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Etiology and Epidemiology of Mini-ring in Ultradwarf Bermudagrass Putting Greens

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ETIOLOGY AND EPIDEMIOLOGY OF MINI-RING IN ULTRADWARF BERMUDAGRASS PUTTING GREENS

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by
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ABSTRACT

Mini-ring is a disease in ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens caused by *Waitea zeae* (Voorhees) J.A. Crouch & Cubeta, (formerly *Rhizoctonia zeae*). Symptoms typically resemble frog-eye patches that are 10 to 40 cm in diameter with a bronze to orange outer ring and green center. In the southeastern United States, mini-ring symptoms appear in late-summer and generally persist until UDBG dormancy in late-fall. Mini-ring is often problematic in UDBG when nitrogen (N) fertility is reduced to manage organic matter production and improve putting green performance and perceived green speed. While *W. zeae* is most frequently reported as the causal agent, other species of *Waitea* have been isolated from UDBG exhibiting mini-ring symptoms. *Waitea zeae* causes visible leaf lesions and basal sheath rot in other turfgrasses; however, in UDBG, dieback of leaf tissue occurs in the absence of leaf lesions and sheath rot. Although *W. zeae* has been isolated from UDBG leaf tissue throughout the growing season, it is unclear if other plant tissues—e.g., root, rhizomes, and stolons—may be possible infection courts and when *W. zeae* infection most likely occurs. The objectives of these studies were to: I) investigate the impact of N source and N rate on mini-ring disease development and severity in UDBG; II) determine what plant tissues *W. zeae* can infect and when infection is most likely to occur; and III) collect and characterize isolates of *Waitea* spp. recovered from symptomatic UDBG putting greens.

To study the impact of N on mini-ring disease severity, ammonium sulfate (AMS) [$(NH_4)_2SO_4$] and urea (CH_4N_2O) were applied weekly to 'P18' (MiniVerde) and 'TifEagle' UDBG at rates of 4.9, 9.8, and 14.7 kg N ha^{-1}. Mini-ring severity increased with increasing rates of AMS whereas disease symptoms in plots treated with urea remained relatively low.

Cores from a UDBG putting green located in Florence, SC were collected monthly from June to October in 2016 and 2017. Isolation of *W. zeae* occurred in all months, isolation frequencies were greatest in August and September.

In a growth chamber bioassay, UDBG roots, stolons, and leaves were infected by *W. oryzae* or *W. zeae* when inoculum when growing medium was infested with representative isolates.

Nineteen *Waitea* species isolates were recovered from UDBG putting greens expressing mini-ring symptoms in North Carolina and South Carolina. Isolates of *W. prodiga*, *W. oryzae*, and *W. zeae* represented 5, 16, 79% of isolates collected. Isolates were characterized by sequencing the rDNA-internal transcribed spacer region, and these sequences clustered with *Waitea* species isolate sequences deposited in GenBank and previously described. These studies demonstrate the primary causal agent of mini-ring in UDBG is *W. zeae*, while other species of *Waitea* are likely involved to a lesser extent.

An effective mini-ring management strategy should include regular N applications using N sources other than AMS, such as urea, to promote UDBG growth and recuperative potential and applications of fungicides mid-summer before symptom development. Fungicides should be applied in a manner that encourages movement of active ingredients into the putting green rootzone to reduce *W. zeae* infection of UDBG roots and stolons.

DEDICATION

As iron sharpens iron, so one person sharpens another.

— Proverbs 27:17

This dissertation is dedicated to all individuals who have sharpened me as a father, co-worker, and friend. I wish I could name you all. Below are just a few individuals that have a made life as good as good as it could be.

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CHAPTER ONE

LITERATURE REVIEW

Mini-ring and leaf and sheath spot

The disease formerly known as Rhizoctoina leaf and sheath spot, now leaf and sheath spot (LSS) or mini-ring in hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens, is caused by the pathogen *Waitea zeae* (Voorhees) J.A. Crouch & Cubeta, (formerly *Rhizoctonia zeae* Voorhees*;* teleomorph = *Waitea circinata* Warcup & Talbot) (Dant & Martin, 2023; Kerns et al, 2017; Smiley et al., 2005). *Waitea oryzae* J.A. Crouch & Cubeta (formerly *R. oryzae* Ryker & Gooch; teleomorph: *W. circinata*) has also been associated with LSS symptoms, but *W. zeae*, appears to play a more prominent role in turfgrass disease development (Elliott, 1999; Kerns et al., 2017; Martin & Lucas, 1983; Martin & Lucas, 1984). Rhizoctoina leaf and sheath spot does not describe the predominate symptoms visible to the turfgrass practitioner; however, this disease common name was likely adapted from sheath spot, which is the common name for disease caused by *W. oryzae* in rice (B. Martin & J. Kerns, personal communication, November 2022). Additionally, *R. zeae* and *R. oryzae* have recently been reclassified within the genus *Waitea*, further reducing the utility of the disease name Rhizoctoina leaf and sheath spot (Stalpers et al., 2021).

For the purposes of this review, *Rhizoctonia oryzae* and *R. zeae* are used interchangeably with *Waitea oryzae* and *W. zeae*. Additionally, *R. oryzae* and *R. zeae* are the predominate nomenclature used in the references cited section of this review.

Although the epidemiology of LSS is not well understood, it is considered a mid-summer, hot weather disease as mycelium growth of *W. zeae* occurs rapidly between 28 to 36 °C, with an optimum temperature of 30 to 32 °C (Burpee & Martin, 1992; Elliott, 1999; Kammerer, 2011; Kerns et al., 2017). *Waitea oryzae*, which is also associated with symptoms of LSS, prefers temperatures near 30°C as well. (Burpee & Martin, 1992). For reference, the temperature optimum for *R. solani*, the pathogen which

causes brown patch and large patch, is approximately 18-28°C (Burpee & Martin, 1992; Martin & Lucas, 1983; Smiley et al., 2005). Reproduction of *W. zeae* and *W. oryzae* in turf is not well understood; however, it is believed to be primarily through vegetative means, as asexual spores are not produced and sexual structures are rarely visible in nature, especially in turfgrass systems. (Burpee & Martin, 1992; Martin, 2009). Both *W. zeae* and *W. oryzae* likely overwinter and survive unfavorable environment conditions as sclerotia. Both species produce abundant sclerotia in culture and sclerotia of *W. zeae* can be present within the canopy of symptomatic turf (Burpee & Martin, 1992; Dant, unpublished data, 2022). It is also possible mycelium within leaf tissue or decaying organic matter are sources of inoculum and germinate when conditions are favorable (Burpee & Martin, 1992).

