	1.37(0.38)	0.93(0.34)	0.64(0.08)	0.88(0.12)	0.52(0.03)	5.11xy
W4	0.50(0.00)	1.42(0.25)	1.11(0.15)	0.77(0.03)	0.50(0.00)	0.50(0.00)	0.50(0.00)	0.50(0.00)	0.50(0.00)	5.79x
NGF F12/P1	0.54(0.01)	1.73(0.16)	2.13(0.11)	0.60(0.09)	0.50(0.00)	0.50(0.00)	0.53(0.06)	0.50(0.00)	0.85(0.15)	7.19w
W7	0.50(0.00)	0.73(0.13)	0.94(0.10)	1.58(0.18)	1.88(0.10)	1.33(0.38)	0.50(0.00)	0.50(0.00)	0.50(0.00)	7.97vw
LP3	0.50(0.00)	1.48(0.18)	1.82(0.19)	1.33(0.25)	0.86(0.06)	1.35(0.25)	0.58(0.09)	0.53(0.06)	0.59(0.06)	8.49v
E10	0.50(0.00)	1.80(0.13)	1.41(0.38)	2.83(0.10)	2.02(0.23)	1.10(0.22)	0.50(0.00)	0.50(0.00)	0.50(0.00)	10.66u
W9	0.50(0.00)	1.06(0.09)	1.47(0.28)	2.85(0.13)	2.50(0.36)	1.58(0.15)	0.52(0.03)	0.53(0.04)	0.50(0.00)	10.99tu
LP4	0.57(0.03)	1.58(0.38)	1.47(0.18)	2.58(0.35)	1.78(0.19)	1.62(0.58)	0.98(0.21)	0.83(0.03)	1.08(0.14)	11.68stu
W11	0.50(0.00)	0.86(0.13)	0.98(0.15)	2.35(0.49)	1.73(0.28)	1.28(0.15)	2.02(0.13)	1.77(0.08)	1.70(0.26)	12.09st
MCC3	0.59(0.01)	1.24(0.12)	0.89(0.16)	0.50(0.00)	0.50(0.00)	0.50(0.00)	3.56(0.16)	3.63(0.08)	2.68(0.57)	12.47rs
MBG(M)	0.50(0.00)	0.88(0.06)	1.46(0.18)	2.15(0.53)	2.67(0.18)	2.47(0.06)	1.88(0.54)	1.42(0.45)	0.73(0.12)	13.54qr
LP2	0.74(0.01)	2.13(0.13)	2.23(0.24)	2.82(0.41)	2.75(0.22)	2.23(0.20)	0.50(0.00)	0.50(0.00)	0.50(0.00)	13.79pq
W23	0.59(0.07)	1.53(0.35)	2.53(0.23)	3.32(0.10)	3.67(0.08)	2.23(0.10)	0.50(0.00)	0.50(0.00)	0.50(0.00)	14.82p
W10	0.64(0.01)	1.58(0.21)	3.03(0.49)	2.95(0.13)	3.62(0.08)	3.88(0.16)	1.50(0.00)	1.40(0.09)	1.13(0.10)	18.86o
N5	0.62(0.05)	1.43(0.16)	1.63(0.28)	2.75(0.35)	3.07(0.31)	3.77(0.19)	2.98(0.21)	2.28(0.24)	1.55(0.10)	18.99o
CGC F18(C)	0.50(0.00)	1.18(0.13)	1.55(0.09)	2.63(0.20)	3.23(0.28)	3.70(0.35)	4.03(0.16)	3.88(0.31)	1.92(0.06)	21.43n
ACC P3(C)	0.50(0.00)	1.60(0.33)	1.28(0.22)	2.92(0.51)	3.83(0.58)	3.25(0.53)	3.97(0.08)	3.77(0.32)	2.12(0.10)	21.92n
W14	0.91(0.13)	1.65(0.54)	2.43(0.11)	2.62(0.25)	3.02(0.36)	4.62(0.20)	3.05(0.09)	3.60(0.10)	1.32(0.10)	22.09n
W6	0.60(0.05)	2.07(0.06)	1.64(0.18)	2.93(0.14)	3.83(0.59)	5.05(0.22)	4.02(0.42)	4.20(0.22)	1.92(0.15)	25.00m
ACC P3(A)	0.64(0.06)	2.12(0.20)	2.63(0.14)	3.30(0.13)	4.35(0.15)	4.53(0.21)	4.92(0.19)	4.47(0.19)	2.27(0.18)	27.76l
NGC F18/P1	0.65(0.05)	1.88(0.08)	3.00(0.27)	2.93(0.03)	3.82(0.06)	5.58(0.25)	4.77(0.06)	4.75(0.43)	1.55(0.17)	27.84kl
3-RF	0.51(0.01)	2.17(0.03)	2.78(0.08)	3.45(0.27)	4.43(0.34)	4.15(0.18)	4.81(0.21)	4.85(0.33)	2.33(0.33)	28.06jkl
ACC P3(B)	0.59(0.01)	2.00(0.10)	2.71(0.09)	3.68(0.21)	5.18(0.28)	5.08(0.19)	4.92(0.29)	4.12(0.20)	1.88(0.08)	28.93ijk
ACC P3(D)	0.58(0.08)	2.25(0.30)	2.82(0.20)	3.42(0.14)	4.88(0.20)	5.13(0.47)	4.68(0.73)	4.65(0.18)	1.99(0.09)	29.12hij
SGGC2	0.59(0.08)	1.92(0.12)	2.47(0.35)	3.27(0.12)	3.99(0.06)	5.92(0.28)	5.60(0.18)	5.47(0.28)	2.07(0.10)	29.95ghi
Rh 146	0.65(0.05)	2.15(0.05)	2.62(0.06)	2.88(0.10)	4.07(0.28)	6.20(0.13)	5.10(0.10)	5.45(0.63)	2.57(1.03)	30.08gh
NGC F18/P2	0.53(0.04)	2.03(0.10)	3.28(0.07)	3.63(0.23)	5.10(0.15)	6.48(0.32)	4.77(0.33)	4.03(0.03)	1.27(0.08)	30.23fg
6-RF	0.65(0.05)	2.23(0.15)	2.42(0.35)	3.78(0.20)	4.62(0.25)	6.03(0.97)	5.50(0.29)	4.77(0.13)	1.93(0.15)	30.64efg
9-RF	0.58(0.00)	2.33(0.13)	3.02(0.12)	3.53(0.18)	4.73(0.32)	6.28(0.10)	4.98(0.08)	4.75(0.44)	1.80(0.18)	30.82efg
CGC F7	0.68(0.03)	2.30(0.10)	2.98(0.08)	3.75(0.23)	5.17(0.14)	6.78(0.34)	4.55(0.57)	4.55(0.18)	1.77(0.08)	31.31def
SGGC1(B)	0.73(0.04)	2.00(0.00)	2.50(0.10)	3.62(0.13)	4.48(0.15)	4.98(0.13)	6.22(0.16)	6.15(0.22)	2.13(0.08)	31.38de
LP 17Li	0.68(0.04)	2.08(0.10)	2.82(0.10)	3.68(0.18)	5.08(0.31)	5.80(0.15)	5.77(0.25)	5.52(0.36)	1.91(0.19)	32.04d
RF4	0.75(0.09)	2.46(0.09)	2.65(0.16)	3.75(0.09)	5.27(0.08)	5.72(0.20)	5.30(0.23)	5.57(0.18)	2.02(0.13)	32.09d
NGF F12/P2	0.63(0.12)	2.55(0.09)	3.32(0.03)	3.90(0.09)	5.18(0.21)	7.08(0.23)	4.53(0.19)	4.70(0.10)	1.27(0.13)	32.21d
11-RF	0.57(0.03)	1.88(0.15)	2.52(0.21)	3.88(0.03)	4.87(0.13)	6.57(0.10)	5.90(0.31)	5.70(0.00)	1.53(0.03)	32.37d
RF6	0.50(0.00)	1.97(0.21)	3.15(0.13)	3.85(0.05)	5.47(0.14)	5.78(0.13)	6.18(0.16)	6.08(0.13)	1.86(0.19)	33.66c
CGC 7PT	0.60(0.00)	2.20(0.13)	3.13(0.05)	4.12(0.13)	5.67(0.78)	7.50(0.09)	5.87(0.14)	4.90(0.23)	1.47(0.12)	34.42c
MBG(Z)	0.63(0.03)	2.30(0.18)	3.37(0.01)	4.08(0.43)	6.00(0.13)	7.67(0.16)	5.77(0.13)	4.28(0.26)	1.32(0.12)	34.44c
ACC2	0.73(0.04)	1.87(0.13)	3.47(0.42)	4.25(0.61)	6.15(0.30)	7.73(0.26)	4.83(0.08)	5.03(0.20)	1.80(0.13)	34.60c
SJCC T16	0.78(0.08)	2.90(0.09)	3.67(0.06)	4.55(0.49)	5.97(0.06)	6.60(0.46)	5.07(0.23)	4.83(0.41)	1.50(0.10)	34.73c
CGC F18(D)	0.68(0.08)	2.60(0.09)	3.48(0.11)	4.95(0.23)	6.63(0.10)	7.87(0.25)	5.00(0.30)	4.93(0.25)	1.47(0.18)	36.54b
ACC3	0.85(0.09)	3.20(0.31)	3.58(0.07)	4.70(0.18)	6.13(0.13)	6.12(0.13)	5.57(0.03)	6.03(0.23)	1.74(0.04)	36.62b
ACC1	0.68(0.15)	3.03(0.18)	3.83(0.03)	4.42(0.23)	6.68(0.40)	8.10(0.13)	6.22(0.23)	5.82(0.55)	1.83(0.21)	39.35a

*Values represent the average colony diameter of three replicate PDA plates per isolate per location for each temperature. Values in parenthesis represent standard deviations of the means.

**Area Under Mycelial Growth Curve (AUMGC). Calculated as $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where i= 1,2,3,…,n-1, y_i is the colony diameter, and t_i is temperature at the time of the ith rating. Values followed by the same letters are not statistically different (P=0.05).

Chapter 3 - Effect of cultivation and timing of nitrogen fertilization on large patch disease of zoysiagrass

Abstract

Large patch is a common disease of zoysiagrass (*Zoysia* spp.) and is caused by the fungus *Rhizoctonia solani* Kühn AG 2-2 LP. The effects of summer cultivation (core-aerification, verticutting and sand topdressing) and spring and fall versus summer nitrogen (N) fertilization on large patch symptoms were investigated from 2008 to 2011 at three sites: Manhattan, Haysville, and Olathe, Kansas. The effect of summer cultivation on thatch temperature, soil temperature, and soil water content was measured in Manhattan. Established stands of 'Meyer' zoysiagrass maintained at fairway height and inoculated with *R. solani* were used for the study. Disease was assessed by direct measurement of patch sizes or by analysis of digital images of affected plot areas to determine the percentage of damaged turfgrass within the patches. Thatch temperature, soil temperature, and soil water content were not significantly different between cultivated and non-cultivated plots. Summer cultivation did not result in consistent or significant reductions in patch sizes nor average weekly rate of patch size increase among the plots at the three experimental locations. Turf recovery from the disease in early summer was also not significantly different between the cultivated and non-cultivated plots. However, spring and fall N fertility was often associated with lower percentages of diseased turf within affected plot areas in Manhattan and Haysville as estimated by digital image analysis.

INTRODUCTION

Zoysiagrass (*Zoysia japonica* Steud. and *Z. matrella* (L.) Merr.) is a warm-season (C4), perennial turfgrass that is widely used on golf courses in the "transition-zone" of the United

States, a region that includes Kansas and states eastward to Virginia and North Carolina. Compared to certain cool-season (C3) turfgrass species such as creeping bentgrass (*Agrostis stolonifera* L.), zoysiagrass has lower water, fertilizer, and pesticide requirements for maintenance while maintaining a high-quality surface (Fry *et al*., 2008). Large patch, caused by *Rhizoctonia solani* anastomosis group AG 2-2 LP, is the most common and severe disease of zoysiagrass.

In Kansas, symptoms of large patch appear during spring (April-May) and autumn (Sept-Oct) as roughly circular light-brown to straw-colored patches with slightly matted areas of bright-orange discoloration that eventually fade to a tan or dull brown color with bright orange margins (Smiley *et al*., 2005; Tisserat *et al*., 1994; Green *et al*., 1993). Patches can range in size up to 6 meters or more in diameter (Tisserat *et al*., 1994; Green *et al*., 1993) with healthy turf sometimes scattered within the patches. Symptoms on individual plants occur as reddish-brown to black lesions on the basal leaf sheaths. Infection of the leaf sheath results in the girdling of shoots, which cuts off water and nutrient supplies to the upper parts of the plant and culminates in the bright orange discoloration characteristic of the disease (Tisserat *et al*., 1994). During summer conditions, regrowth within patches often results in full turf recovery though weeds can encroach while the turfgrass is thinned.

In laboratory tests, a temperature range of 15 to 25°C was found to be optimum for infection of zoysiagrass by the fungus (Green *et al*., 1993). It has been suggested that the lack of symptoms during summer months is because thatch and soil temperatures often exceed 30°C, potentially hindering growth of the fungus while at the same time favoring zoysiagrass root and shoot growth (Green *et al*., 1993). Large patch symptoms may, however, develop in shaded areas during unusually cool, moist summers (Green *et al*., 1993). Additionally, soil moisture and leaf

wetness are important factors in large patch development and severity. Severe large patch symptoms on zoysiagrass have been associated with compacted and poorly drained soils as well as with periods of excessive rain (Green *et al*., 1993). Brown patch, caused by the related fungus *R. solani* AG 2-2 IIIB, has also been reported to be more severe in poorly drained soils (Haygood *et al*., 1989; Piper and Oakley, 1921; Fidanza *et al*., 1996).

Currently, management of large patch is primarily by fungicide application, and there have been few studies of the effects of cultural practices on the disease. Green *et al*. (1994) studied the effects of mowing height, nitrogen (N) source, and N application rates on large patch development and severity on zoysiagrass. They found that lower mowing heights resulted in more severe disease. In addition, large patch was not affected by N source (urea, urea formaldehyde, poultry litter, sewage sludge, and bovine waste) or the two different application rates of 74 kg and 148 kg of N per hectare per year. They did not, however, study the effect of different fertilization timings. In an effort to promote faster emergence from winter dormancy some turfgrass managers practice early spring fertilization. Similarly, N fertility is applied by some during late fall to extend the duration of green color retention by zoysia, thereby delaying the onset of dormancy (Fry *et al*., 2008).

In the turfgrass industry, cultivation refers to aerification (punching solid or hollow cores in the soil to create holes) and verticutting (slicing into the turf canopy and roots with vertical blades). These practices lead to improved soil moisture and oxygen conditions, resulting in improved root growth as well as increased microbial activity that is essential to the biodegradation of thatch (Christians, 2004). On zoysiagrass turf, aerating and verticutting fairways when large patch is active has been anecdotally reported to result in the development of

new satellite infections on healthy areas of turf by infected cores (Spurlock and Milus, 2009), but the effects of cultivation in summer is not known.

The effect of timing of nitrogen (N) fertilization and cultivation on large patch development and severity in zoysiagrass is not known, although turfgrass managers have associated severe large patch outbreaks to excessive N fertilization (Green *et al*., 1994). Similarly, high N applications were also associated with increased susceptibility of cool-season turfgrasses to *Rhizoctonia* brown patch (Smiley *et al*., 1992; Cook *et al*., 1964). Furthermore, the influence of the interplay between cultivation and timing of fertilization, if any, on large patch remains to be determined. The objectives of this study were to (1) evaluate the effect of cultivation on soil moisture, thatch temperature, and soil temperatures; (2) evaluate the effects of core-aerification, verticutting, and sand topdressing on large patch development; and (3) evaluate the effect of timing of fertilization on large patch development.