Waitea zeae is an economically important pathogen of both creeping bentgrass (*Agrostis stolonifera* L.) and tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] (Burpee & Martin, 1992; Inguagiato & Martin, 2015; Martin & Lucas, 1983). Researchers have demonstrated pathogenicity in other cool-season turfgrasses including Kentucky bluegrass (*Poa pratensis* L.), roughstalk bluegrass (*Poa trivialis* L.) and perennial ryegrass (*Lolium perenne* L.); however, epidemiology within these turfgrass species is not well understood (Burpee & Martin, 1992; Inguagiato & Martin, 2015; Kammerer et al., 2011; Martin & Lucas, 1983). In tall fescue and creeping bentgrass, *W. zeae* causes yellow to orange to brown rings or patches, very similar in appearance to symptoms of brown patch (causal agent: *R. solani*) (Burpee & Martin, 1992, Koehler & Shew, 2017, Smiley et al., 2005). In creeping bentgrass, *W. zeae* does not normally form a “smoke-ring”, which is a characteristic of brown patch (Burpee & Martin, 1992). *Rhizoctonia solani* and *W. zeae* cause similar leaf lesions on tall fescue and patch-like symptoms caused by both pathogens are undistinguishable; therefore, isolation is necessary to determine which pathogen is responsible for inciting disease on these hosts. (Burpee & Martin, 1992; Koehler & Shew, 2017; Smiley et al., 2005).

Nearly all warm-season grasses cultivated for aesthetic purposes are potential hosts for *Waitea zeae*. When *W. zeae* infects centipedegrass [*Eremochloa ophiuroides* (Munro) Hack], or St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze], the predominate symptoms are leaf lesions and basal sheath rot (Burpee & Martin, 1992; Haygood & Martin, 1990). In seashore paspalum, (*Paspalum vaginatum* Swartz), symptoms caused by *W. zeae* are not well described, but generally manifest as chlorosis and necrosis of lower leaf blades (Kammerer et al., 2011). *Waitea zeae* causes similar symptoms in *Cynodon* spp. where leaf blighting and lower leaf dieback occurs and leaves lack lesions; however, on a macrolevel, the diseases forms "frog-eye" patches 10 to 30 cm in diameter (Kerns et al., 2017; Martin, 2009). Symptoms described as frog-eye patches are generally limited to *Cynodon* putting greens planted to dwarf or ultradwarf bermudagrass (UDBG). *Rhizoctonia* leaf and sheath spot (now leaf and sheath spot) is rarely used to describe the symptoms caused by *W. zeae* in UDBG (except in academic publications) instead the name "mini-ring" has been coined because it more accurately describes the appearance of diseased turf. For consistency and based on historical reference, turfgrass pathologists and practitioners should use the common name "mini-ring" to refer to the disease caused by *W. zeae* (and possibly *W. oryzae)* in dwarf or ultradwarf bermudagrass.

Mini-ring became problematic in the late-1990s and early-2000s as UDBG became a popular putting surface for golf courses located within the transition zone and subtropical climates of the United States (Elliott, 1999; Kerns et al., 2017; Martin, 2009). As stated above, symptoms generally mimic a frog-eye patch, where the leading-edge of the patch is bleached or straw-to-tan in color and the center of the patch contains green turf which visually, appears unaffected (Inguagiato & Martin, 2015; Kerns et al., 2017; Martin, 2009). In some cases, faint partial rings, bronze or orange in color, may develop prior to the onset of the frog-eye-like symptoms (Martin, 2009). Mini-ring symptoms are most apparent in late-summer and fall in the transition zone and southeast United States. Yet, they can develop in early-

summer if temperatures are consistently warm and bermudagrass is compromised due to abiotic or biotic stress (Inguagiato & Martin, 2015; Kerns et al., 2017; Martin, 2009).

Managing mini-ring in UDBG with fungicides has been a challenge. Several researchers have shown that *W. zeae* and *W. oryzae* are insensitive to benzimidazole fungicides – e.g., benomyl and thiophanate-methyl (Burpee & Martin, 1992; Elliott, 1999; Kerns et al., 2017, Martin et al., 1984). Kerns et al (2017) demonstrated that *W. zeae* was sensitive to several demethylation inhibitor (DMI), quinone outside inhibitor (QOI) and succinate dehydrogenase inhibiting (SDHI) fungicides when isolates were exposed *in vitro*. Results from fungicide efficacy and performance trials conducted in the field have been variable and fungicides performance was inconsistent over multiple locations and years (Dant, unpublished data, 2022). Poor and inconsistent fungicide efficacy when applied in the field is likely due to a poor understanding of disease etiology and epidemiology, as well as other factors, such as aggressive agronomic practices, inadequate or incorrect fertility, nematodes and environmental factors that limit UDBG recuperative potential (Dant, unpublished data, 2022; Kerns et al., 2017; Martin, 2009)

Waitea zeae **and the genus** ***Waitea***

Voorhees (1934) first characterized *R. zeae* after it was isolated from rotten ears of field corn (*Zea mays* L.) grown in Florida. *Rhizoctonia zeae* reduced corn emergence and infected corn seedlings when inoculum was placed in soil or on the soil surface and it infected and colonized artificially inoculated ears of corn. Voorhees (1934) concluded the fungus was a new species and described it as having multi-septate hyphae with constrictions at the septa. The hyphae branched from parent hyphae at right angles and ranged from 4 to 10 µm in width. In culture, hyphae were initially hyaline but mycelium became reddish-brown as the culture matured. Voorhees (1934) documented that *R. zeae* readily produced sclerotia 0.1 to 1.0 mm in diameter and white in color when young but when mature, turned brown to dark-brown. Based on the above description, Voorhees (1934) concluded the fungus belonged to the genus *Rhizoctonia*.