MATERIALS AND METHODS

Pathogen isolation, storage, and inoculation

Rhizoctonia solani 2-2 LP isolates were recovered from large patch-infected zoysiagrass samples from Kansas. Leaf sheath sections measuring 1 to 2 cm with blight symptoms were removed from infected plants, surfaced-sterilized with 0.5% NaOCl for about 2 min, blotted dry, and placed in a 9-cm (diameter) Petri plate containing 6 g per liter potato dextrose agar amended with chloramphenicol (10 mg/L) and streptomycin (10 mg/L) (designated here as "1/4 PDA^{++}") (Biotech Research Grade, Fisher Scientific Inc., New Jersey). Cultures were maintained at 23°C in the dark. Identification of *R. solani* from hyphal-tipped cultures was based on hyphal characteristics, nuclear conditions (nuclear counts) (Martin, 1987), hyphal anastomosis with a known tester isolate belonging to the anastomosis group AG-2-2 LP on agar-coated glass slides (Martin and Lucas, 1984; Carling, 1996), and by polymerase chain reaction (PCR) using the AG 2-2 LP-specific primer P22-LP developed by Carling *et al*. (2002) (see Chapter 2). One large patch isolate was used to infest oat kernels as described by Tisserat *et al*. (1989). One hundred and fifty grams of oat kernels were mixed with 150 ml of distilled water in a glass jar and autoclaved twice at 121°C for 30 min. After cooling, several plugs from an actively growing *R. solani* AG 2-2 LP culture on ¼ PDA^{++} were placed into each jar. The glass jars containing the inoculated oat kernels were shaken every 1-3 days to ensure even distribution. After about 14 days of incubation, the infested oat kernels were subsequently used, without drying, for inoculation of field plots.

Experimental field plots of established Meyer zoysiagrass stands at Manhattan, Olathe, and Haysville, in Kansas were inoculated on 25 September, 2 and 3 October of 2008, respectively (Table 1). Stands were divided into 3.7 m × 3.1 m plots that were each subdivided

into four quadrants. The center of each quadrant was inoculated by placing 8-10 g of infested oat kernels in a small furrow of about 5 to 7 cm-diameter, made using a hand trowel, between the turf and thatch layer. Plots were subsequently irrigated daily for about 10 days following inoculation to promote the establishment of disease at the inoculated foci (Figure 1). In addition to the inoculated patches, turf at the Manhattan site had some disease symptoms arise from a pre-existing natural large patch infection.

Summer cultivation and fertility treatments

The effects of cultivation (core-aerification, verticutting and sand topdressing) and timing of N applications on large patch development were examined in 2008 at Manhattan using natural infection and again in 2009, 2010, and 2011 at Manhattan, Olathe, and Haysville using inoculated patches. Soil at Manhattan was a Chase silt loam (fine montmorillonitic, mesi, Aquic, Argiudolls) with a pH of 7.3. At Olathe, the soil was a Kennebec silt loam with a pH of 7.0. At Haysville, the soil was a Canadian-Waldeck fine sandy loam with a pH of 6.8. At each site, treatment plots were arranged in a randomized complete block split-plot design with cultivation (versus non-cultivation) as the whole plot (3.7 m × 6.2 m) and timing of fertilization as the split-plot (3.7 m × 3.1 m). There were four blocks, leading to four replicate plots per treatment combination. There was a 0.6 m alley between the blocks. Each 3.7 m × 3.1 m split-plot contained four inoculation foci, as described above. Cultivation was carried out once every year (Table 1) at each of the three experimental locations. In Haysville, a Ryan Greensaire 24 aerator (Ryan, Johnson Creek, WI) with a core spacing of 5 cm and core depth of 7.6 cm was used. At Olathe, a Plugr PL800 aerifier (SourceOne, Inc., Lincoln, NE) with a core spacing of 20.3 cm and core depth of 5.7 cm was used, and at Manhattan, a John Deere aerifier (Deere & Company, Moline, IL) with a core spacing of 5.1 cm and core depth of 3.8 cm was used. At each location,

around 640 core-holes per square meter were made during aerification. After aerification, the cores were allowed to air-dry for several hours and subsequently broken up using a BlueBird verticutter (BlueBird, Charlotte, NC). Approximately 0.65 cm of dry sand was then applied as top-dressing and incorporated into the turf canopy with a cart-mounted brush driven across the plot. At the end of the study, soil cores were collected from the cultivated plots to a depth of about 15 cm and examined to measure the average height of any accumulated sand profile.

All plots received a total of 90 kg N/ha/yr. In Olathe and Haysville, plots receiving the spring/fall timing were treated with urea (46-0-0) in two separate applications of 45 kg N/ha. In Manhattan, the spring and fall treatments were each split into two applications of 22.5 kg N/ha. Plots receiving summer fertilization were treated with polymer-coated urea (41-0-0) (Pursell Technologies Inc., Sylacausa, AL) in a single midsummer application at 90 kg N/ ha. Dates are shown in Table 1. Turfgrass stands were mowed twice per week with a triplex reel mower at a mowing height of 14.3 mm in Manhattan and Wichita, and 25.4 mm in Olathe.

Disease assessment

During spring and fall when patch symptoms were visible with distinct margins, patch sizes were measured weekly, to the nearest centimeter, and expressed as the average patch diameter along two consistent perpendicular axes. The individual patches served as subsamples in each plot. In Manhattan, patch sizes were adjusted due to the unequal sizes of the pre-existing naturally occurring patches within experimental plots. Adjustments were similarly made for Manhattan in subsequent years in addition to patches from inoculation carried out in the fall of 2008. The patch sizes were rescaled to reflect a common percentage size origin (100%) at the start of each season. Increases thereafter were reflected as percentage increases over the initial adjusted size of 100% and expressed as 'relative patch size'. The rate of increase in patch sizes,

estimated as the average weekly increases in patch sizes, was determined at all three locations in 2009.

During early summer, patches become less visible with indistinct margins, making patch size measurements difficult. At this time and whenever patch margins were not clearly defined, starting in spring of 2009, disease was quantified by digital image analysis of the patches. Patch symptoms within a 65 cm × 75 cm grid in the center of each plot were photographed weekly using the automatic settings of a Nikon D70s digital camera (Nikon Inc., Japan) at 1.2 m above the turf canopy. Plots were manually brushed and air-blown with a motorized blower to remove dead grass clippings and fallen leaves prior to being photographed. If cultural practices were performed on the same date, photographs were taken first. Digital images were analyzed with SigmaScan Pro version 5.0 software (SPSS, Chicago, IL) using a SigmaScan Pro macro named "Turf Analysis" by Karcher and Richardson (2005) for batch analysis of the digital images. The threshold settings of Karcher and Richardson (2005) were adjusted to Hue: 0 to 53, and saturation: 0 to 57. These threshold settings allowed for estimation of pixels (expressed as percentages) that represented large patch-diseased turf (percentage of diseased, or non-green, turf), relative to healthy (green) turf. The data obtained allowed for quantification of turf recovery in the different treatment plots during early summer, as well as in spring and fall while the disease was active but patch margins were not clearly defined.

Data analysis

Statistical analysis of data for whole-plot, split-plot, and interaction effects were performed with Minitab version 16 (Minitab Inc., Pennsylvania) statistical software. Data obtained for patch size measurements and digital image analysis were tested for normality, and subjected to analysis of variance (ANOVA). Treatment means were compared using Fisher's

individual error rate at $P \leq 0.05$. The overall effect of each treatment combination on large patch, for a given set of data points, was calculated and expressed as the "Area Under Disease Progress Curve" (AUDPC). The AUDPC values for both patch size and percentage of diseased turf were calculated using the method of Madden *et al*. (2007) with the formula $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n\text{-}1$, y_i is the amount of disease (patch size or percentage of diseased turf), and t_i is the time of the *i*th rating.

Microclimate measurements

To evaluate the effect of cultivation practices on soil moisture, soil temperature and thatch temperature, dual probes and thermocouple sensors connected to an analog data logger were installed, one each in four cultivated and non-cultivated plots, at the Manhattan site in September of 2008 and left in place through the fall of 2010. The soil-encapsulated thermocouple (SET) sensors were assembled following the method of Ham and Senock (1992) and installed in the thatch layer of the turf to measure thatch temperatures at hourly intervals. The volumetric soil water content (ratio of water volume to soil volume: cubic meters water per cubic meter soil) at 13 cm below the thatch layer was measured using the dual-probe heat-pulse (DPHP) technique (Campbell *et al*., 1991; Tarara and Ham, 1997; Song *et al*., 1998). DPHP sensors were fabricated as described by Basinger *et al*. (2003) and Bremer (2003). Measurements were automated and logged once daily at 0626 CST. All data acquisition and controls were accomplished with a micrologger and accessories (CR10x, two AM16/32's, and one AM25T, Campbell Scientific, Logan, UT). Dual probes and thermocouples were installed in four replicated cultivated and non-cultivated whole plots. Data were collected, tested for normality, and subjected to analysis of variance (ANOVA) using Minitab statistical software. Mean comparisons was performed using the Fisher's individual error rate function in Minitab.

Volumetric soil water content and soil and thatch temperature data were collected in the treatment plots from May to September and April to July of 2009 and 2010, respectively.

RESULTS

Effects of summer cultivation on soil and thatch temperatures and volumetric soil water content

At the end of the study, accumulated sand profiles measured from soil cores collected from the cultivated plots were diffuse in the soil profile but reached a depth of up to 3.5 cm from the soil surface. There was no significant effect of cultivation on soil or thatch temperatures in 2009 or 2010 (Figure 2). There was no effect of position (soil vs thatch) on temperature in 2009 (Figure 2) or 2010 (data not shown). Though soil volumetric water content tended to be slightly lower in cultivated plots there was no significant difference (Figure 3).

Effects of summer cultivation and timing of nitrogen application on large patch

Manhattan – 2008

Whole-plot cultivation treatment, split-plot N fertilization timing, and their interaction had no significant effect on weekly relative patch size or AUDPC values for the spring or fall 2008 epidemics (Table 2, Figure 4). After 21 May, no disease symptoms were visible in the non-cultivated/summer fertility plots. In all treatments, large patch symptoms were again visible with distinct margins and orange-yellow borders by 14 October when data collection was commenced for the fall season of 2008. Patch sizes at this time were larger than were last recorded in early summer, indicating pathogen spread over summer (Table 2, Figure 4). Again, there was also no significant difference among relative patch sizes in the treatment plots in fall. No data were collected after the week of 23 October due to the onset of winter dormancy.

Manhattan – 2009

In spring 2009, in addition to natural infection, all experimental plots had fairly uniform large patch symptoms following inoculation of plots in the fall 2008. After 19 June, patch margins were indistinct and digital image analysis was conducted to determine patch severity, measured by digital image analysis (Figure 5) as the percent of pixels representing diseased turf. Cultivation, fertility, and their interaction had no significant effect on weekly relative patch size, AUDPC, or patch severity as measured by digital image analysis (Table 3, Figure 6). The average rate of increase in patch size averaged 6.4 cm and 10.3 cm per week in the cultivated and non-cultivated plots, respectively, but were not significantly ($P = 0.05$) different. Cool and dry weather conditions during the fall resulted in poor establishment of disease symptoms, as well as the early onset of turf dormancy. As a consequence, autumn patch data could not be collected.

Manhattan – 2010

After the zoysiagrass greened up and disease symptoms were visible in spring, patches within most of the experimental plots had enlarged and coalesced to cover most of the plots so patch size measurement was no longer feasible (Table 4, Figure 7). Weekly assessment of treatment effects on large patch was therefore carried out solely through analysis of digital images of the plots. Cultivation, fertility, and their interaction did not have any significant effect on weekly measures of percent diseased turf or the AUDPC calculated based on the weekly values (Table 4). Following the onset of fall large patch symptoms, plots that received spring and fall applications of N had significantly lower percentages of diseased turf compared with those that received summer applications (Table 4).

Manhattan – 2011

As measured by digital image analysis, fertility and the fertility × cultivation interaction had a significant effect on large patch. Consistent with the fall data of 2010, plots which received spring and fall applications of N had significantly lower percentages of diseased turf compared with their corresponding plots which received only summer applications of N (Table 4).

Haysville – 2009

In spring 2009, all experimental plots had large patch symptoms of approximately uniform sizes following inoculation of the plots during the fall of 2008. Cultivation, fertility, and their interaction had no significant effect on weekly patch size or AUDPC (Figure 8, Table 5). Additionally, there was no significant difference in the percentage of diseased turf measured by image analysis among the treatment plots by 26 June (Table 5). The average rate of increase in patch size averaged 18.2 cm and 13.4 cm per week in the cultivated and non-cultivated plots respectively but were not significantly ($P = 0.05$) different. Similar to Manhattan, cool and dry weather conditions during the fall resulted in poor establishment of disease symptoms, as well as the early onset of turf dormancy. As a consequence, fall disease data could not be collected.

Haysville – 2010

Emergence from dormancy was delayed due to prolonged cool temperatures following the harsh 2009/2010 winter, but which resulted in no visible winter-kill. Patch size data could not be collected due to poorly defined margins, and data were collected solely by analysis of digital images of plots on 22 June and 7 July. Spring and fall applications of N resulted in significantly lower percentages of diseased turf on 22 June compared with those that received summer applications of N (Table 5).

Haysville – 2011

Patch data were collected only on 3 June, when symptoms were most visible. Again, only the split-plot factor of timing of fertility had a significant effect on disease severity. The cultivated plots that received spring and fall applications of N had significantly lower percentage of diseased turf compared with the non-cultivated plots that were summer fertilized (Table 5).

Olathe – 2009

All experimental plots had large patch symptoms following inoculation of the plots during the fall of 2008. In the spring, cultivation, fertility, and their interaction had no significant effect on large patch (Table 6, Figure 9). The average rate of increase in patch size averaged 3.7 cm and 5.1 cm per week in the cultivated and non-cultivated plots, respectively, and were not significantly (P = 0.05) different.

Olathe – 2010

On 1 June, the percentage of diseased turf was significantly lower in the non-cultivated plots compared to their corresponding cultivated plots (Table 6). This difference was no longer significant by 21 June.

Olathe – 2011

Again, symptom expression in the treatment plots during this spring lacked clearly defined margins for direct measurement of patch sizes and digital image analysis was conducted on 7 June. Cultivation, fertility, and their interaction had no significant effect on large patch (Table 6).

DISCUSSION

Two aspects of large patch disease symptoms, patch size and percentage of blighted tissues within a patch, were evaluated to better understand the effects of cultivation and timing of fertilization of zoysiagrass on large patch disease. In a practical sense, both reductions in patch size and increases in green tissue in the patch area would be beneficial and relevant to turfgrass managers. A large diameter patch with a high percentage of green tissue, which recovers quickly, may be less objectionable than a small patch in which most of the turfgrass is blighted, potentially requiring a longer time to recovery.

Soil micro-climate data collected through the summer of 2009 and 2010 showed the maximum soil and thatch temperatures recorded were around 29°C and 28°C respectively, despite air temperatures above 32°C. Studies of in vitro mycelial growth of *R. solani* AG 2-2 LP have shown that the fungus is capable of near-optimum growth at 30°C (Green *et al*., 1993; Obasa, 2012, Chapter 2). Therefore, the absence of disease symptoms during summer during the study period was not directly due to suppressive soil or thatch temperatures. It is not clear at this point what other factor(s) might be involved in disease suppression during summer. Like other C4 grasses, zoysiagrass has optimum growth at higher temperatures and in hot summer conditions it may outgrow the pathogen. Alterations in zoysiagrass gene expression at high temperatures could also influence disease development. Poor drainage has been associated with more severe large patch damage in the field. Three years of cultivation and sand topdressing did not contribute to significantly improved drainage (as measured by volumetric water content) in the cultivated plots compared with the non-cultivated. Finally, the summer cultivation practice, compared with non-cultivation, did not result in any detectable change in soil and thatch temperatures. And based on our findings, it appears unlikely that the lack of symptoms during

summer is a direct consequence of summer temperatures. Indeed, the findings of Aoyagi *et al*. (1998) and Kobayashi (1980) involving the isolation of *R. solani* AG 2-2 LP from sheath tissues with no obvious symptoms suggested that the fungus is present in zoysiagrass at all times, but disease symptoms only occur when climatic conditions are favorable to the pathogen but not the plant.

Although zoysiagrass recovers from the disease during summer, it is also possible for symptoms to persist during the summer months in shaded and moist areas, especially during unusually cool midsummer weather (Green *et al*., 1993). It has been suggested that high summer temperatures suppress large patch, and that the fungus spends the summer in thatch or on stolons as sclerotia (Tisserat *et al.*, 1994). The patch size data for the fall of 2008 in Manhattan suggests that although symptoms were not visible during the summer of that year, proliferation of the pathogen's mycelia likely continued through the thatch and or soil, resulting in the larger patch sizes when symptoms reappeared in the fall, following the onset of favorable environmental conditions for disease development. The estimated weekly rate of patch size increase ranged from 3.7 to 18.2 cm per week, similar to the rate determined by Aoyagi *et al*. (1998) of 1.5 cm per day, under optimum temperature for *R. solani* AG 2-2 LP. While the rate of patch size increase varied somewhat from site to site, there were no differences between cultivated and non-cultivated plots. This finding is in contrast to greater severity of *Rhizoctonia* root rot disease in wheat under reduced tillage (Bockus and Shroyer, 1998) and root and crown rot in sugar beet where disease caused by *R. solani* AG 2-2 IIIB was reduced in cultivated compared to non-cultivated treatments (Buhre *et al.*, 2009). However, large patch is a sheath blighting pathogen, not a root pathogen, and cultivation practices in turfgrass are less disruptive to soil structure than tillage in crops.