Warcup and Talbot (1962) characterized the teleomorph of *R. zeae* and placed it in newly created genus *Waitea* and gave it the specific epithet, *W. circinata.* Their isolate, described as saprophytic, was collected from soil at the Waite Institute in Adelaide, South Australia. Warcup and Talbot (1962) described their isolate as having "*Rhizoctonia*-like" hyphae mostly submerged in culture and produced no clamp connections. Cultures were pink to white and formed pink to orange sclerotia that were up to 2 mm in diameter. Basidia were characterized as being somewhat cylindrical in shape, supporting four horn-like sterigmata, with elliptical-shaped hyaline spores often developing one or two septa. Warcup and Talbot (1962) suggested many characteristics of their isolate were similar to *Thanatephorus cucumeris* (teleomorph of *R. solani*), but also noted there were several differences. Basidiospores of *T. cucumeris* have no septa and frequently germinate; in contrast, *W. circinata* basidiospores may contain one or two septa dividing the spore horizontally, and do not germinate with regularity (Warcup and Talbot, 1962). Additionally, *W. circinata* sterigmata are "horn-like" and short whereas sterigmata of *T. cucumeris* are long and tube-like, widening at the base (Warcup and Talbot, 1962).

The taxonomy of the anamorph *W. zeae* and teleomorph *W. circinata* is complex, in part due to their association and relative similarities to *R. solani*. Gunnell (1986), Oniki et al. (1985), and Windham (1985) appear to be the first to link the teleomorph, *W. circinata,* to the anamorph, *R. zeae*. Gunnell (1986) further suggested three *W. circinata* varieties (*W. circinata* var. *circinata*, var. *oryzae* and var. *zeae*) be created to accommodate anamorphs *R. circinata*, *R. oryzae*, and *R. zeae*. Gunnell's proposal was only published in her Ph.D. dissertation (1986) and never formally published in a manuscript or accepted. A year later, Moore (1987), attempted to define and segregate *Rhizoctonia*-like anamorphs based on morphology of speta in hyphae and, in doing so, recommended a new genus *Moniliopsis,* be created to accommodate anamorphs species of the teleomorphs *Waitea* and *Thanatephorus*. In this classification system, *R. zeae* was renamed *Moniliopsis zeae.* Although this change was procedurally

correct, it further complicated the taxonomic classification of *R. zeae* because the genus *Rhizoctonia* was well established (Burpee & Martin, 1992). Citing the work conducted by Gunnell (1986), and their own research, Leiner and Carling (1994) suggested it should be classified as *R. circinata var. zeae* rather than referring to the anamorph as *R. zeae*. This would allow for the anamorphs *W. oryzae* (formerly *R. oryzae* Ryker & Gooch) and *W. circinata* (no valid anamorph at the time of the Leiner and Carling publication) to be grouped with *R. zeae* as *R. circinata* var. *oryzae* and *R. circinata* var. *circinata* and in part, retain the well-recognized *Rhizoctonia* genus designation. This classification would also link the anamorphs to the teleomorph *W. circinata*; however, linking three distinct anamorphs as varieties to a single teleomorph was not ideal. Also, *R. zeae* and *R. oryzae* had been used extensively in previous literature and *R. circinata* had not been utilized prior to the Leiner and Carling publication in 1994, which created more confusion and discord in the plant pathology community.

Staplers & Anderson (1996) agreed with Moore's (1987) classification of *R. zeae*; however, Andersen (1996) later suggested the anamorphs of *Waitea* spp. be removed from the genus *Moniliopsis*. Stalpers and Andersen (1996) proposed creating the genus *Chrysorhiza*, to accommodate *Waitea* spp. anamorphs. Ultimately, *Chrysorhiza* and *Moniliopsis* genera did not receive widespread acceptance. In 1998, Stalpers et al. published a paper to conserve the genus *Rhizoctonia* and establish the type species as *R. solani*, the anamorph of *T. cucumeris*. Unfortunately, Stalpers et al. (1998) only mentioned the *Waitea* anamorphs are "considered to belong to *Rhizoctonia* s.l."; therefore, taxonomic confusion within the genus *Waitea* (and forementioned anamorphs) remained.

From the late-1990's until 2021, the anamorph *W. zeae* was referred to as *Chrysorhiza zeae*, *R. zeae, W. circinata* var. *zeae* with the latter two used most frequently (Aydin et al., 2013; Chen et al., 2009; de la Cerda et al., 2007; Elliott, 1999; Gürkanli et al., 2016; Kammerer, 2011; Kammerer et al., 2011; Kerns et al., 2017; Okubara et al., 2008; Toda et al., 2005; Toda et al., 2007; Tomaso-Peterson & Trevathan, 2007; Vojvodić et al., 2021). During this same timeframe, two new *W. circinata* varieties, var.

prodigus and var. *agrostis*, were established outside of taxonomic convention and are now invalid (Kammerer, 2011; Kammerer et al., 2011; Sharon et al., 2008; Stalpers et al., 2021). The anamorph of *W. circinata* (synonym = *W. circinata* var. *circinata*) was studied extensively during this same period, although the anamorph was never named (Aydin et al., 2013; Chen et al., 2009; de la Cerda et al., 2007; Gürkanli et al., 2016; Kammerer, 2011; Kammerer et al., 2011; Toda et al., 2005; Toda et al., 2007; Vojvodić et al., 2021). In 2021, Stalpers et al. identified one scientific name per fungus in the taxon *Agaricomycotina*, a subdivision of *Basidiomycota* and addressed the *Waitea* teleomorphs and their corresponding *Rhizoctonia* anamorphs. Four new species were created within the preserved *Waitea* genus (Stalpers et al., 2021). *Waitea circinata* was also preserved and selected as the type species for the genus (Stalpers et al., 2021). Table 1.1 summarizes the new taxonomic classification scheme within the genus *Waitea* as well as select references to previously used invalid naming conventions.