The cultural practices were conducted over several years to examine the potential impact over time. By 2010, the timing of N application appeared to affect the percentage of diseased turf within affected areas of plots regardless of cultivation status. Fertilization in spring and fall was associated with lower percentages of diseased turf at Manhattan and Haysville, but not Olathe, in 2010 and 2011. Applications of N during spring and fall when zoysiagrass growth is not optimal might have promoted more shoot re-growths within affected areas.

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Figure 3.1 Experimental plots at Manhattan in the spring of 2009 following inoculation of plots during the fall of 2008, showing large patch symptoms of approximate uniform size in the inoculated plots and symptoms from pre-existing natural infections. Treatment plots were arranged in a split-plot design with cultivation (versus non-cultivation) as the whole plot (3.7 m × 6.2 m) and timing of fertilization as the split-plot (3.7 m × 3.1 m). Each split-plot had four inoculation foci.

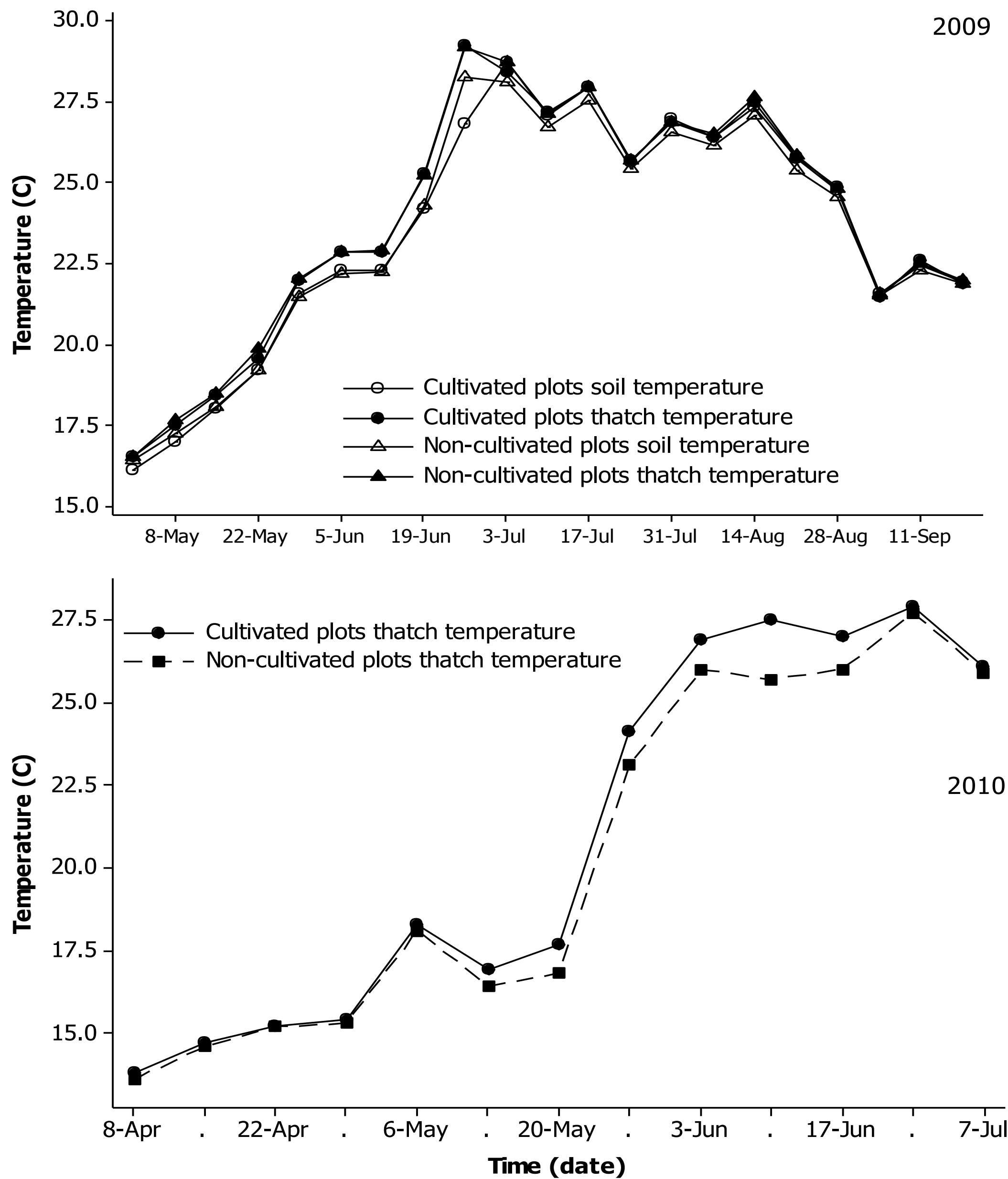

Figure 3.2 Effect of core-aerification, verticutting and sand topdressing on soil and thatch temperatures at Manhattan in 2009 and 2010. Comparison of soil and thatch temperatures as measured by dual probes (13 cm below the thatch layer), and thermocouples (in the thatch layer) in cultivated and non-cultivated plots respectively. Each point represents the average weekly temperature from four plots per treatment. There were no significant difference (*P* = 0.05) between soil and thatch temperature.

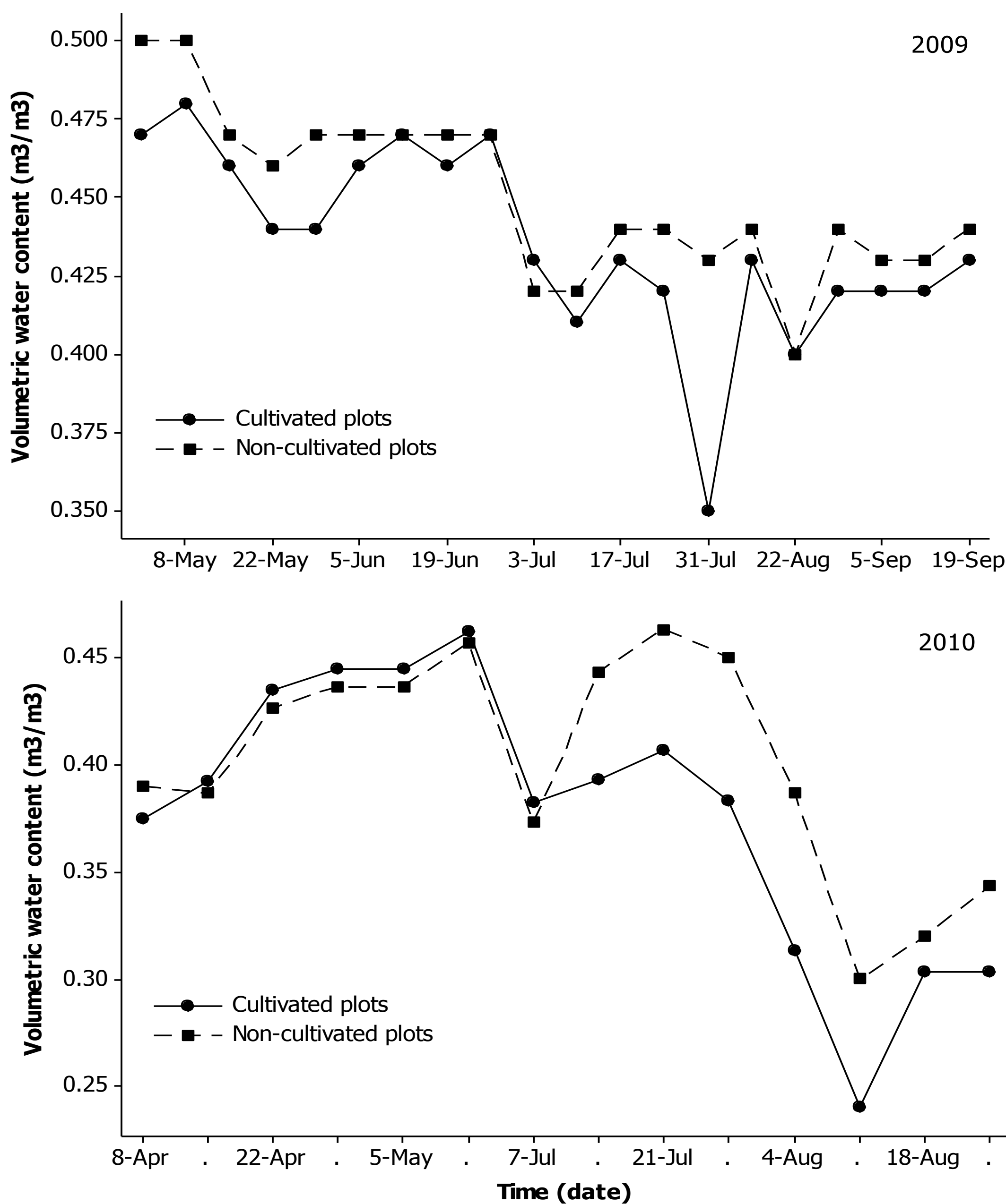

Figure 3.3 Effect of core-aerification, verticutting and sand topdressing on volumetric soil water content, measured with dual probes installed at 13 cm below the thatch layer, in cultivated and non-cultivated plots at Manhattan in 2009 and 2010. Each point represents the average weekly volumetric water content (cubic meters water per cubic meter soil) of four plots per treatment. There was no significant difference (*P* = 0.05) in volumetric soil water content between cultivated and non-cultivated plots.

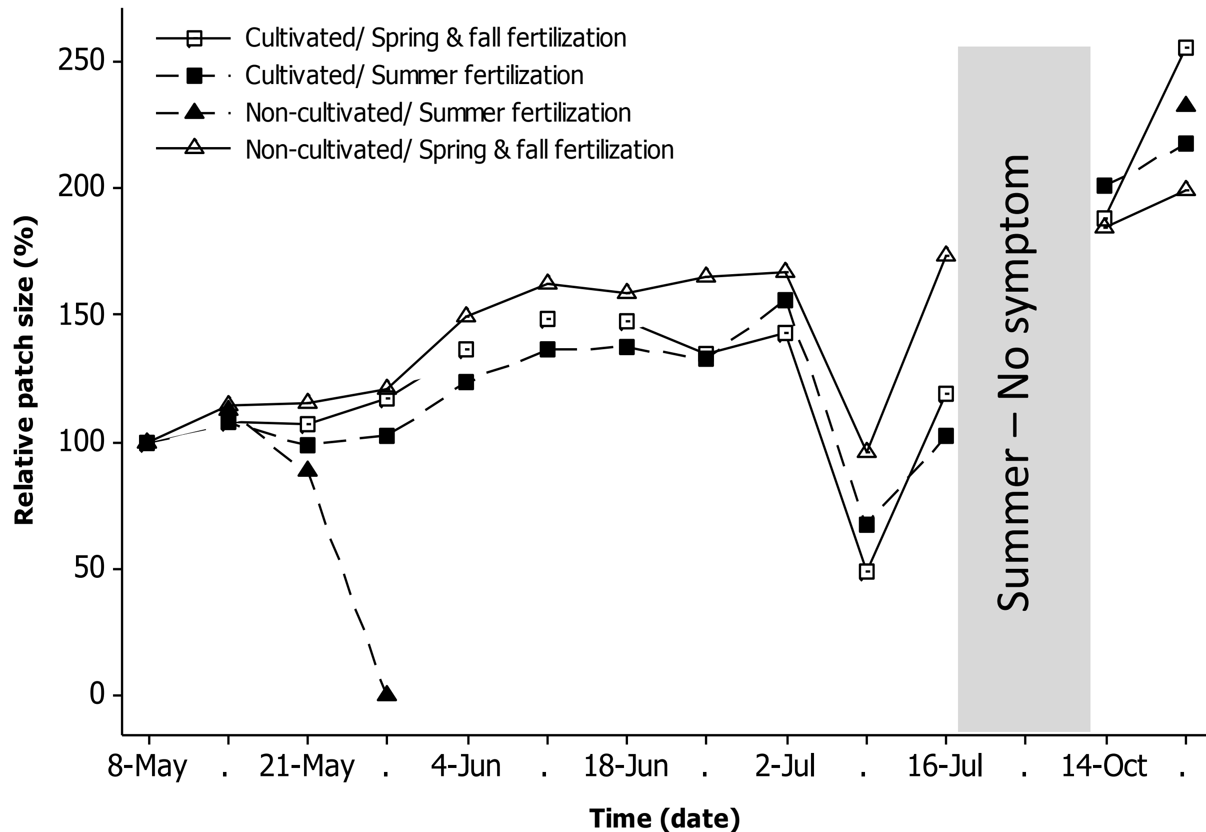

Figure 3.4 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2008 at Manhattan, KS. Since plots had varying amounts of natural infection at start of experiment, initial patch sizes where set to 100% and relative increases or decreases were calculated. Spring fertility was applied as urea at 22.5 kg N/ha on 28 April and 8 May. Summer fertility was applied on 27 June as polymer-coated urea at 90 kg N/ha.

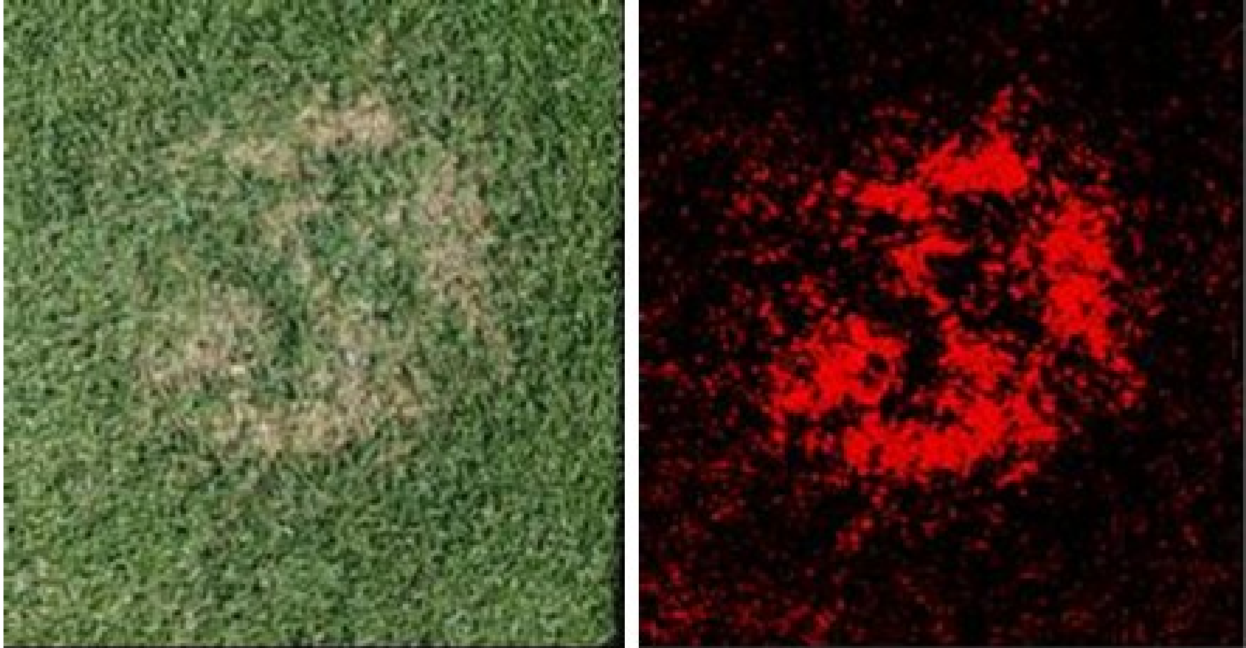

Figure 3.5 Analysis of digital image of plot with large patch symptoms showing image before (left) and after (right) analysis with the SigmaScan Pro 5 (SPSS 5) image analysis software.

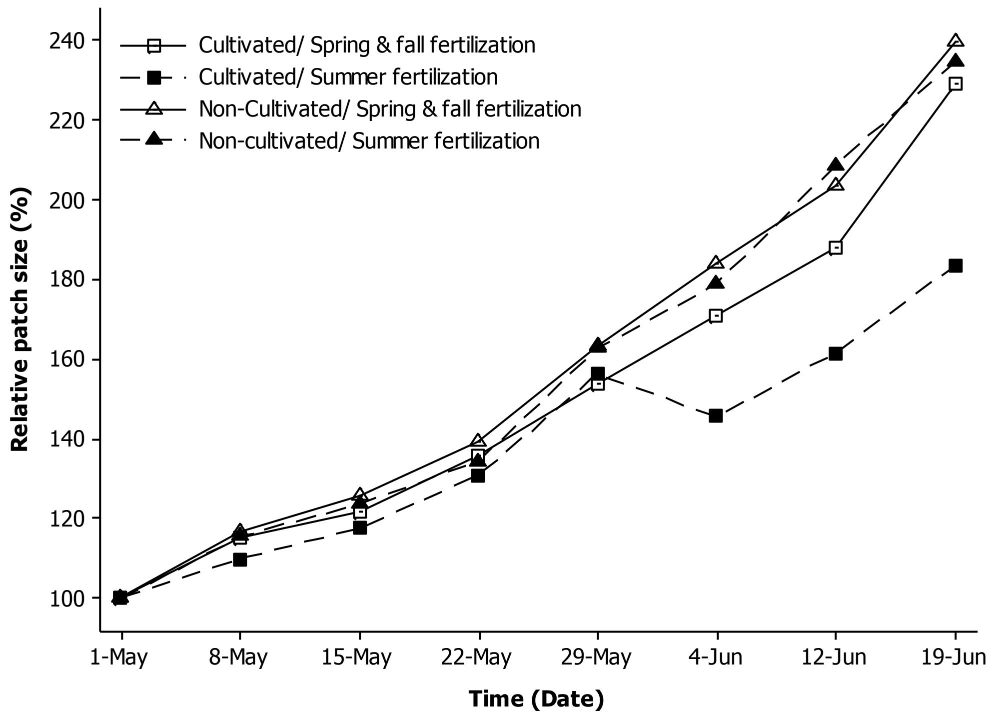

Figure 3.6 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2009 at Manhattan, KS. Each point represents the average of sixteen patches from four replicate plots. Points followed by similar letters are not statistically different (*P* = 0.05).

Spring fertility was applied as urea at 22.5 kg N/ha on 27 April and 28 May. Summer fertility was applied on 23 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008. In Manhattan, patch sizes were adjusted due to the unequal sizes of the pre-existing naturally occurring patches within experimental plots. The patch sizes were rescaled to reflect a common percentage size origin (100%) at the start of each season. Increases thereafter were reflected as percentage increases over the initial adjusted size of 100% and expressed as 'relative patch size'.

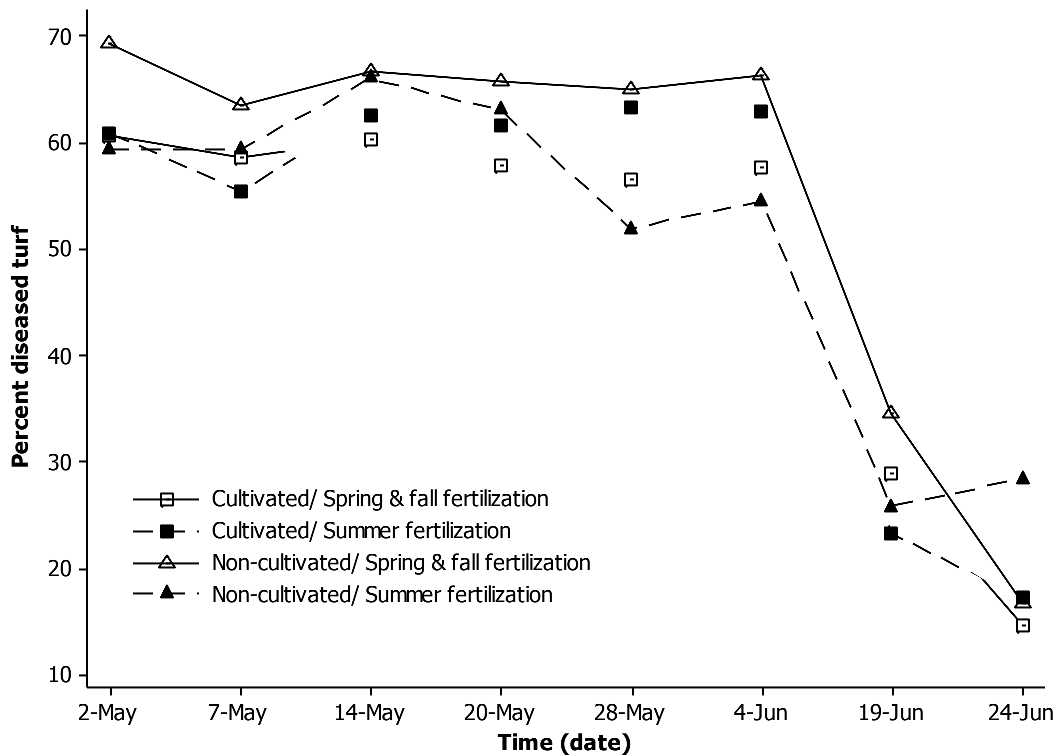

Figure 3.7 Effect of summer cultivation and timing of nitrogen application on large patch disease symptoms during the spring of 2010 at Manhattan, KS. Percent diseased turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software. Each point represents the average of sixteen patches from four replicate plots. Spring fertility was applied as urea at 22.5 kg N/ha on 30 April and 1 June. Summer fertility was applied on 30 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008.

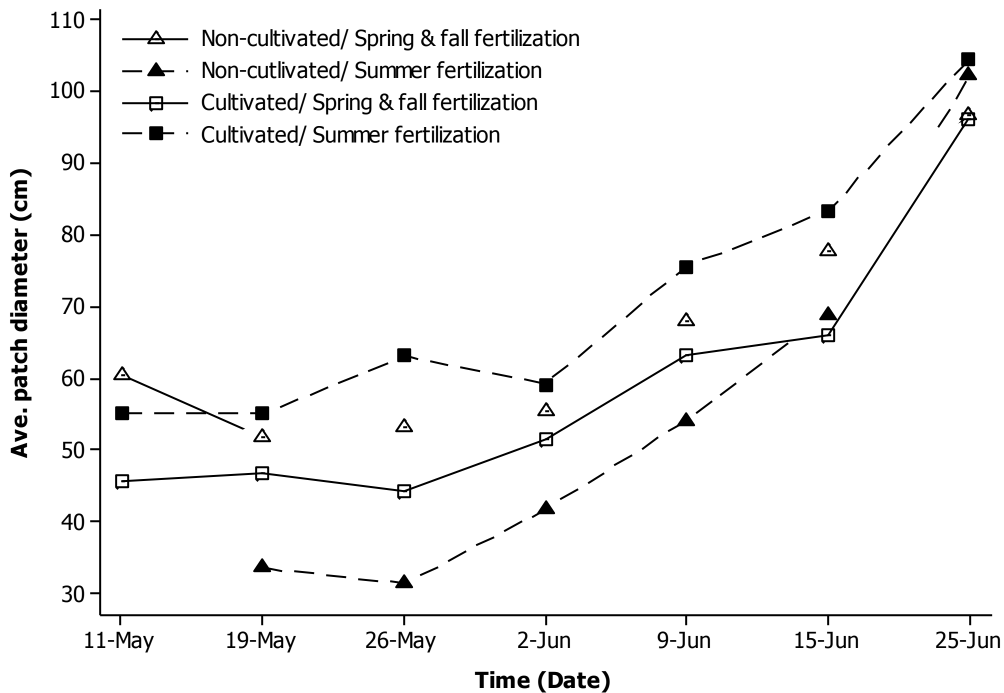

Figure 3.8 Effect of summer cultivation and timing of nitrogen application on large patch during spring 2009 at Haysville, KS. Each point represents the average of sixteen patches from four replicate plots. Spring fertility was applied as urea at 45 kg N/ha on 4 May. Summer fertility was applied on 26 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008.

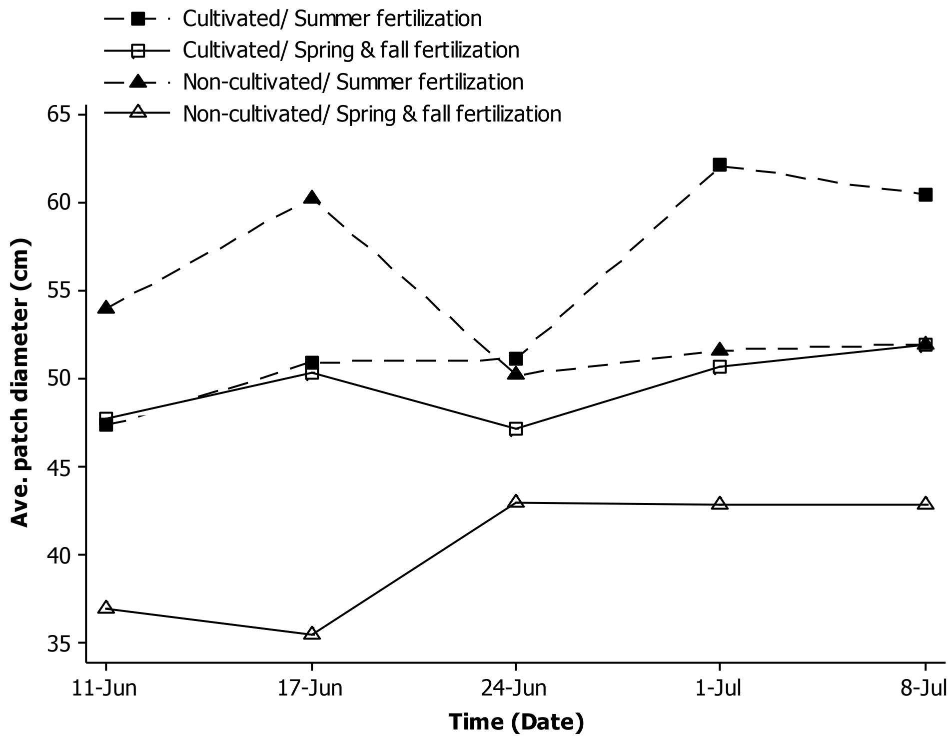

Figure 3.9 Effect of summer cultivation and timing of nitrogen application on large patch during spring of 2009 at Olathe, KS. Each point represents the average of sixteen patches from four replicate plots. Spring fertility was applied as urea at 45 kg N/ha on 1 May. Summer fertility was applied on 8 July as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008.

Table 3.1 Experimental locations with schedule of cultural practices.

Year	Activity*	Manhattan	Olathe	Haysville
2008	Spring fertilization	28-April & 8-May	1-May	29-April
	Summer fertilization	27-June	8-July	14-July
	Fall fertilization	22-Sep. & 23-Oct.	24-September	25-September
	Cultivation	27-June	8-August	14-August
	Plot inoculation	25-September	2-October	3-October
2009	Spring fertilization	27-April & 28-May	30-April	4-May
	Summer fertilization	23-June	24-June	26-June
	Fall fertilization	26-Aug. & 25-Sep.	28-August	4-September
	Cultivation	22-June	24-June	26-June
2010	Spring fertilization	30-April & 1-June	3-May	5-May
	Summer fertilization	30-June	21-June	22-June
	Fall fertilization	1-Sep. & 4-Oct.	16-September	15-September
	Cultivation	8-July	21-June	22-June
2011	Spring fertilization	28-April & 27-May	26-April	27-April
	Summer fertilization	6-June	2-June	3-June

*Cultivation included core-aerification, verticutting, and sand topdressing.
Spring and fall fertility was applied as urea (46-0-0) at a rate of 45 kg N/hectare (ha) each season for an annual total of 90 kg N/ha. In Manhattan, the spring and fall applications were each split into two applications of 22.5 kg N/ha per season. Summer fertility was applied as polymer-coated urea (41-0-0) at 90 kg N/ha.

Table 3.2 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by relative patch size during spring 2008 at Manhattan, KS.

	Large patch relative size[z]													
	May				June				July			Oct		
Treatment[y]	8	14	21	28	4	11	18	25	2	9	16	14	23	AUDPC[x]
Whole plot														
Cultivated	100	108.6	102.4	110.2	130.4	142.5	142.9	134.1	153.7	130.7	134.9	187.4	241.2	880.5
Non-cultivated	100	112.8	102.2	121.4	150.0	163.1	159.4	165.7	167.4	96.3	173.4	184.5	225.7	696.1
Split plot														
Summer	100	110.3	96.2	102.7	124.1	136.4	137.5	133.2	156.1	67.4	102.3	201.1	208.1	527.4
Spring & Fall	100	111.1	108.4	119.6	146.7	155.8	153.9	150.3	155.2	73.0	146.3	186.5	252.1	1049.3
Whole plot × Split plot														
Cultivated Summer N	100	108.5	100.3	102.7	124.1	136.4	137.5	133.2	156.1	67.4	102.3	201.1	204.0	856.1
Non-cult. Summer N	100	112.1	92.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	212.2	198.6
Cultivated Spring & Fall N	100	108.7	104.4	117.7	136.7	148.5	148.3	134.9	142.9	49.6	119.1	188.5	278.4	904.9
Non-cult. Spring & Fall N	100	113.4	112.4	121.4	150.0	163.1	159.4	165.7	167.4	96.3	173.4	184.5	225.7	1193.6
ANOVA[w]														
Whole-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Whole-plot × Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[z]Since plots had varying amounts of natural infection at start of experiment, initial patch sizes were set to 100% and relative increases or decreases were calculated;

[y]Spring fertility was applied as urea at 22.5 kg N/ha on 28 April and 8 May and fall fertility on 2 September and 23 October, for an annual total of 90 kg N/ha. Summer fertility was applied on 27 June as polymer-coated urea at 90 kg N/ha;

[x]Area under disease progress curve. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (relative patch size), and t_i is the time of the ith rating;

[w]Analysis of variance. NS – Not significant. * - Significant.

Table 3.3 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by relative patch size during spring and early summer of 2009 at Manhattan, KS.

	Large patch relative size[y]									Percent diseased turf	
	May					Jun				Jun	Jul
Treatment[z]	1	8	15	22	29	4	12	19	AUDPC[x]	20	3
Whole plot											
Cultivated	100	111.8	117.6	130.6	148.3	162.1	176.5	199.6	928.6	17.1	6.4
Non-cultivated	100	114.9	123.0	135.1	159.5	176.4	199.7	228.5	1065.1	13.6	3.6
Split plot											
Summer	100	112.5	119.1	130.9	150.0	166.9	184.3	210.2	958.9	14.5	5.4
Spring & Fall	100	114.1	121.5	134.6	157.8	171.6	191.8	217.7	1034.7	16.2	4.6
Whole-plot × Split-plot											
Cultivated Summer N	100	110.8	116.3	128.7	145.4	161.0	173.2	197.3	899.6	14.3	7.6
Non-cult. Summer N	100	114.3	121.8	133.2	154.5	172.7	195.4	223.2	1018.3	14.7	3.2
Cultivated Spring & Fall N	100	112.7	118.9	132.4	151.1	163.1	179.7	201.8	957.5	19.9	5.2
Non-cult. Spring & Fall N	100	115.5	124.1	136.9	164.5	180.1	203.9	233.7	1111.8	12.5	3.9
ANOVA[w]											
Whole-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Whole-plot × Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[z]Spring fertility was applied as urea at 22.5 kg N/ha on 27 April and 28 May. Summer fertility was applied on 23 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since June 2008. Summer cultivation was performed on 22 June;

[y]Each value represents the average of sixteen patches from four replicate plots. Values in a column followed by the same letters are not statistically different (P = 0.05). On 20 and 3 July, percent diseased (non-green) turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software;

[x]Area under disease progress curve. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (relative patch size), and t_i is the time of the ith rating;

[w]Analysis of variance. NS – Not significant. * - Significant.

Table 3.4 Effect of summer cultivation and timing of nitrogen application on large patch disease symptoms as measured by digital image analysis during spring 2010, fall 2010, and spring 2011 at Manhattan, KS.