Since their discovery, morphological characteristics, (hyphal diameter, sexual spore and basidium shape and size, colony color, and sclerotium size, shape and color), nuclear number, and later anastomosis reactions have been used to differentiate members of the genus *Waitea*, frequently following techniques used to identify isolates of *R. solani* (Aydin et al., 2013; Burpee & Martin, 1992; Haygood & Martin, 1990; Kammerer et al., 2011). Morphological characteristics are variable, shape and color descriptions are subjective and vary with medium and *Waitea* spp. infrequently produce basidia; therefore, classification beyond the species level has proven difficult and some authors have questioned whether morphological differences (mainly among anamorphs) constitute the need for separate species (Aydin et al., 2013; Gunnell, 1986; Leiner & Carling, 1994). Anastomosis reactions have been helpful in distinguishing strains of *R. solani*, but reactions can be inconsistent and have only been utilized on a limited basis within the genus *Waitea*; consequently, anastomosis groups (AG) and tester isolates are not well-established (Leiner & Carling, 1994; Oniki et al., 1985; Toda et al., 2007; Vigalys & Cubeta, 1994). Molecular methods were first used in the late-1980s and early-1990s to discriminate among *R.*

solani populations (Cubeta et al., 1996). These methods included DNA hybridization, restriction fragment length polymorphism (RFLP), and ribosomal DNA (rDNA) amplification and sequence analysis (Cubeta et al., 1996).

Anderson (1996) appears to be one of the first researchers to use molecular techniques to categorize species of the genus *Waitea*, when he used RFLP and concluded that *W. zeae* and *W. circinata isolates* were similar, yet markedly different from isolates of *R. solani* included in the study. Mazzola et al., also in 1996, used amplified rDNA sequences of the internal transcribed spacer region (ITS) to distinguish isolates of *W. oryzae* from isolates of *R. solani* isolated primarily from wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.) and designed two specific primers capable of detecting *W. oryzae* infection in plant tissue. The first extensive molecular study focused on members of the genus *Waitea* was published in 2005 by Toda et al. and used both random amplified polymorph DNA-polymerase chain reaction (RAPD-PCR) and RFLP. Toda et al. (2005) showed both RFLP and RAPD-PCR could be used alone and in combination to identify and group species of *Waitea*. In 2007, Toda et al. successfully sequenced the rDNA-ITS region of *W. agrostidis* (an unidentified *Waitea* species at time of publication), *W. circinata*, *W. oryzae*, and *W. zeae*. After the Toda et al. (2007) publication, rDNA-ITS region sequence comparisons, morphological characteristics and mycelial growth rate were used as primary factors for speciation of isolates in the genus *Waitea* (Aydin et al., 2013; Chen et al., 2009; de la Cerda et al., 2007; Kammerer, 2011; Kammerer et al., 2011; Kerns et al., 2017; Vojvodić et al., 2021). Gürkanli et al. (2016), amplified the 18S gene and β-*tubulin* gene of three *Waitea* species, but did not sequence the ITS region; therefore, it cannot be concluded from their work if 18S or B-tubulin gene sequences provide more taxonomic value than sequences of the ITS region.

More recently, Vojvodić et al. (2021) sequenced four genomic regions including the ITS region and part of the large RNA subunit (LSU), as well as the β-*tubulin*, and RNA polymerase II, subunit B (RPB2) genes. They concluded:

> "Direct sequencing of four targeted genome regions (ITS, LSU, RPB2, and β-tubulin) proved that only the ITS region is reliable for variety-level (now species-level, i.e., *W. circinata* var. *zeae* = *W. zeae*) identification. Due to the small number of available sequences in GenBank, only species-level (now genus-level, i.e. *Waitea*) identification is achievable by using direct sequences of the LSU, RPB2, and β-tubulin genomic regions" (Vojvodić et al., 2021, p. 794).

Increasing the number of available sequences from *Waitea* species isolates of genes or regions other than ITS is required to examine if these alternative molecular sequences can provide taxonomic resolution within the genus *Waitea* that the ITS region cannot.

Ultradwarf bermudagrass

The predominate golf course turf in the southern agronomic region of the United States is bermudagrass (*Cynodon* spp.) (Lyman et al., 2007). Bermudagrass is a C_4 grass, which aggressively produces both rhizomes and stolons which make an ideal turf for sod production and high-traffic sites due to its recuperative potential (Beard, 1973; Cui et al., 2021; McCarty, 2018). Bermudagrass can thrive in a range of soil types and is heat, drought, and salt tolerant (Beard, 1973; McCarty, 2018). Leaf color, texture, and density of bermudagrass varies significantly among and within species, as does tolerance of low cutting heights. Consequently, bermudagrass can successfully be maintained on all golf course playing surfaces (Christians, 2004; Reasor et al., 2018). The inability of bermudagrass to thrive in low-light settings and its low tolerance of cold temperature are significant limitations (Christians, 2004; McCarty, 2018). Bermudagrass growth ceases at approximately 10°C and death can occur at temperatures below -7°C (McCarty, 2018; Reasor et al.,2018).

Based on survey data, Lyman et al. (2007), calculated that bermudagrass is planted on over 195 thousand hectares of golf course turf, which represents 32% of total maintained golf course turf area in the US. In the southeast, southwest, and transition zone agronomic regions of the US, where the climate is suitable for bermudagrass growth, 67% of maintained golf course turf is planted to it (Lyman

et al., 2007). Both common bermudagrass [*Cynodon dactylon* (L.) Pers var. *dactylon*], and hybrid bermudagrass, a cross between common bermudagrass and African bermudagrass [*C. transvaalensis* (Burtt-Davy)], are used on tee boxes, fairways, and rough. Only the hybrid is used as putting green turf. As of 2007, bermudagrass putting greens represented 43% of putting green turf in the southern agronomic region (southeast, southwest, and transition zone) (Lyman et al., 2007). This percentage is higher given the continued conversion of creeping bentgrass putting greens to bermudagrass in the transition zone since 2007 (L. B. McCarty, personal communication, 2022).

The genus *Cynodon* is believed to have originated in southeastern Africa and was dispersed by foraging hoofed animals to the present-day countries of Afghanistan, Australia, China, India, and South Africa (Beard, 2013; Cui et al., 2021; Forbes & Burton, 1963; Harland and de Wet, 1969; McCarty, 2018; Taliaferro, 1995). It is likely Spanish conquistadors unknowingly introduced common bermudagrass to the United States, in the 1600s but possibly earlier (Beard, 2013; McCarty, 2018). Common bermudagrass was the predominate bermudagrass turf used on golf courses until the mid-1900s when several cultivars of hybrid bermudagrass were introduced (McCarty, 2018).