	Percent of diseased turf[y]										
	2010										2011
	May					Jun				Oct	Jun
Treatment[z]	2	7	14	20	28	4	19	24	AUDPC[x]	25	1
Whole plot											
Cultivated	60.9	57.1	61.5	59.8	60.0	60.4	26.2	16.1	363.3	47.6	24.6
Non-cultivated	64.4	61.5	66.5	64.5	58.6	60.5	30.3	22.7	382.6	47.7	28.6
Split plot											
Summer	60.2	57.5	64.4	62.4	57.7	58.8	24.6	22.9	364.1	53.4a	33.8a
Spring & Fall	65.0	61.2	63.6	61.9	60.9	62.1	31.9	15.8	381.9	41.9b	19.3b
Whole-plot × Split-plot											
Cultivated Summer N	61.0	55.5	62.7	61.6	63.3	62.9	23.3	17.4	368.5	53.2	30.9ab
Non-cult. Summer N	59.4	59.4	66.1	63.1	52.0	54.6	25.9	28.5	359.6	53.5	36.7a
Cultivated Spring & Fall N	60.7	58.7	60.3	57.9	56.7	57.8	29.0	14.8	358.1	41.9	18.2c
Non-cult. Spring & Fall N	69.3	63.6	66.8	65.9	65.1	66.4	34.7	16.8	405.6	41.9	20.4bc
ANOVA[w]											
Whole-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*
Whole-plot × Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*

[z]In 2010, spring fertility was applied as urea at 22.5 kg N/ha on 30 April and 1 June. Summer fertility was applied on 30 June as polymer-coated urea at 90 kg N/ha, fall fertility was applied as urea at 22.5 kg N/ha on 1 Sept and 4 Oct for an annual total of 90 kg N/ha. In 2011, spring fertility was applied as urea at 22.5 kg N/ha on 28 April and 27 May. Summer fertility was applied on 6 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008;

[y]Percent diseased(non-green) turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software. Each value represents the average of sixteen patches from four replicate plots. Values in a column followed by the same letters are not statistically different (P = 0.05);

[x]Area under disease progress curve. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where i = 1,2,3,…,n-1, y_i is the amount of disease (percent diseased turf), and t_i is the time of the ith rating.

[w]Analysis of variance. NS – Not significant. * - Significant.

Table 3.5 Effect of summer cultivation and timing of nitrogen application on large patch symptoms as measured by patch size (2009) and digital image analysis (26 June 2009, 2010 and 2011) at Haysville, KS.

	Large patch symptoms											
	2009[y]									2010[x]		2011[x]
	May			Jun						Jun	Jul	Jun
Treatment[z]	11	19	26	2	9	15	25	26	AUDPC[w]	22	7	3
Whole plot												
Cultivated	50.5	50.8	53.6	55.4	69.4	74.6	100.3	32.9	379.1	29.4	27.3	55.6
Non-cultivated	60.3	42.6	42.2	48.5	61.0	73.4	99.5	28.5	332.4	27.2	18.6	63.9
Split plot												
Summer	55.2	44.3	47.2	50.4	64.9	76.1	103.3	29.7	348.1	36.3a	27.0	64.1a
Spring & Fall	53.0	49.2	48.6	53.5	65.5	71.9	96.4	31.7	363.3	20.6b	18.8	55.4b
Whole-plot × Split-plot												
Cultivated Summer N	55.2	55.0	63.1	59.1	75.6	83.2	104.4	30.6	415.8	38.1	30.3	60.3
Non-cult. Summer N	-	33.5	31.3	41.6	54.1	68.9	102.2	28.7	280.5	33.9	23.7	67.8
Cultivated Spring & Fall N	45.7	46.6	44.1	51.6	63.1	66.0	96.1	35.1	342.3	20.7	24.2	50.9
Non-cult. Spring & Fall N	60.3	51.7	53.1	55.3	67.9	77.8	96.7	28.2	384.3	20.5	13.4	59.9
ANOVA[v]												
Whole-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*
Whole-plot × Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[z]In 2009, spring and fall fertility was applied as urea at 45 kg N/ha on 4 May and 4 September, respectively , while summer fertility was applied on 26 June as polymer-coated urea at 90 kg N/ha. In 2010, spring and fall fertility was applied as urea at 45 kg N/ha on 5 May and 15 September, respectively, while summer fertility was applied on 22 June as polymer-coated urea at 90 kg N/ha. In 2011, spring fertility was applied as urea at 45 kg N/ha on 27 April, while summer fertility was applied on 3 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been conducted since 2008;
[y]Each value except for 26 June, 2009 represents the average diameter (cm) of sixteen patches from four replicate plots. On 26 June, 2009 large patch was estimated from the plots as percent diseased turf by analysis of digital images within a 65 cm × 75 cm grid;
[x]Percent diseased (non-green) turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software. Each value represents the average of sixteen patches from four replicate plots. Values in a column followed by the same letters are not statistically different (P = 0.05);
[w]Area under disease progress curve based on patch sizes from 11 May to 25 June 2009. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (patch diameter), and t_i is the time of the ith rating;
[v]Analysis of variance. NS – Not significant. * - Significant.

Table 3.6 Effect of summer cultivation and timing of nitrogen application on large patch as measured by patch size (2009) and digital image analysis (2010 and 2011) at Olathe, KS.

	Large patch									
	2009[y]						2010[x]			2011[x]
	Jun			Jul			May[w]	Jun		Jun
Treatment[z]	11	17	24	1	8	AUDPC[v]	26	1	21	7
Whole plot										
Cultivated	47.5	50.7	49.1	56.4	56.2	208.0	64.9	56.0a	19.2	22.9
Non-cultivated	45.5	47.9	46.6	47.2	47.4	188.1	72.2	45.6b	22.4	24.7
Split plot										
Summer	50.6	55.6	50.7	56.9	56.2	216.5	64.9	51.5	23.3	21.7
Spring & Fall	42.4	43.0	45.2	46.8	47.4	179.6	72.3	50.2	18.3	26.0
Whole-plot × Split-plot										
Cultivated Summer N	47.3	50.9	51.1	62.1	60.4	218.0	64.1	57.0	23.3	22.5
Non-cult. Summer N	53.9	60.3	50.2	51.6	51.9	215.0	65.6	45.9	23.2	20.9
Cultivated Spring & Fall N	47.7	50.4	47.1	50.7	51.9	198.0	65.8	55.0	15.1	23.5
Non-cult. Spring & Fall N	37.0	35.5	42.9	42.8	42.8	161.1	78.8	45.3	21.5	28.5
ANOVA[u]										
Whole-plot	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Whole-plot × Split-plot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[z]In 2009, spring fertility was applied as urea at 45 kg N/ha on 1 May, while summer fertility was applied on 8 July as polymer-coated urea at 90 kg N/ha. In 2010, spring fertility was applied as urea at 45 kg N/ha on 3 May while summer fertility was applied on 21 June as polymer-coated urea at 90 kg N/ha. In 2011, spring fertility was applied as urea at 45 kg N/ha on 26 April, while summer fertility was applied on 2 June as polymer-coated urea at 90 kg N/ha. Note that cultivation and N treatments had been done since 2008;
[y]Each value represents the average diameter (cm) of sixteen patches from four replicate plots;
[x]Percent diseased (non-green) turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software. Each value represents the average of sixteen patches from four replicate plots. Values in a column followed by the same letters are not statistically different (P = 0.05). Plot digital images were collected before cultivation was performed on 21 June, 2010;
[w]The percentage of each plot affected by large patch was determined by visual assessment;
[v]Area under disease progress curve. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (patch diameter), and t_i is the time of the ith rating;
[u]Analysis of variance. NS – Not significant. * - Significant.

Chapter 4 - Evaluation of spring and fall fungicide applications for large patch management in zoysiagrass

Abstract

The efficacy of spring and fall preventive application timings of azoxystrobin, flutolanil, and triticonazole for the control of large patch disease caused by *Rhizoctonia solani* Kühn AG 2-2 LP were evaluated under field conditions. The study was conducted on fairway stands of 'Meyer' zoysiagrass from 2008 to 2010 at the Rocky Ford Turfgrass Research Center, Kansas State University. Experimental plots were inoculated and had established large patch symptoms prior to the start of the studies. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 206.8 kPa in water equivalent to 816 liters ha^{-1}. Disease assessment was performed by direct measurement of patch sizes or by analysis of digital images of affected plot areas from the different application timings. In general, two fall applications of fungicide did not reduce disease compared to one fall application. Fall applications made when thatch temperatures ranged from 17.8°C to 23.2°C reduced disease compared to untreated controls. Similarly, two spring applications of fungicide did not reduce disease compared to one spring application. The earliest spring applications of azoxystrobin and triticonazole, made after the turf had broken dormancy and plots were mostly green, resulted in the lowest amount of disease.

INTRODUCTION

Zoysiagrass (*Zoysia japonica* Steud. and *Z. matrella* (L.) Merr.) is a warm-season (C4), perennial turfgrass that is widely used on golf courses in the "transition-zone" of the United

States, a region that includes states such as Kansas eastward to Virginia and North Carolina. Some desirable characteristics of zoysiagrasses include good density and resistance to pests (Fry and Huang, 2004). Also, compared to certain cool-season (C3) turfgrass species such as creeping bentgrass (*Agrostis stolonifera* L.), zoysiagrass has lower water, fertilizer, and pesticide requirements for maintaining a high-quality surface (Fry *et al*., 2008).

An important consideration in the adoption and widespread use of zoysiagrass is large patch disease. Large patch is a serious problem for turfgrass managers, particularly along the northern range of zoysiagrass adaptation in North America (Green *et al*., 1993), but also represents a major problem everywhere zoysiagrass is utilized. The disease is caused by the fungus *Rhizoctonia solani* belonging to the anastomosis group (AG) 2 and intra-specific group (ISG) 2-2 LP (Hyakumachi *et al*., 1998). The disease also affects other warm-season turfgrasses including bermudagrass (*Cynodon dactylon* (L.) Pers.) (Martin and Lucas, 1984) and St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) (Hurd and Grisham, 1983; Haygood and Martin, 1990).

In Kansas, large patch symptoms appear during spring (April to early June) and autumn (late September through October). Typical symptoms appear as light-brown to straw-colored sunken patches up to 6 m or more with or without bright-orange margins (Smiley *et al*., 2005; Tisserat *et al*., 1994; Green *et al*., 1993). Healthy turf tissues are sometimes scattered within the patches. Patches have fewer living tillers and a reduced rate of leaf growth. Lower leaf sheaths of affected grasses appear water-soaked, with reddish brown or black lesions, and affected tillers subsequently turn orange to orange-yellow (Aoyagi *et al*., 1998; Tisserat *et al*., 1994).

Large patch symptom development in zoysiagrass is favored by relatively cool and humid weather. Thatch temperatures of 15 to 25°C, compacted and poorly drained soils, and excessive

and prolonged wetness near the leaf surface are optimal conditions for the development of large patch symptoms (Green *et al*, 1993). However, symptoms are thought to be suppressed during summer by high thatch and soil temperatures, usually above 30°C (Green *et al.*, 1993). During summer, growth of new shoots from living stolons and rhizomes often result in full turf recovery, though weeds may encroach while turfgrass is recovering.

In addition to environmental conditions, several cultural practices such as mowing height (Green *et al*., 1994) and water management (Green *et al*., 1994) can also influence large patch development. However, because cultural management practices do not provide an acceptable level of disease control, fungicides are often applied during spring and/or fall. Fall applications are intended to be preventative in nature. Spring applications are generally used to target areas that were missed by fall applications. For example, some turf managers map areas known to develop large patch each year, and those areas are targeted for fungicides rather than treating all zoysiagrass areas.

Several classes of fungicides including the sterol biosynthesis demethylation inhibitors (DMI), quinone outside inhibitors (QoI, respiration inhibitors), polyoxins (chitin inhibitors), carboxamides (respiration inhibitors), and aromatic hydrocarbons (lipid and membrane synthesis inhibitors) are labeled for large patch. While fungicide applications can be useful for suppressing large patch, optimum timing of application remains uncertain. Preventative fungicide applications made before the development of large patch symptoms have been demonstrated to provide better disease control than applications made after the onset of disease symptoms (Tisserat *et al*., 1993). Preventative applications made during fall not only inhibit fall symptoms, but also suppress or delay disease development during the following spring (Tisserat *et al*., 1994). As a general rule, the recommendation for the timing of the first fungicide application is

when thatch temperatures drop below 21°C. However, neither the optimum thatch or soil temperatures for fungicide application nor the number of applications required for optimum control is known. It is not uncommon for turf managers to plan applications based on calendar dates. However, changing environmental conditions may influence the efficacy of such applications. The objective of this study was to evaluate large patch control efficacy with several spring and fall fungicide application timings for azoxystrobin (QoI), flutolanil (carboxamide), and triticonazole (DMI). We measured thatch temperatures at the time of application to determine potential optimum temperature for application.

MATERIALS AND METHODS

Inoculum preparation

Rhizoctonia solani AG 2-2 LP isolates were recovered from large patch-infected zoysiagrass samples from Kansas in 2008. Leaf sheath sections measuring 1 to 2 cm with blight symptoms were removed from infected plants, surfaced-sterilized with 0.5% NaOCl for about 2 min, blotted dry, and placed on $^{1}/_{4}$ potato-dextrose agar (Difco Laboratories, Maryland) amended with tetracycline (10 mg/L) and streptomycin (10 mg/L) (designated here as "1/4 PDA^{++}") (Biotech Research Grade, Fisher Scientific Inc., New Jersey). Hyphal tips were obtained from cultures and maintained at 23°C in the dark. Identification of *R. solani* from cultures was based on in vitro hyphal phenotypic characteristics (right angle branching of hyphae, absence of sclerotia and zonation, and aerial hyphae) (Hyakumachi *et al*., 1998), nuclear conditions (multinucleate) using DAPI staining (Martin, 1987), hyphal anastomosis with known tester isolates belonging to AG-2-2 LP (Martin and Lucas, 1984), and by polymerase chain reaction (PCR) using the AG 2-2 LP-specific primer P22-LP (Carling *et al*., 2002) (see Chapter 2). One large patch isolate was then selected from the collection and used to infest pre-sterilized oat kernels in glass jars (Tisserat *et al*., 1989). One hundred and fifty grams of oat kernels were mixed with 150 ml of distilled water (1:1) in a glass jar and sterilized by steam autoclaving, twice at 121°C for 30 min. Plugs of colonized agar were added to cooled oats. The glass jars were shaken every few days to ensure even colonization by the growing mycelia. After about 14 days of incubation, the infested oat-kernels were used, without drying, for inoculation of field plots as described below.

Site description and inoculation of study plots

The study was conducted on two stands of the cultivar 'Meyer' zoysiagrass at the Rocky Ford Turfgrass Research Center in Manhattan, Kansas (39.128 N longitude, 96.358 W latitude). Soil at the site was a Chase silt loam (fine montmorillonitic, mesi, Aquic, Argiudolls) with a pH of 7.3.

Plots in the two stands of zoysiagrass were inoculated on 26 and 25 September of 2007 and 2008, respectively. A knife was used to slice a small slot in the thatch layer at 1.5 m spacings, and 8-10 grams of infested oat kernels were placed in each slot. Plots were irrigated daily for about 10 days following inoculation to promote the establishment of disease. The plots of the fairway inoculated in the fall of 2007 served for the evaluation of fall 2008 and 2009 fungicide applications, while those inoculated during the fall of 2008 were used for the evaluation of spring 2009 and 2010 applications.

In-ground irrigation was used to prevent stress and supplement rainfall to provide 2.5 cm water per week, and plots were maintained at fairway height by mowing two or three times weekly at 14 mm. Urea (46-0-0) was applied in July and August of each year to provide an annual total of 90 kg N ha^{-1}.

Fungicide timing studies

Fungicide application timing studies were conducted in 2008, 2009 and 2010 (Table 1). In all studies, plots measured 1.5 × 1.5 meters (each containing one inoculation point) and were arranged in a randomized complete block design (RCB) with four replicates. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1}. In all cases, unsprayed inoculated plots served as the control.

Fall applications, 2008

During the fall of 2008, single applications of flutolanil (ProStar 70WP, Bayer Crop Science) at 4.7 kg a.i. ha^{-1} were made on 9, 16, 23, or 30 September. Plots receiving sequential applications were treated on 9 + 23 September, 16 + 30 September, or 23 September + 7 October. All applications were completed prior to turf dormancy. On 15 and 22 May, 2009, patch sizes were measured to the nearest centimeter, and expressed as the average patch diameter along two perpendicular axes. Patch size data were natural log (ln)-transformed prior to analysis. Plots were not re-randomized in future spray-applications of the fungicides.

Spring applications, 2009

Following plot inoculation in September 2008, fungicide applications in spring 2009 were initiated on 1 May after the turf had broken dormancy and plots were mostly green, and when fifty percent of the individual plots expressed large patch symptoms, which were patches around 30 cm in diameter. Single applications of flutolanil, azoxystrobin, and triticonazole were made on 1 and 8 May, whereas plots receiving sequential applications were treated on 1 + 15 May, or 8 + 22 May. Plots were not re-randomized in future years. Patch diameters were measured weekly from 8 to 29 May until patch margins became indistinct as the turfgrass recovered. On 26 June, disease was assessed by quantifying the percentage of diseased turf within each plot using digital image analysis of each plot. Patch symptoms within a 65 cm × 75 cm area in the center of each plot were photographed weekly using the automatic settings of a Nikon D70s digital camera (Nikon Inc., Japan) at 1.2 m above the turf canopy. Plots were manually brushed and air-blown with a motorized blower to remove dead grass clippings and fallen leaves prior to being photographed. Digital images were then analyzed with SigmaScan Pro version 5.0 software (SPSS, Chicago, IL) using a SigmaScan Pro macro named “Turf Analysis” by Karcher and Richardson (2005) for batch analysis of the digital images. The

threshold settings of Karcher and Richardson (2005) were adjusted to Hue: 0 to 53, and saturation: 0 to 57. These threshold settings allowed for estimation of pixels (expressed as percentages) that represented large patch-diseased turf (percentage of diseased turf), relative to healthy (green) turf.

Fall applications, 2009

In the fall of 2009, single applications of flutolanil (ProStar 70WP, Bayer Crop Science) at 4.7 kg a.i. ha^{-1}, azoxystrobin (Heritage 50WDG, Syngenta) at 305 g a.i. ha^{-1}, and triticonazole (Trinity 1.69SC, BASF) at 980 g a.i. ha^{-1} were made on 3, 10, 17 or 24 September of 2009. All applications were completed prior to turf dormancy. Patches had expanded beyond the 1.5 × 1.5 m plot size, so on 28 May of 2010, patch symptoms within each plot were assessed by visually estimating percentage of turf within each plot area with large patch symptoms. Patch data were ln-transformed prior to analysis.

Spring applications, 2010

After turf began to break dormancy and plots were mostly green, single applications of flutolanil, azoxystrobin, and triticonazole were made at the above rates on 16, 23, and 30 April and 7 May 2010. Most plots had become completely covered with large patch so that patch size measurement was no longer useful. Consequently, weekly evaluation of disease severity was assessed by analysis of digital images of plots as described above.

Soil temperature measurement

Thatch temperatures at the time of fungicide applications were measured using soil-encapsulated thermocouple (SET) sensors installed in the thatch layer of turf. Sensors were installed on 19 September, 2008 and remained in the plots through the entire experiment. The SET sensor was assembled following the method of Ham and Senock (1992), and measured

thatch temperatures at hourly intervals. All data acquisition and control were accomplished with a micrologger and accessories (CR10x, two AM16/32, and one AM25T, Campbell Scientific, Logan, UT). Condition at the time of application was expressed as the average daily thatch temperature for the week (7 days) prior to the application date.

Data analysis

Statistical analysis of data was performed with Minitab version 16 (Minitab Inc., Pennsylvania) statistical software. Data obtained for patch size measurements and digital image analysis were tested for normality, and then subjected to analysis of variance (ANOVA). Treatment means were compared using Fisher's individual error rate at $P \leq 0.05$. The overall effect of any given treatment on large patch, for a given set of data points, was calculated and expressed as the "Area Under Disease Progress Curve" (AUDPC). The AUDPC values for both patch size and percentage of diseased turf were calculated using the method of Madden *et al*. (2007) with the formula $\Sigma\ [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n\text{-}1$, y_i is the amount of disease (patch size or percentage of diseased turf), and t_i is the time of the ith rating.

RESULTS

Efficacy of fall 2008 applications on large patch in spring 2009

Average thatch temperatures for the single applications ranged from 19.6 to 21.2°C. All the single applications of flutolanil, except the 9 September application, resulted in significantly reduced patch sizes compared to the untreated control in the spring 2009 (Table 2). On 15 May there were no differences among the 16, 23, and 30 September application timings. On 22 May, the 16 September timing had significantly smaller patches than the 30 September timing. In general there was little difference in patch size between plots receiving the single or sequential applications of flutolanil. On both rating dates, the 9 + 23 September applications reduced patch size compared to 9 September alone but not compared to 23 September alone. The 16 + 30 September treatment did not reduce patch size beyond either single application on 15 May, but it did reduce disease compared to 30 September alone on the 22 May assessment date. On both rating dates, the 23 September + 7 October application did not reduce patch size compared to the 23 September application.

Efficacy of spring 2009 applications

Average thatch temperatures ranged from 16.4 to 22.7°C for the spring 2009 applications. Based on AUDPC values calculated from weekly patch size measurements, all the application regimes, with the exception of azoxystrobin applied on 8 May, resulted in significantly reduced patch sizes compared with the untreated control (Table 3). In general, sequential fungicide applications did not reduce AUDPC compared to single fungicide applications.

For the single applications made on 1 May, triticonazole had a significantly lower AUDPC than flutolanil but not azoxystrobin. Additionally, the AUDPC value for the single

application of triticonazole made on 1 May was significantly lower than for both sequential applications of the same product.

The single application of azoxystrobin made on 1 May had a lower AUDPC than its single application made on 8 May and sequential azoxystrobin applications on 8 + 22 May. The AUDPC of treatments receiving sequential applications of azoxystrobin or flutolanil on 1 + 15 May was not significantly different from the corresponding single application on 1 May. Furthermore, the AUDPC in plots receiving sequential applications of either fungicide on 8 + 22 May was not significantly different from that in plots receiving the single application on 8 May (Table 3).

On 26 June, when patch margins were indistinct and digital analysis was used, plots which received a single azoxystrobin, triticonazole or flutolanil application on 1 May, or a sequential application on which the first treatment occurred 1 May, had significantly lower percentages of diseased turf (Table 3). The sequential applications of flutolanil and azoxystrobin on 8 + 22 May also reduced disease compared to the untreated control. Disease in turf receiving sequential applications was not significantly different from that in turf receiving one fungicide application.

Fall applications, 2009

Thatch temperatures started at 21.6°, rose to 23.2°, then fell to 20.4° and 17.8°C for the four application dates, respectively (Table 4). The percentage of plot areas showing large patch symptoms in the spring season of 2010 was significantly lower compared with the untreated plots for all the application timings for flutolanil, azoxystrobin, and triticonazole with the exception of the single application of flutolanil made on 3 September (Table 4). Disease control for the single applications of triticonazole on 3 and 10 September was significantly better than

for the single application of the same product on 24 September (Table 4). There were no significant differences in large patch for the different application timings for azoxystrobin (Table 4).

Spring applications, 2010

Average thatch temperatures varied little, ranging from 16.1 to 17.6°C for the four application timings (Table 5). The AUDPC values for large patch, calculated from digital image analysis, of single applications of flutolanil, azoxystrobin, and triticonazole on 16 and 23 April indicated lower percentages of diseased turf compared to the untreated control (Table 5). Zoysiagrass receiving a single application of flutolanil on 16 April had a significantly lower AUDPC than all the applications of triticonazole, with the exception of the application made on 23 April. For applications made on 30 April and 7 May, only azoxystrobin consistently resulted in significantly lower overall (AUDPC) percentages of diseased turf compared with the untreated control (Table 5).

DISCUSSION

The timing of fungicide applications for turfgrasses can be scheduled based on calendar dates, weather, scouting, and combinations of those factors. Environmental conditions and soil microclimate, which may influence the efficacy of applied fungicides, vary from year to year. For instance, thatch temperatures were slightly higher in the fall of 2009 compared with similar periods in 2008 during the fall fungicide application-timing studies (Tables 2 and 4). Temperatures also fluctuated in unpredictable ways. For example, in fall 2008 average thatch temperature was 20.7°C on 9 September, fell to 19.6°C on September 16, and then rose to 21.2°C on September 30 (Table 2). In fall 2009, average thatch temperature was 21.6°C on 3 September, rose to 23.2°C on 10 September, then fell again to 20.4 and 17.8°C, respectively, on 17 and 24 September (Table 4).

The general guideline for the timing of fall fungicide deployment in the management of large patch disease is to apply fungicides when thatch temperatures reach 21-24°C (Corwin *et al*., 2007; Kennelly, 2011; Tisserat *et al*., 1994). However, the single applications of flutolanil on 9 September 2008 and 3 September 2009, when the thatch temperature averaged 20.7°C and 21.6°C respectively, failed to achieve a significant reduction in patch sizes compared with the untreated controls. In contrast, single applications of azoxystrobin and triticonazole made on 3 September 2009 resulted in significantly reduced patch sizes compared with the untreated control. Aside from the failure of the first fall flutolanil applications to manage large patch, fall application timings at thatch temperatures ranging from 17.8 to 23.2°C across the two years of the study reduced disease compared to the untreated control. All single applications of flutolanil made when the thatch temperature averaged 20°C, in 2008 (19.6°C) and 2009 (20.4°C), as well as sequential applications involving a first application made when thatch temperature averaged

about 20°C in 2008, had the least disease. Using sequential fungicide applications in the fall of 2008 also did not increase disease control compared to single applications.

Since the fungicides are locally systemic, the first spring applications were made after the turf had broken dormancy and plots were mostly green, to allow for the uptake of the fungicides by the growing plants. The results of the 2009 and 2010 spring application studies showed that the earlier fungicide applications provided better control of large patch symptoms. In the spring of 2009, the earliest single applications of azoxystrobin and triticonazole, made when the thatch temperature was 16.4°C, resulted in similar or lower disease than subsequent applications. Additionally, results from our spring application-timing studies also demonstrate potential differences in how fungicides can affect the expression of large patch symptoms. For example, in 2009, the earliest application of triticonazole reduced the AUDPC based on patch size compared to flutolanil. In contrast, in 2010, the earliest application of flutolanil reduced AUDPC compared with triticonazole based on percent symptomatic turf within the patch as determined by digital image analysis. Both patch size and the intensity of blighting within a patch area (and the resulting differences in turf recovery in the patch) are of interest to turfgrass managers.

This study did not use pre-determined specific thatch temperatures as triggers for application timing. Additional studies using more targeted thatch temperatures and other environmental factors as a guide for fungicide deployment in the management of large patch is encouraged to further determine its suitability, as well as applicability to the different classes of fungicides used in the management of the disease. The mode of action of each fungicide, fungicide rate, the ability of the plant to take up the fungicides at different temperatures, and/ or environmental factors not considered in this study may play a role.

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Table 4.1 Application-timing schedule for flutolanil, azoxystrobin, and triticonazole for the management of large patch on 'Meyer' zoysiagrass.

	Fall		Spring	
Fungicide*	**2008**	**2009**	**2009**	**2010**
Single application				
flutolanil	9, 16, 23, 30 Sept	3, 10, 17, 24 Sept	1, 8 May	16, 23, 30 Apr, 7 May
azoxystrobin		3, 10, 17, 24 Sept	1, 8 May	16, 23, 30 Apr, 7 May
triticonazole		3, 10, 17, 24 Sept	1, 8 May	16, 23, 30 Apr, 7 May
Sequential applications				
flutolanil	9 + 23 Sept		1 + 15 May	
	16 + 30 Sept		8 + 22 May	
	23 Sept + 7 Oct			
azoxystrobin			1 + 15 May	
			8 + 22 May	
triticonazole			1 + 15 May	
			8 + 22 May	

*Flutolanil (ProStar 70WP, Bayer Crop Science) was applied at 4.7 kg a.i. ha^{-1}, azoxystrobin (Heritage 50WDG, Syngenta) was applied at 305 g a.i. ha^{-1}, and triticonazole (Trinity 1.69SC, BASF) was applied at 980 g ha^{-1}. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1}.

Table 4.2 Effect of single and sequential fall 2008 applications of flutolanil on large patch in the spring of 2009.

Fungicide application[z] timing and thatch temperature	Patch size[x] (cm)	
	15-May	22-May
Single applications		
9 Sept (20.7°C)[y]	77.7a	82.4a
16 Sept (19.6°C)	0.4b	0.4d
23 Sept (19.6°C)	6.1b	6.5cd
30 Sept (21.2°C)	15.3b	25.6bc
Sequential applications		
9 + 23 Sept (20.7 + 19.6°C)	13.3b	15.3cd
16 + 30 Sept (19.6 + 21.2°C)	0.4b	0.8d
23 Sept + 7 Oct (19.6 + 17.6°C)	0.8b	1.9cd
Untreated control	70.5a	75.9a

[z]Flutolanil (ProStar 70WP, Bayer Crop Science) was applied at 4.7 kg a.i. ha^{-1} with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1};
[y]Temperature indicates the average thatch temperature during the preceding 7 days as measured by soil-encapsulated thermocouple (SET) sensors installed in the thatch layer;
[x]Patch sizes were measured to the nearest centimeter, and expressed as the average patch diameter along two consistent and perpendicular axes. Values were ln-transformed for analysis, but actual patch sizes are shown, and represent the mean of four replicate plots per treatment. Values followed by the same letter are not statistically different (P=0.05).

Table 4.3 Effect of single and sequential spring 2009 applications of flutolanil, azoxystrobin, and trinity on large patch.

Application timing[z]	Fungicide	Patch size[y] (cm)					Percentage of diseased turf[w]
		May 8	May 15	May 22	May 29	AUDPC[x]	June 26
Single applications							
1 May (16.4°C)[v]	flutolanil	42.3bc	45.4bcd	46.3bcd	56.7ab	134.1bc	5.8b
	azoxystrobin	32.9bc	34.8cd	38.7de	39.3bc	109.6cd	5.3b
	triticonazole	27.1c	30.3d	32.8e	33.3c	93.2d	8.1b
8 May (21.4°C)	flutolanil	39.8bc	45.0bcd	49.4abc	50.0bc	139.4bc	11.9ab
	azoxystrobin	48.3ab	50.5ab	53.7ab	56.4b	156.5ab	10.8ab
	triticonazole	37.1bc	42.6bcd	45.2bcd	47.5bc	130.1bcd	12.4ab
Sequential applications							
1 & 15 May (16.4 + 19.7°C)	flutolanil	36.9bc	39.6bcd	41.3cde	42.6bc	120.7bcd	8.0b
	azoxystrobin	39.1bc	41.6bcd	43.9bcd	45.8bc	127.9bcd	5.8b
	triticonazole	41.8bc	44.2bcd	46.0bcd	45.7bc	133.9bc	8.0b
8 & 22 May (21.4 + 22.7°C)	flutolanil	37.6bc	42.0bcd	46.3bcd	41.8bc	117.6bcd	7.9b
	azoxystrobin	43.5b	48.3bc	51.8ab	54.4b	149.1b	8.2b
	triticonazole	42.7bc	46.7bc	52.8ab	53.4b	147.5bc	11.2ab
	Untreated control	63.1a	65.6a	58.7a	76.1a	193.9a	18.3a

[z]Flutolanil (ProStar 70WP, Bayer Crop Science) was applied at 4.7 kg a.i. ha^{-1}, azoxystrobin (Heritage 50WDG, Syngenta) was applied at 305 g a.i. ha^{-1}, and triticonazole (Trinity 1.69SC, BASF) was applied at 980 g ha^{-1}. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1};

[y]Patch sizes were measured weekly, to the nearest centimeter, and expressed as the average patch diameter along two consistent and perpendicular axes. Values represent the mean of four replicate plots per treatment. Values followed by similar letters are not statistically different ($P = 0.05$);

[x]Area under disease progress curve (AUDPC). Calculated as $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (patch size), and t_i is the time of the ith rating. Values followed by similar letters are not statistically different ($P = 0.05$);

[w]Percent diseased turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software;

[v]Temperature indicates the average thatch temperature during the preceding 7 days as measured by soil-encapsulated thermocouple (SET) sensors installed in the thatch layer.

Table 4.4 Effect of single fall 2009 applications of flutolanil, azoxystrobin, and triticonazole on large patch in the spring of 2010.

Fungicide application[z] timing, and thatch temperature	Percentage of plot with disease[x] 28 May
3 Sept (21.6°C)[y]	
flutolanil	29.8a
azoxystrobin	0.9c
triticonazole	0.8c
10 Sept (23.2°C)	
flutolanil	3.0bc
azoxystrobin	1.0bc
triticonazole	0.8c
17 Sept (20.4°C)	
flutolanil	0.8c
azoxystrobin	1.5bc
triticonazole	7.0bc
24 Sept (17.8°C)	
flutolanil	4.0bc
azoxystrobin	2.0bc
triticonazole	8.5b
Untreated control	42.0a

[z]Flutolanil (ProStar 70WP, Bayer Crop Science) was applied at 4.7 kg a.i. ha^{-1}, azoxystrobin (Heritage 50WDG, Syngenta) was applied at 305 g a.i. ha^{-1}, and triticonazole (Trinity 1.69SC, BASF) was applied at 980 g ha^{-1}. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1};
[y]Temperature indicates the average thatch temperature during the preceding 7 days as measured by soil-encapsulated thermocouple (SET) sensors installed in the thatch layer;
[x] Values were ln-transformed for analysis, but actual percentages of plot area affected are shown, and represent the mean of four replicate plots per treatment. Values followed by similar letters are not statistically different ($P = 0.05$).

Table 4.5 Effect of single spring 2010 applications of flutolanil, azoxystrobin, and triticonazole on large patch in the spring of 2010.