'Tiffine' (Tifton 127) a sterile bermudagrass hybrid, was released in 1953 by breeder Dr. Glenn W. Burton, a United States Department of Agriculture geneticist located at the University of Georgia Coastal Plain Experiment Station in Tifton, Georgia, and is believed to be the first hybrid bermudagrass produced specifically for putting green use (Burton, 1991; Robinson & Burton, 1953). Based on limited references in the literature and because 'Tifgreen' was released shortly after, in 1956, 'Tiffine' did not become a widely planted bermudagrass putting green cultivar. Also, a common and African bermudagrass hybrid, Burton (1991) characterized 'Tifgreen' (Tifton 328) as a better putting green grass then 'Tiffine'. Planted on hundreds of golf courses within the United States, 'Tifgreen' could be mowed daily at a height of 6.4 mm (Burton, 1964). Burton (1964) described 'Tifgreen' as a low-growing, rapid spreading, disease-resistant hybrid with fine leaves that created a dense turf ideal for putting greens.

Drs. Burton and J. Earl Elsner released a vegetative mutant of 'Tifgreen' in 1965 and named it 'Tifdwarf' (Burton & Elsner, 1965). When compared to 'Tifgreen', 'Tifdwarf' was touted as a superior putting green turf due to its darker green color, greater density and ability to withstand mowing heights as low as 4.8 mm (Burton & Elsner, 1965). The term "dwarf"—used to describe low growing, dense bermudagrass hybrids, capable of withstanding mowing heights less than 5 mm—appears to coincide with the release of 'Tifdwarf' (Burton & Elsner, 1965; Moncrief, 1967).

From the introduction of 'Tifdwarf' in 1965, until the late-1990s, little improvement occurred in bermudagrass cultivars for use on golf course putting greens (Reasor et al., 2016). 'Tifdwarf', and to some extent, 'Tifgreen' became the standard cultivars for bermudagrass putting greens during this approximate 30-year period. Somatic mutations or "off-type" bermudagrass cultivars from 'Tifgreen' and 'Tifdwarf' were continually being selected, evaluated, and released by various bermudagrass breeding programs across the southeastern United States; however, none were widely accepted until the mid- to late-1990s (McCarty, 2018; Reasor et al., 2018; Reasor et al., 2016). The term "ultradwarf" became popular during this time as a collective adjective to describe bermudagrass cultivars with fine leaf blades capable of forming a dense turf canopy when maintained at heights at or below 3.2 mm (McCarty, 2018; Reasor et al., 2016). Some of the most popular UDBG cultivars include 'Floradwarf', 'Champion Dwarf' (also known as 'Champion'), 'MiniVerde' (originally registered as cultivar 'P-18'), and 'TifEagle'. Both 'Champion' and 'MiniVerde' were selected from existing Tifdwarf putting greens, while Floradwarf is purportedly an off-type originally derived from 'Tifgreen' (Reasor et al., 2016). 'TifEagle' is the product of a radiation-induced mutation from 'Tifway II' or 'Tifgreen' and appears to be more genetically stable than the other commercially produced UDBG cultivars (Hanna & Elsner, 1999; Harris-Shultz et al., 2011; McCarty, 2018; Reasor et al., 2016; Zhang et al., 1999).

Shortly after the introduction and widespread adoption of UDBG by the golf course industry several agronomic challenges were identified. Due to the aggressive lateral growth and high shoot

density of UDBG, thatch and other forms of organic matter accumulation can become problematic on putting greens if not addressed (Hollingsworth et al., 2005; Rowland et al., 2009a; Rowland et al., 2009b; Unruh et al., 2005; White et al., 2004). Frequent vertical mowing (aka, "grooming") in combination with sand topdressing is required to manage thatch and maintain acceptable putting green quality (McCarty, 2018; McCarty & Canegallo, 2005). Additionally, aggressive hollow-tine aerification followed by sand topdressing must be conducted periodically to remove thatch and organic matter (McCarty & Canegallo, 2005). Turf managers have also limited nitrogen (N) fertility to reduce thatch accumulation and increase ball roll distance (Inguagiato & Martin, 2015; McCullough et al., 2006). Depending on growing season length, root zone composition and other site-specific characteristics, research has shown between 6 to 13.5 kg N ha^{-1} wk^{-1} is required to produce high-quality UDBG putting greens (Cisar et al., 2005; Inguagiato & Martin, 2015; McCarty & Canegallo, 2005; McCullough et al., 2006; Unruh et al., 2005; White et al., 2004). However, turf manages tend to provide N fertility at the lower end of the range and potentially as low as 3 to 4.5 kg N ha^{-1} wk^{-1} (Inguagiato & Martin, 2015; Lowe, 2012; Lowe, 2013). Low N fertility reduces UDBG stress tolerance and increases susceptibility to fungus pathogens and plant-parasitic nematodes white limiting recuperative potential.

Outside of the transition zone, in the southern most regions of the United States, leaf spot caused by *Bipolaris* spp. [*Bipolaris cynodontis* (Marignoni) Shoemaker, *B. spicifera* (Bainier) Subram and *B. sorokiniana* (Sacc.) Shoemaker] is likely the most significant fungus disease of UDBG putting greens (Inguagiato & Martin, 2015; Vines et al., 2017). Bipolaris leaf spot can be a problem in the transition zone, but it is less destructive compared to mini-ring. In the transition zone, mini-ring generally causes turf decline in the late-summer and early-fall prior to dormancy, leaving little chance for UDBG to recover (Kerns et al., 2017). Mini-ring can be an issue in the very southern US, but UDBG has the opportunity to recover when the pathogen is suppressed and agronomic practices are modified appropriately. Bermudagrass decline is problematic in UDBG putting greens during warm, cloudy, and

rainy periods, especially in states along the Gulf Coast of the United States (Elliott, 1991; Inguagiato & Martin, 2015; Martin, 2017; Stevens, 2021; Vines, 2015). Bermudagrass decline symptoms have been associated with root-infecting fungi belonging to several genera (e.g., *Gaeumannomyces*, *Candidacolonium* and *Magnaporthiopsis*) and results in thinning and general decline of the turf with symptomology lacking a leading edge or border (Elliott, 1991; Inguagiato & Martin, 2015; Martin, 2017; Stevens, 2021; Vines, 2015). Take-all root rot is caused by a similar group of pathogens, however; large circular patches of declining turf, white-to-gray in color appear in the fall and late-spring and symptoms are most common in the transition zone, near the northern range of where UDBG is grown (Stevens, 2021; Stevens et al., 2022). Bermudagrass decline and take-all root-rot appear to be caused by similar pathogens; however, symptom appearance and environmental conditions favorable for disease development differ; therefore, these diseases should be considered separately (Stevens, 2021). Spring dead spot [*Ophiosphaerella korrae* (J. Walker & A. M. Sm. bis.) Shoemaker; *O. herpotricha* (Fr.) J. Walker; and *O. narmari* (J. Walker & A.M. Sm. bis) H.C. Wetzel, Hulbert & Tisserat] is likely the most destructive disease of bermudagrass in the transition zone, where bermudagrass is dormant for three to five months (Tredway et al., 2009). As the use of UDBG cultivars increases and pushes into the northern range of this turf's adaptability, lack of cold temperature tolerance in combination with pathogens infecting roots in late-summer and fall, such as those pathogens associated with mini-ring, take-all root rot and spring dead spot are becoming increasingly problematic (Kerns et al., 2017; Inguagiato & Martin, 2015; McCarty, 2018).

Golf course putting greens root zones are constructed of sand or native soil modified with sand; and, therefore, are an excellent habitat for plant-parasitic nematodes. Furthermore, many golf courses in the southern United States are located where native soil is high in sand; hence nematodes are naturally present. Ultradwarf bermudagrass is somewhat unique in than it is grown almost exclusively on putting greens and established primarily by sprigging or less commonly by sod. With either

establishment method, the potential of transporting plant-parasitic nematodes, by means of contaminated plant tissue, to a rootzone ideal for nematode reproduction is high (McCarty, 2018, Inguagiato & Martin, 2015). Ultradwarf bermudagrass cultivars are more susceptible to nematode damage than the preceding hybrid bermudagrass cultivars and creeping bentgrass because they generally develop comparably shallow root systems (Inguagiato & Martin, 2015; Kerns et al., 2017; McCarty & Canegallo, 2005). Luc and Crow (2004) reported 87% of Florida golf courses had nematode populations capable of damaging turfgrass. Zeng et al. (2012) conducted a turf nematode survey and found 21 species of plant-parasitic nematodes were present in samples taken from bermudagrass turf in South Carolina, while in North Carolina, 15 species were present. In contrast, only 10 and 12 species of plant-parasitic nematodes were present in samples collected from creeping bentgrass turf from South and North Carolina, respectively. In South Carolina bermudagrass samples, the most prevalent nematodes were ring (*Mesocriconema xenoplax* Raski), spiral (*Helicotylenchus dihystera*), sting (*Belonolaimus longicaudatus* Rau), root-knot (*Meloidogyne graminis*), stubby-root (*Paratrichodorus minor*), and lance (*Hoplolaimus galeatus*) nematodes, present in 79, 58, 56, 50, 49 and 42% of bermudagrass samples, respectively. Root-knot (58%), spiral (54%), sting (50%) and ring (46%) were commonly found in bermudagrass samples from North Carolina. Although the relationship between fungal pathogens and plant-parasitic nematodes has not been well studied, the presence of both groups of plant pathogens makes management of UDBG challenging (Gu & Crow, 2016; McCarty, 2018).

Fungicides are frequently and readily used on golf course putting greens to manage pathogenic fungi. Currently, fungicides from nine unique Fungicide Resistance Action Committee (FRAC) groups are available for use in the golf course maintenance market (Latin, 2011). Demethylation inhibitors (DMI), a sub-class of the sterol biosynthesis inhibitors, represent a large group of fungicide active ingredients registered for use on turfgrass (Latin, 2011). They manage a broad spectrum of pathogenic fungi and are generally considered acropetal penetrants, although mobility in the plant and soil varies by active

ingredient (Latin, 2011). The DMI fungicides are especially useful for managing root-infecting fungi, such as those that cause mini-ring, spring dead spot and take-all root rot (Inguagiato & Martin, 2015; Kerns et al., 2017). Unfortunately, many DMI fungicides can be injurious to UDBG—e.g. phytotoxicity and growth regulation—therefore, they must be used judiciously and/or when some bermudagrass injury can be tolerated. Recently, four DMI fungicide active ingredients (difenoconazole, flutriafol, mefentrifluconazole, and prothioconazole,) have been registered for golf course use and exhibit greater bermudagrass safety. Additionally, managing soilborne pathogens can be difficult as fungicide active ingredients do not readily move into the root zone due to the dense canopy and thatch layer produced by UDBG (Latin, 2011; Stephens et al., 2021).

Managing nematodes in UDBG putting greens is often more difficult than managing diseases cause by fungi. There are multiple synthetic and naturally derived nematicides available for turf use; however, their activity spectrum is limited. Furthermore, nematicides generally reduce populations for short period of time, then nematodes rebound when environmental conditions are favorable (Crow, 2005; Crow, 2021b; Crow et al., 2017; McCarty, 2018). As with fungicides, it is difficult to move nematicides past turf and thatch layers, into the rootzone of a UDBG putting green (Crow et al., 2017; Crow 2021a; McCarty, 2018). Currently, a programmatic approach to nematode management, implementing beneficial cultural practices to promote turf health, in combination with applications of synthetic and naturally-derived nematicides appears as the best strategy for managing nematodes in UDBG putting greens (Crow, 2005; Crow et al., 2017; McCarty, 2018).

In summary, UDBG cultivars have significantly changed the management of golf course putting greens in the southern region of the United States and their use continues to increase in the transition zone. Although UDBG has a high tolerance for abiotic stress, specifically, high heat and humidity, managing thatch production, pathogenic fungi, and plant-parasitic nematodes continue to be a challenge.

The objectives of this research project are three-fold: 1) Determine the impact of nitrogen fertilization on mini-ring symptom development and severity; 2) Collect isolates of *Waitea* spp. from multiple UDBG putting greens displaying mini-ring symptoms and using molecular techniques, identify isolates to the species level, 3) Study the epidemiology of mini-ring in UDBG and determine what part of the UDBG plant is infected and when infection most likely occurs. The overall goal of this research project is to deliver practical information to turf practitioners to aid them in successfully managing mini-ring in golf course putting greens.