Application timing	Fungicide	Percentage of diseased turf[y]								
		Apr 30	May 7	May 14	May 20	May 28	Jun 4	Jun 19	Jun 24	AUDPC[x]
Apr 16	flutolanil	33.1bc	41.2d	42.9b	33.2d	29.2e	30.3cd	20.7b	10.1ab	219.0e
(16.1°C)[w]	azoxystrobin	34.7bc	61.4abc	54.8ab	37.9cd	36.2cde	29.8cd	17.1b	9.3ab	259.2de
	triticonazole	40.3abc	62.4abc	63.5a	46.3bcd	46.0bcd	28.8d	24.8ab	12.5ab	298.1bcd
Apr 23	flutolanil	40.7abc	53.6bcd	54.3ab	45.5bcd	23.2e	29.8cd	17.5b	12.5ab	250.6de
(17.3°C)	azoxystrobin	45.8abc	62.0abc	50.2ab	48.9bc	38.7bcde	37.3bcd	18.9b	14.2ab	286.0bcde
	triticonazole	45.8abc	46.9cd	52.8ab	54.7ab	30.7de	25.0d	23.1ab	13.4ab	262.8de
Apr 30	flutolanil	50.8ab	73.1a	62.2a	62.2a	54.0ab	45.2abc	21.9b	13.7ab	350.8ab
(17.7°C)	azoxystrobin	47.4abc	47.7cd	51.9ab	43.6bcd	38.7bcde	30.0cd	31.3ab	12.3ab	273.0cde
	triticonazole	54.7a	70.4a	60.5a	63.0a	46.2bcd	40.3bcd	24.9ab	23.4ab	339.3abc
May 7	flutolanil	50.8ab	52.5bcd	61.7a	56.3ab	33.5de	36.6bcd	25.1ab	11.0ab	296.5bcd
(17.6°C)	azoxystrobin	31.1c	61.6abc	61.7a	51.6ab	38.2bcde	27.4d	19.6b	8.7b	280.0cde
	triticonazole	43.3abc	58.9abc	61.9a	55.6ab	50.0abc	50.3ab	27.7ab	15.6ab	333.9abc
	Untreated control	44.3abc	64.6ab	62.1a	63.9a	62.8a	57.0a	36.9a	18.1a	378.5a

[z]Flutolanil (ProStar 70WP, Bayer Crop Science) was applied at 4.7 kg a.i. ha^{-1}, azoxystrobin (Heritage 50WDG, Syngenta) was applied at 305 g a.i. ha^{-1}, and triticonazole (Trinity 1.69SC, BASF) was applied at 980 g ha^{-1}. All fungicide applications were made with a CO_2-powered boom sprayer with XR Tee Jet 8003VS nozzles at 207 kPa in water equivalent to 816 liters ha^{-1};

[y]Percent diseased turf was estimated by analysis of digital images within a 65 cm × 75 cm grid within plots using SPSS 5 image analysis software. Values followed by similar letters are not statistically different ($P = 0.05$);

[x]Area under disease progress curve (AUDPC). Calculated as $\sum[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (percentage of diseased turf), and t_i is the time of the ith rating. Values followed by similar letters are not statistically different ($P = 0.05$);

[w]Temperature indicates the average thatch temperature during the preceding 7 days as measured by soil-encapsulated thermocouple (SET) sensors installed in the thatch layer.

Chapter 5 - Evaluation freeze-tolerant zoysiagrass genotypes for susceptibility to large patch disease caused by *Rhizoctonia solani* AG 2-2 LP.

Abstract

Large patch, caused by the fungus *Rhizoctonia solani* Kühn anastomosis group (AG) 2-2LP, is the most common and severe disease of zoysiagrass (*Zoysia* spp). Despite the importance of this disease, few studies have examined cultivar susceptibility. Fourteen new zoysiagrass germplasm lines from parental crosses including *Z. japonica*, *Z. matrella*, and *Z. pacifica* were evaluated for susceptibility to large patch under growth chamber and field conditions and compared with 'Meyer', the most widely utilized cultivar in the transition zone of the United States. All progeny had similar disease levels compared to Meyer in the growth chamber, but only 6 consistently had disease levels as low as Meyer in the field. Growth chamber results did not correlate to field results.

INTRODUCTION

Zoysiagrass (*Zoysia* spp.) is a warm-season (C4) turfgrass that is popular in the transition zone of the United States (Fry *et al*., 2008; Patton *et al*., 2007; Dunn and Diesburg, 2004). Some desirable characteristics of zoysiagrasses include good density and resistance to pests (Fry and Huang, 2004). Zoysiagrass is also relatively easier and cheaper to maintain compared with a cool-season turfgrass species such as creeping bentgrass (*Agrostis stolonifera* L.) (Fry *et al*., 2008).

Large patch, caused by *Rhizoctonia solani* AG 2-2 LP is the most common and severe disease of zoysiagrass in the transition zone, and everywhere zoysiagrass is utilized. It can cause

large areas of blighted turf in the spring and fall as zoysiagrass breaks and enters winter dormancy. Typical symptoms appear as light-brown to straw colored sunken patches with or without bright-orange margins (Smiley *et al*., 2005; Tisserat *et al*., 1994; Green *et al*., 1993). Patches can range in size up to 6 meters or more in diameter (Tisserat *et al*., 1994; Green *et al*., 1993) with healthy turf tissues sometimes scattered within the patches. During summer conditions, growth of new shoots from living stolons and rhizomes within patches often results in full turf recovery.

Along with large patch, the relative lack of an acceptable level of winter hardiness and long period of winter dormancy are limiting factors in the widespread use of zoysiagrass cultivars in the transition zone. The level of winter injury suffered varies widely among zoysiagrass genotypes (Patton and Reicher, 2007). ‘Meyer’ Zoysiagrass (*Zoysia japonica* Steud.), a vegetatively propagated zoysiagrass cultivar, has been the most widely used cultivar on golf courses in the transition zone since 1952 (Fry *et al*., 2008). Meyer and ‘Zenith’ zoysiagrass (*Z. japonica*), which is seed-propagated, have better freeze-tolerance than cultivars such as Zorro, Diamond, and Royal, which are *Z. matrella* (Patton *et al*., 2007). However, Meyer is slow to establish and recover, and it is coarser in texture than *Z. matrella* cultivars (Patton and Reicher, 2007; Fry and Dernoeden, 1987).

Since 2004, turfgrass researchers at Kansas State University have evaluated over 600 new zoysiagrass progeny for winter survival and quality (Zhang and Fry, 2006; Okeyo *et al*., 2011). These progeny were the result of genotypic crosses made at Texas A&M-Dallas, most of which involved one parent from *Z. japonica* and one from a *Z. matrella* cultivar or Emerald (*Z. japonica* × *Z. pacifica*). The crosses were made in an effort to develop one or more cultivars with freezing tolerance as good as or better than ‘Meyer’, as well as having good density, finer leaf

texture, and quality. In a recent study, Okeyo *et al*. (2011) found zoysiagrass progeny associated with reciprocal crosses of *Z. matrella* (L.) Merr. × *Z. japonica* or 'Emerald' × Meyer, 'Cavalier' (*Z. matrella*), and DALZ 0102 (*Z. japonica*) showed freezing tolerance comparable with Meyer. Furthermore, some also were superior to Meyer in autumn green color retention, but not spring green color retention (Okeyo *et al*., 2011).

The objective of this study was to evaluate the susceptibility to large patch of fourteen new freeze-tolerant zoysiagrass progeny, and Meyer, under growth chamber and field conditions. The fourteen progeny are a subset of selections made from evaluations of the original 600 zoysiagrass progeny for cold tolerance and the other traits listed above at Kansas State University.

MATERIALS AND METHODS

Pathogen isolation and storage

Rhizoctonia solani AG 2-2 LP isolates were recovered from large patch-infected zoysiagrass samples from Kansas in 2008. Leaf sheath sections measuring 1 to 2 cm with blight symptoms were removed from infected plants, surfaced-sterilized with 0.5% NaOCl for about 2 min, blotted dry, and placed on one-fourth strength potato-dextrose agar (Difco Laboratories, Maryland) amended with tetracycline (10 mg/L) and streptomycin (10 mg/L) (designated here as "1/4 PDA^{++}") (Biotech Research Grade, Fisher Scientific Inc., New Jersey). Cultures were maintained at 23°C in the dark. Identification of *R. solani* from cultures was based on hyphal characteristics, nuclear conditions (multinucleate) as described by Martin (1987), hyphal anastomosis and fusion frequency with a known tester isolate belonging to the anastomosis group AG-2-2 LP on agar-coated glass slides (Martin and Lucas, 1984; Carling, 1996), and by polymerase chain reaction (PCR) using the AG 2-2 LP-specific primer P22-LP developed by Carling *et al*. (2002) (See Chapter 2). One large patch isolate was then selected from the collection and used to infest oat kernels in glass jars according to the method described by Tisserat *et al*. (1989). To prepare inoculum, 150 g of oat kernels mixed with 150 ml of distilled water in a glass jar were sterilized by steam autoclaving, twice at 121°C for 30 min. sterilized oats were inoculated with several agar cubes from plates of ¼ PDA^{++}. The glass jars containing the inoculated oat kernels were shaken from time to time, as required, to ensure even distribution. After about 14 days of incubation, the infested oat kernels were used, without drying, for inoculation of established progeny turfgrasses in pots and in the field.

Growth chamber studies

Plant inoculation and disease assessment

Stolons of the fourteen new lines, and Meyer (Table 1) were collected in October of 2008 and November of 2009 respectively, from the edges of established field plots measuring 1.5 × 1.5 meters with three replicate plots each, at Rocky Ford Turfgrass Research Station in Manhattan, KS (Okeyo *et al*., 2011). Stolons were rinsed under tap water to remove soil debris, surface sterilized with 0.5% NaOCl for 3 min, and finally rinsed in two changes of distilled water. Prepared stolons were subsequently propagated in potting media (Metro Mix 510, SUN GRO, Washington) contained in 5 × 5 cm plastic pots and kept under an intermittent mist system in the greenhouse at 25°C for about two months. Pots with stolon sections containing 3-10 shoots were subsequently removed from the mist chamber and maintained at 28°C and 16 h photoperiod, achieved with supplemental lighting of up to 580 µmol m^{-2} s^{-1} at the canopy level (Zhang, 2007), in a greenhouse for an additional 3 months before inoculation. Grasses were fertilized once with urea to provide N at 49 kg ha^{-1} immediately following transfer to the greenhouse. Pots were watered twice a week and grasses were maintained at a height of about 2 cm using scissors.

Each zoysia line was inoculated with eight to ten infested or non-infested sterile oat kernels by placing the kernels on the soil surface in each pot. Pots were then arranged in separate plastic trays filled with water to a depth of 2 cm, and covered with a clear plastic lid to maintain a high relative humidity. The plastic trays were arranged in a complete randomized design and maintained at 25°C and a 13 h photoperiod in a Conviron ATC60 growth chamber (Conviron, Canada).

After 5 days, and at 5-day intervals thereafter for 25 days, three pots of each line were randomly selected and removed from the growth chamber for destructive sampling. They were rated for disease incidence by determining the percentage of individual shoots in each pot with distinct, water-soaked brown lesions on the leaf sheath according to the method of Green *et al*. (1993). To verify the causal pathogen, representative samples of infected tissues of each line were surface-sterilized and incubated on $^1/_4$ PDA^{++} at 25°C for re-isolation of *R. solani*. After 25 days, the three uninoculated pots of each zoysia line were also removed from the growth chamber and similarly rated for disease incidence. This study was conducted in 2009 and repeated in 2010.

Field studies

Plot inoculation and disease assessment

The study was conducted at the Rocky Ford Turfgrass Research Center in Manhattan, Kansas (39.128 N longitude, 96.358 W latitude). Soil at the site was a Chase silt loam (fine montmorillonitic, mesi, Aquic, Argiudolls) with a pH of 7.3. Plots of the new zoysiagrass progeny and Meyer measuring 1.5 m × 1.5 m were used for the study. Each plot was established from sixteen 6 cm-diameter plugs planted in a 30.5 cm × 30.5 cm area at the center of each plot in 2007 (Okeyo *et al*., 2011). The plots were arranged in a randomized complete block design (RCB) with three replicates per line, and a 0.6 m alley between each plot. The plots were mowed twice every week at 1.4 cm. In-ground irrigation was used to prevent stress and supplement rainfall to provide 2.5 cm of water per week. Fertilization was done in July and August of each year with urea to provide a total of 49 kg N ha^{-1} per year.

For the establishment of large patch disease, all the plots were inoculated in September of 2008. Inoculation was performed by placing 8-10 grams of infested oat kernels in a small furrow

of about 5 to 7 cm-diameter, made using a hand trowel, between the turf and thatch layer at the center of each plot. Plots were subsequently irrigated daily for about 10 days following inoculation to promote the establishment of disease.

In the spring of 2009, patch sizes in progeny field plots were measured weekly using a meter rule and expressed as the average patch diameter along two perpendicular axes. Additionally, analysis of digital images of plots was carried out. Patch symptoms within a 65 cm × 75 cm rectangle in the center of plots were photographed weekly using the automatic settings of a Nikon D70s digital camera (Nikon Inc., Japan) at 1.2 m above the turf. Plots were manually brushed and air-blown with a motorized blower to remove dead grass clippings prior to being photographed. This was necessary in order to avoid error in large patch estimations due to contributions from the color of the dead clippings. The digital images were subsequently analyzed with SigmaScan Pro version 5.0 software (SPSS, Chicago, IL) using a SigmaScan Pro macro for batch analysis of digital images named "Turf Analysis" by Karcher and Richardson (2005). The threshold settings of Karcher and Richardson (2005) were adjusted (Hue: 0 to 53 and Saturation: 0 to 57) to select for pixels representing patch symptoms within each digital image. In the spring of 2010, however, large patch assessment within the plots was carried out only by the method of digital analysis of patch images, due to poorly defined patch margins which prevented accurate size measurements. Data collected by digital image analysis represented the percentage of diseased turf within each plot.

Data analysis

Statistical analysis of data was performed with Minitab version 16 (Minitab Inc., Pennsylvania) statistical software. The areas under the disease progress curve (AUDPC) for each zoysia line from the growth chamber and field studies were calculated using the method of

Burpee (1992) with the formula $\Sigma[(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease incidence (growth chamber) or the percentage of diseased turf (field), and t_i is the time of the *i*th rating, providing an estimate of cumulative disease severity over time. Data obtained following the analysis of the digital images were subjected to analysis of variance (ANOVA). Mean comparisons were performed using the Fisher's individual error rate at $P \leq 0.05$.

Correlation analysis between the average AUDPC values for the growth chamber and field studies in 2009 and 2010, respectively was performed using Pearson's product moment correlation coefficient. A similar analysis was performed to determine the correlation between the growth chamber AUDPC values for each progeny in 2009 and 2010, and their respective field AUDPC values in both years.

RESULTS

Growth chamber inoculation and disease assessment

At all rating dates except 5 days after inoculation in 2009, there were no significant differences among any lines (Table 2). At 5 days after inoculation (DAI), the percentage of individual shoots in each pot with distinct, water-soaked brown lesions on the leaf sheath was significantly higher for 5324-32 compared with Meyer and many other lines (Table 2). None of the new lines had AUDPC values significantly different from Meyer. The AUDPC values for DALZ 0102, 5313-71, 5334-59, and 5324-32 were not statistically different from Meyer, but were significantly lower than those of 5325-11 and 5312-55 (Table 2).

In the 2010 study, the overall performance of each zoysiagrass for the duration of the study, represented by their respective AUDPC values, again was not significantly different from that of Meyer (Table 3). Consistent with the 2009 result, line 5324-32 had a significantly higher disease incidence at 5 DAI compared with Meyer and all of the other entries. Additionally, at 10 DAI, progeny 5313-46 also had a significantly higher disease incidence compared with Meyer, while 5324-26 at 15 dai, and 5321-9 at 25 DAI had significantly lower disease than Meyer (Table 3).

Field experiments

2009

Patch sizes in the new lines were not significantly different from those of Meyer on June 12 and 19 (Table 4). Furthermore, the percentage of diseased turf, as measured by image analysis, was not significantly different among the new lines and Meyer, with the exceptions of 5312-55 on June 21, 5311-16 on June 27, and 5321-9 on July 3, which were higher all than

Meyer (Table 4). No progeny had an AUDPC value significantly different from Meyer. Progeny 5313-71 had a significantly lower AUDPC value than 5312-55.