Table 1. Species within the preserved genus of *Waitea* (Stalpers et al., 2021).

***Waitea* species**	**Teleomorph synonym**	**Anamorph synonym(s)**	**Turfgrass disease common name**	**Selected references**
W. circinata Warcup & Talbot[a]	*W. circinata* var. *circinata*	--	Brown ring patch[b]	Warcup & Talbot 1962 Toda et al., 2005 de la Cerda et al. 2007 Chen et al., 2009
W. agrostidis J.A. Crouch & Cubeta	*W. circinata* var. *agrostis*	--	*Waitea* reddish-brown patch disease	Toda et al., 2007 Sharon et al., 2008 Kammerer et al, 2011
W. prodiga J.A. Crouch & Cubeta	*W. circinata* var. *prodigus*	--	Basal leaf blight	Kammerer, 2011 Kammerer et al., 2011
W. oryzae J.A. Crouch & Cubeta	*W. circinata* var. *oryzae*	*Rhizoctonia oryzae* *Moniliopsis oryzae*	Leaf and sheath spot[c]	Ryker & Gooch, 1938 Haygood & Martin, 1990 Burpee & Martin, 1992 Smiley et al., 2005
W. zeae (Voorhees) J.A. Crouch & Cubeta	*W. circinata* var. *zeae*	*R. zeae* *Chrysorhiza zeae* *M. zeae*	Mini-ring[d] Leaf and sheath spot¶	Voorhees, 1934 Burpee & Martin, 1992 Smiley et al., 2005 Martin, 2009 Kerns et al., 2017

[a] Type species assigned to the genus *Waitea* by Stalpers et al. (2021).

[b] Also known as *Waitea* patch.

[c] Formerly, Rhizoctonia leaf and sheath spot.

[d] Symptoms caused by *W. zeae* in bermudagrass putting greens are referred to as the disease mini-ring

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CHAPTER TWO

NITROGEN SOURCE IMPACTS RHIZOCTOINA LEAF AND SHEATH SPOT SEVERITY IN ULTRADWARF BERMUDAGRASS

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Abstract

The disease *Rhizoctonia* leaf and sheath spot (RLSS), caused by *Rhizoctonia zeae* Voorhees, is problematic in ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens. Typically, RLSS symptoms are most severe in late-summer and appear as frog-eye patches 10 to 40 cm in diameter. In UDBG, RLSS is often called "mini-ring" because of the small brown rings that are produced when symptoms are prominent. The effect of N source and N rate on RLSS severity was investigated in 2016 and 2017. Nitrogen sources, ammonium sulfate (AMS) [$(NH_4)_2SO_4$] and urea (CH_4N_2O) were applied weekly during the growing season at rates of 4.9, 9.8, and 14.7 kg N ha^{-1}. The study was conducted on (MiniVerde) and 'TifEagle' UDBG cultivars maintained as research putting greens at the Clemson University Pee Dee Research and Education Center in Florence, SC. For both cultivars and in both years, urea decreased RLSS severity when compared to AMS. In November of 2016, RLSS severity increased with increasing rates of AMS, however a rate response was not evident in 2017. In November of 2016 and 2017, RLSS severity was 25 and 30% when AMS was applied at 14.7 kg N ha^{-1} wk^{-1} verses 3 and 1% for UDBG receiving applications of urea at that same rate. These studies demonstrate that utilizing urea as a N source, rather than AMS, is an effective strategy to manage RLSS in UDBG.

Introduction

Rhizoctonia zeae Voorhees is a well-documented pathogen of turfgrass, causing the disease formally referred to as *Rhizoctonia* leaf and sheath spot (RLSS). Martin and Lucas, (1983) demonstrated pathogenicity of *R. zeae* on centipedegrass [*Eremolchloa ophiuroides (*Munro) Hack], common bermudagrass [*Cynodon dactylon* (L.)], creeping bentgrass (*Agrostis stolonifera* L.), Kentucky bluegrass (*Poa pratensis* L.), perennial ryegrass (*Lolium perenne* L.), and tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh.). Haygood and Martin (1990) documented that *R. zeae* caused sheath rot and induced foliar leaf lesions in centipedegrass and St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze]. More recently, Elliott (1999) confirmed pathogenicity of *R. zeae* on hybrid bermudagrass; and Kammerer et al. (2011) isolated *R. zeae* from seashore paspalum (*Paspalum vaginatum* Swartz). Until the 1990s, most host-pathogen research with *R. zeae* had been conducted with cool-season turfgrasses as a host (Martin & Lucas, 1983; Martin et al., 1983; Martin & Lucas, 1984). Isolation of *R. zeae* from bermudagrass putting green turf became frequent in the late-1990's and early-2000's with the adoption of ultradwarf bermudagrass (UDBG) as a putting surface (Kerns et al., 2017; Martin, 2009). *Rhizoctonia zeae* was most often isolated from bermudagrass exhibiting frog-eye patch symptoms ranging from 10 to 40 cm in diameter, possibly larger, in which an outer ring was bleached and tan in color, with the turfgrass in the center of the patch generally unaffected or green (Martin, 2009). Although the disease is formally referred to as RLSS, many in the turfgrass industry have adopted the name "mini-ring" based on symptom appearance in bermudagrass putting greens. In the Southeast United States, symptoms can develop as early as mid-July, but more frequently appear in September or October and persist until dormancy (Kerns et al., 2017; Inguagiato & Martin, 2015).

Ultradwarf bermudagrass is an interspecific hybrid bermudagrass commonly used for putting green surfaces located in hot and humid climates. First introduced in the 1990s, UDBG cultivars exhibit finer leaf texture and produce greater leaf density than preceding bermudagrass cultivars (Beard &

Sifers, 1996; Reasor et al., 2016). When intensively managed, UDBG can tolerate low mowing heights, at or below 4 mm, and has similar texture, density and playability as creeping bentgrass (Hanna, 1996; Hannah & Elsner, 1999). This has driven the conversion of creeping bentgrass playing surfaces to UDBG in the transition zone of the United States due to the superior heat and summer stress tolerance of UDBG (Kerns et al., 2017; McCarty, 2018). The practice of "no-till" conversions from creeping bentgrass to UDBG have reduced transitional costs and speed-of-establishment, making it possible for low- and high-budget golf courses to transition to UDBG (O'Brien & Hartwiger, 2011). Additionally, reduced labor costs, lower pesticide inputs and improved summer performance have been reported as major benefits of UDBG versus creeping bentgrass (McCarty, 2018; O'Brien & Hartwiger, 2011).