2010

The percentage of diseased turf was significantly lower for Meyer compared with nine new lines (5313-71, 5313-46, 5327-67, 5325-11, 5324-32, DALZ 0102, 5324-26, 5321-9 and 5313-23) on May 1, six new lines (5313-71, 5313-46, 5327-67, 5325-11, DALZ 0102, and 5321-9) on May 7, and seven new lines (5313-71, 5313-46, 5327-67, 5325-11, 5324-32, 5324-26, and 5321-18) on May 28 (Table 5). By June 24, there was no difference in the percentage of diseased turf among the new lines compared with Meyer, with the exception of 5313-71 with a significantly higher percentage (Table 5). The AUDPC of Meyer was also significantly lower compared with eight (5313-71, 5313-46, 5327-67, 5325-11, 5324-32, DALZ 0102, 5324-26, and 5321-9) of the new lines (Table 5). The remaining six of the thirteen new lines (5313-23, 5313-34, 5321-18, 5334-59, 5312-55 and 5311-16) had AUDPC values that were not statistically different from that of Meyer.

Correlation analysis

We compared the results for the performance of the progeny, based on their average AUDPC values for 2009 and 2010, under growth chamber and field conditions respectively, and found no significant correlation ($r = 0.08$, $P = 0.79$).

Additionally, there was also no significant correlation ($r = 0.46$, $P = 0.09$) between the performance of the progenies under growth chamber conditions in 2009 and 2010, and there was no significant correlation between the 2009 and 2010 field studies ($r = -0.39$, $P = 0.16$).

DISCUSSION

Large patch is widely considered as the most important disease of zoysiagrasses, and particularly in the transition zone of the United States (Green *et al*., 1993). Given that these zoysiagrass progeny were not bred specifically for resistance to large patch disease, it was particularly important to evaluate and compare their susceptibility to large patch with that of Meyer, the most widely used zoysiagrass cultivar in the transition zone, and which is considered moderately resistant to large patch (Metz *et al*., 1993; Reicher, 2004, 2006; Brunneau, 2005). In a separate study, Fry and Cloyd (2011) assessed the susceptibility of the fourteen zoysiagrass progeny and Meyer to bluegrass billbug larval damage. The study found that 5313-71, DALZ 0102, 5321-18 and 5334-59 suffered significantly lower larval damage compared with Meyer during the two years of the study in 2009 and 2010.

Under the growth chamber conditions, designed to be optimal for fungal growth, all progeny performed similar to Meyer in both experiments. In the growth chamber, two progeny, 5313-71 and DALZ 0102, had lower AUDPC values than 5312-55 and 5325-11 in 2009, and 5313-34 and 5313-46 in 2010. The parents of 5313-71 are associated with reciprocal crosses involving *Z. japonica* and *Z. matrella*, both of which were identified as moderately resistant to large patch (Metz *et al*., 1993; Reicher, 2004; Brunneau, 2005). However, in the field, 5313-71 and DALZ 0102 did not consistently have disease levels as low as Meyer. Although all progeny performed comparably with Meyer under field conditions in 2009, results from the 2010 field study indicated that only six progeny (5311-16, 5312-55, 5313-23, 5313-34, 5321-18, and 5334-59) had AUDPC large patch levels as low as Meyer.

This study represents the first report of the evaluation of these new zoysiagrass progeny lines for their susceptibility to large patch disease. Additional studies are required to further evaluate their performance under disease conditions and at other locations.

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Table 5.1 Backgrounds of the zoysiagrass genotypes

Progeny*/Parent
Cavalier × Chinese Common
5311-16
5312-55
Zorro × Meyer
5313-23
5313-34
5313-71
5313-46
Emerald × Meyer
5321-9
5321-18
Meyer × 8501
5324-26
5324-32
Meyer × 8508
5325-11
Meyer × Diamond
5327-67
Emerald × Zenith
5334-59
DALZ 0102
Meyer

* Meyer, DALZ 0102 and Anderson (Chinese common) = *Z. japonica*
Zorro, 8501, 8508 and Diamond = *Z. matrella*

Table 5.2 Large patch (caused by *R. solani* AG 2-2 LP) disease incidence of new zoysiagrass lines and 'Meyer' under growth chamber conditions[z] in 2009.

	Disease incidence (%)[y]					
	Days after inoculation (dai)					
Progeny/Parent	5	10	15	20	25	AUDPC[x]
Cavalier × Chinese Common						
5311-16	2.4c	25.4	51.7	47.5	55.3	153.4bcde
5312-55	2.1c	57.1	60.7	56.7	43.6	197.4ab
Zorro × Meyer						
5313-23	18.3ab	49.1	45.7	55.6	60.2	189.6abc
5313-34	7.3bc	49.5	59.7	46.4	50.0	184.2abcd
5313-71	4.2c	29.3	59.5	49.4	43.5	145.6cde
5313-46	0.0c	42.1	53.1	60.5	46.3	178.9abcd
Emerald × Meyer						
5321-18	n/a	25.0	37.6	52.3	n/a	n/a
Meyer × 8501						
5324-26	0.0c	41.6	62.1	56.5	54.2	187.4abcd
5324-32	20.5a	21.0	44.3	51.6	53.0	144.8de
Meyer × 8508						
5325-11	0.0c	57.3	55.6	55.4	59.1	197.9ab
Meyer × Diamond						
5327-67	12.0abc	40.6	53.9	51.8	62.7	183.7abcd
Emerald × Zenith						
5334-59	0.0c	32.2	41.6	45.2	53.1	145.6cde
DALZ 0102	0.0c	34.4	47.8	50.1	40.1	152.3cde
Meyer	6.6bc	43.6	44.9	58.0	49.5	174.4abcd

[z]Plants were maintained in the growth chamber at 25°C, 13-h photoperiod, and a relative humidity of ~95%;
[y]Expressed as a percentage of individual shoots showing water-soaked sheath blighting symptom averaged across three replicated pots destructively sampled at each time point (DAI) for each zoysiagrass line; n/a = not available.
[x]Area under disease progress curve (AUDPC). Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n\text{-}1$, y_i is the amount of disease (incidence), and t_i is the time of the ith rating. Values followed by same letter in a column are not statistically different ($P = 0.05$). There was no significant difference among progeny and Meyer at 10, 15, 20, and 25 DAI.

Table 5.3 Large patch (caused by *R. solani* AG 2-2 LP) disease incidence of new zoysiagrass lines and 'Meyer' under growth chamber conditions[z] in 2010.

Progeny/Parent	Days after inoculation (DAI)[y]					AUDPC[x]
	5	10	15	20	25	
Cavalier × Chinese Common						
5311-16	0.0b	22.2abcd	39.9ab	33.3	22.6bc	106.8abc
5312-55	0.0b	14.7bcd	30.6ab	38.3	21.4bc	94.3abcd
Zorro × Meyer						
5313-34	0.0b	34.1ab	36.5ab	52.8	21.0bc	133.9ab
5313-71	0.0b	6.1cd	27.3ab	23.3	31.8abc	72.6cd
5313-46	0.0b	40.5a	35.9ab	46.7	27.8abc	137.0a
Emerald × Meyer						
5321-9	0.0b	16.7abcd	39.7ab	35.7	9.4c	96.8abc
5321-18	0.0b	0.0d	18.7bc	17.8	26.8abc	49.9cd
Meyer × 8501						
5324-26	0.0b	0.0d	3.3c	29.0	19.4bc	42.0d
5324-32	5.7a	19.4abcd	47.0a	20.0	36.9ab	107.7abc
Meyer × 8508						
5325-11	0.0b	22.9abcd	35.6ab	34.1	42.4ab	113.8abc
Meyer × Diamond						
5327-67	0.0b	26.7abc	19.8bc	15.3	22.9bc	73.2bcd
Emerald × Zenith						
5334-59	0.0b	0.0d	20.9bc	n/a	21.5bc	n/a
DALZ0102	0.0b	4.8cd	19.3bc	25.7	37.3ab	68.5cd
Meyer	0.0b	12.2bcd	33.7ab	26.4	42.4ab	93.6abcd

[z]Plants were maintained in the growth chamber at 25°C, 13-h photoperiod, and a relative humidity of ~95%;
[y]Values expressed as a percentage of individual plants showing water-soaked sheath blighting symptom averaged across three replicated pots destructively sampled at each time point (DAI) for each zoysiagrass line. n/a = not available.
[x]Area under disease progress curve (AUDPC). Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (incidence), and t_i is the time of the ith rating;
Values followed by similar letter in a column are not statistically different ($P = 0.05$). There was no significant difference among progeny and Meyer at 20 DAI.

Table 5.4 Large patch (caused by *R. solani* AG 2-2 LP) diameter and percentage of diseased turf of new zoysiagrass lines and 'Meyer' under field conditions at Manhattan, KS in 2009.

	Ave patch diam (cm)		Percentage of diseased turf*			
Progeny/Parent	Jun-12	Jun-19	Jun-21	Jun-27	Jul-3	AUDPC**
Cavalier × Chinese Common						
5311-16	109.0	114.0	8.9ab	5.9a	3.0b	16.7ab
5312-55	116.5	116.0	12.2a	3.0ab	4.9ab	31.3a
Zorro × Meyer						
5313-23	128.5	121.5	4.6ab	3.8ab	1.9b	14.7ab
5313-34	105.8	111.8	5.2ab	1.7b	5.8ab	9.2ab
5313-71	118.3	120.3	1.8b	0.5b	2.2b	6.4b
5313-46	99.5	115.5	1.8b	1.7b	2.4b	7.6ab
Emerald × Meyer						
5321-9	103.8	115.8	5.0ab	1.9b	10.7a	12.0ab
5321-18	119.3	119.5	2.8b	1.6b	2.6b	8.2ab
Meyer × 8501						
5324-26	109.5	116.0	6.3ab	2.1ab	7.3ab	20.0ab
5324-32	91.0	118.5	4.0ab	3.4ab	1.4b	12.0ab
Meyer × 8508						
5325-11	104.0	120.2	4.7ab	1.0b	2.9b	8.8ab
Meyer × Diamond						
5327-67	128.8	127.8	3.2ab	0.4b	2.8b	9.6ab
Emerald × Zenith						
5334-59	131.3	113.3	8.1ab	1.5b	7.2ab	17.3ab
DALZ 0102	113.5	96.8	3.4ab	2.4ab	1.4b	11.5ab
Meyer	127.5	117.8	2.3b	1.5b	4.0b	6.8ab

*Values show percentage of pixels representing large patch symptom from digital images taken of plots of each zoysiagrass line and averaged across three replicated plots per line. The plots were inoculated in the fall of 2008.
**Area under disease progress curve for the percentage of diseased turf. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (percentage of diseased turf), and t_i is the time of the ith rating. Values followed by same letter in a column are not statistically different ($P = 0.05$). There was no significant difference in patch size among progeny and Meyer on June 12 and 19.

Table 5.5 Percentage of large patch (caused by *R. solani* AG 2-2 LP) diseased turf of new zoysiagrass lines and 'Meyer' under field conditions at Manhattan, KS in 2010.

	Percentage of diseased turf*				
Progeny/Parent	May-1	May-7	May-28	June-24	AUDPC**
Cavalier × Chinese Common					
5311-16	28.6ef	27.2de	36.6de	17.1bc	86.7ef
5312-55	38.0cdef	39.9cde	47.0bcde	9.5c	110.6def
Zorro × Meyer					
5313-23	61.3abcd	46.1cde	45.3bcde	8.6c	126.4cdef
5313-34	48.4bcdef	43.8cde	48.4bcde	11.6bc	122.2def
5313-71	79.9a	80.2a	77.9a	39.7a	217.9a
5313-46	77.2a	72.2ab	76.8a	12.3bc	193.9ab
Emerald × Meyer					
5321-9	58.1abcd	50.8bcd	43.9cde	15.3bc	131.4cde
5321-18	27.8ef	35.2cde	54.9bcd	18.4bc	113.2def
Meyer × 8501					
5324-26	52.8abcde	45.6cde	53.7bcd	14.3bc	132.8cde
5324-32	61.7abcd	47.8bcde	65.9ab	11.1bc	150.1bcd
Meyer × 8508					
5325-11	64.6abc	58.7abc	60.4abc	18.0bc	160.4bcd
Meyer × Diamond					
5327-67	70.1ab	72.3ab	66.0ab	14.1bc	180.4abc
Emerald × Zenith					
5334-59	35.2def	29.8de	49.4bcde	27.2ab	110.4def
DALZ 0102	53.4abcde	57.3abc	52.1bcde	13.2bc	142.7bcd
Meyer	20.14f	23.5e	31.0e	19.8bc	74.5f

*Values are percentage of pixels representing large patch symptom from digital images taken of plots of each zoysiagrass line and averaged across three replicated plots per line. The plots were inoculated in the fall of 2008;
**Area under disease progress curve for the percentage of diseased turf. Calculated as $\sum [(y_i + y_{i+1})/2][t_{i+1} - t_i]$, where $i = 1,2,3,\ldots,n-1$, y_i is the amount of disease (percentage of diseased turf), and t_i is the time of the ith rating. Values followed by same letter in a column are not statistically different ($P = 0.05$).

Chapter 6 - Conclusions

I characterized 36 *Rhizoctonia* isolates from zoysiagrass exhibiting large patch symptoms collected from different golf courses in Kansas based on anastomosis grouping, in-vitro mycelial growth rates, nuclear counts, virulence, PCR, and amplified fragment length polymorphism (AFLP). I evaluated the effects of cultivation (aerification, verticutting and sand topdressing) on soil temperature, thatch temperature, and volumetric soil water content; the effect of cultivation and time of nitrogen (N) fertilization. In addition, I studied the effects of different preventative application timings for three fungicides- flutolanil, azoxystrobin, and triticonazole in spring and fall on large patch development. Finally, I evaluated fourteen new zoysiagrass lines from crosses involving one parent from *Z. japonica* and one from a *Z. matrella* cultivar or Emerald (*Z. japonica* × *Z. pacifica*) for resistance to large patch disease under growth chamber and field conditions.

All the *R. solani* isolates from large patch-infected zoysiagrass from Kansas belonged to AG 2-2 LP. Variations were observed among the isolates in their average number of nuclei per cell, mycelial growth rates and virulence. Additionally, a significant negative correlation ($P < 0.05$, $r = -0.53$) existed between average number of nuclei per cell and mycelia growth rate. There was also variation in the isolates' amplified fragment length polymorphism (AFLP) DNA fingerprint, suggesting possible underlying genetic differences of biological significance among members of AG 2-2 LP. Further studies are needed to validate the observed variability in the average number of nuclei per cell, mycelia growth rates, and virulence, and the correlation between them, among isolates of *R. solani* AG 2-2 LP. The AFLP protocols developed in this research can be utilized for larger-scale population studies to answer questions related to pathogen spread, relationships among isolates from different host species, etc.

There was no significant (*P*=0.05) difference in soil and thatch temperatures as well as volumetric soil water content between cultivated and non-cultivated plots. Cultivation also did not result in a significant and consistent reduction in patch sizes. From 2010 to 2011, spring and fall N fertility was consistently associated with lower percentages of diseased turf in both cultivated and non-cultivated plots at Manhattan and Haysville. Additional studies evaluating the effects of core-aerification, verticutting, and sand topdressing, as well as timing of fertilization on patch size development and severity of blighting by digital image analysis over multiple years across different locations will be important for understanding what impact summer cultivation and fertilization during spring and fall versus summer has on the disease. Data from such studies will also constitute important considerations toward the formulation of recommendations for cultural practices, especially for golf course superintendents.

Although fungicide deployment for the management of large patch is recommended when thatch temperatures reach 21-24°C, the earliest single applications of flutolanil in the fall of 2008 and 2009, unlike similar applications of azoxystrobin and triticonazole, when the thatch temperature averaged 20.7^{o}C and 21.6^{o}C, respectively, failed to achieve a significant reduction in patch sizes compared with the untreated controls. The results of the 2009 and 2010 spring application timing studies showed the earlier fungicide applications provided better control of large patch symptoms than later spring applications. In both the fall and spring application timing studies, some single applications provided better control than sequential applications of the fungicides. Although we observed differences in large patch control for some of the fungicide application timings we evaluated, further studies involving application timings based on pre-determined thatch temperatures for different classes of fungicides could significantly

improve our knowledge of the optimum application timing for different classes of fungicides, and consequently reduced management costs.

There was no significant correlation between the performance of the progenies under growth chamber conditions and field conditions in 2009 and 2010 respectively. Although all progeny performed comparably with Meyer under growth chamber conditions, only six progeny consistently had AUDPC large patch levels as low as Meyer under field conditions. In future studies, it is recommended to utilize large plot sizes in order to observe patch size increase over multiple years, to evaluate progeny in multiple sites, and to screen resistance to multiple isolates.