As creeping bentgrass and existing dwarf bermudagrass putting greens were being converted to UDBG, management practices to maintain and improve UDBG performance were being developed and refined. Thatch accumulation in UDBG putting greens was identified as a possible challenge, due to the aggressive lateral growth and high shoot density of UDBG (Hollingsworth et al., 2005; Rowland, 2009; Unruh et al., 2005; White et al., 2004). Many UDBG managers reduced N fertility and employed cultural practices such as aerification and verticutting, while applying topdressing sand on a frequent basis to manage thatch. Reducing N fertility has also become a technique to increase ball roll distance and perceived green speed. McCullough (2006b) demonstrated that ball roll on a 'TifEagle' UDBG putting green decreased by 9% when N rate doubled from 6 to 12 kg N ha^{-1} wk^{-1} and decreased by 12% when N rate increased from 6 to 24 kg N ha^{-1} wk^{-1}, respectively. Previous studies have shown that the ideal N fertility range for UDBG is from 6 to 13.5 kg N ha^{-1} wk^{-1} depending on geography, root zone composition and cultural practices employed at a given site (Cisar et al. 2005; Inguagiato & Martin, 2015; McCarty & Canegallo, 2005; McCullough et al., 2006a; Unruh, 2005; White et al., 2004). Although it is difficult to determine current N use rates on UDBG, most turf managers favor the low end of the recommended range, potentially as low as 3 to 4.5 kg N ha^{-1} wk^{-1} (Inguagiato & Martin, 2015; Lowe, 2012; Lowe, 2013).

Low N fertility limits UDBG recuperative potential and may increase UDBG susceptibility to fungal pathogens.

The impact of both N source and N rate on turfgrass diseases has been studied extensively. Dollar spot (*Clarireedia* spp.) is a classic example of a turfgrass disease in which severity increases under low N fertility; conversely, brown patch (*R. solani* Kühn) severity increases as N is applied in excess (Smiley et al., 2005). In cool-season turf, Inguagiato et al., (2008) demonstrated that weekly applications of N at 4.9 kg ha^{-1}, applied to annual bluegrass [*Poa annua* L. f. reptans (Hauskins) T. Koyama] reduced anthracnose [*Colletotrichum cereale* Manns Manns (sensu lato Crouch, Clarke, and Hillman] severity by as much as 24% versus the same rate applied on a monthly interval. Thompson et al., (1995) reported that applications of ammonium sulfate (AMS) [$(NH_4)_2SO_4$] to Kentucky bluegrass reduced summer patch [*Magnaporthiopsis poae* (Landschoot & Jackson) J. Lou & N. Zhang] severity by 75% when compared to calcium nitrate [$Ca(NO_3)_2$] applied to deliver an equivalent level of N.

In warm-season turfgrass, the influence of N source on disease severity has been studied more extensively than N rate. *Ophiosphaerella korrae* (J. Walker & A. M. Sm. bis.) Shoemaker and C. E. Babc, *O. herpotricha* (Fr.) J. Walker, and *O. narmari* (J. Walker & A.M. Sm. bis) H.C. Wetzel, Hulbert & Tisserat cause spring dead spot, primarily in bermudagrass (Tredway et al., 2009b). Applications of ammonium-based fertilizers, AMS and ammonium chloride (NH_4Cl), to bermudagrass in MD decreased bulk soil pH and reduced spring dead spot severity caused by *O. korrae* (Dernoeden et al., 1991). Cottrill et al., (2016) demonstrated that *in vitro* mycelial growth of *O. herpotricha* and *O. korrae* was greater when media was amended with calcium nitrate compared to AMS amended media, supporting the previous findings of Dernoeden et al., (1991). Tredway et al., (2009a) reported that calcium nitrate applied to bermudagrass suppressed *O. korrae*, compared to AMS, urea, and sulfur-coated urea. In contrast, *O. herpotricha*, was suppressed by AMS and sulfur-coated urea, compared to calcium nitrate and urea. The effect of N source on large patch in warm-season turf, (*R. solani* Kühn AG 2-2 LP), has also been studied.

In an *in vitro* test similar to Cottrill et al. (2016), Koehler and Miller (2017), demonstrated that mycelial growth of *R. solani* Kühn AG 2-2 LP increased as the concentration of calcium nitrate in amended media increased. In contrast, *R. solani* growth on AMS and urea amended media decreased as the concentration of each N source increased. In a complimentary field study conducted in KS and MO, AMS applications to zoysiagrass (*Zoysia japonica* Steud) appeared to decrease large patch severity compared to applications of urea and calcium nitrate, although results were inconsistent across site and year (Miller et al.,2016).

The impact of N fertility practices on RLSS development and severity has not been studied. Given that current UDBG management practices favor low N fertility, our objectives were to determine if N rate or N source impacts RLSS severity in UDBG putting greens.

Materials and methods

In 2016 and 2017, two studies, one on 'P18' (MiniVerde), the other on TifEagle UDBG, were conducted at the Clemson University Research and Education Center in Florence, SC a on research putting green that was constructed in 2002 using a 85% sand and 15% peat root zone mix. *Rhizoctonia zeae* was isolated from symptomatic areas of the putting green on multiple occasions and symptoms indicative of RLSS were widespread and becoming more severe during the previous two growing seasons. The green was mowed five times wk^{-1} at 3.2 mm and irrigation was applied to prevent drought stress. A 18-3-16 fertilizer was applied in late-April of both years to deliver 16.1 Kg N ha^{-1} in 2016 and 24.4 kg N ha^{-1} in 2017 to aid in spring transition and recovery from RLSS damage from the previous year. Thiophanate-methyl was applied at 4.6 kg a.i. ha^{-1} as needed to control dollar spot. On 12 Oct. 2017, a fungicide formulated to deliver 460 g a.i. ha^{-1} azoxystrobin and 287 g a.i. ha^{-1} difenoconazole was applied along with 19.4 kg ha^{-1} N in the form of urea to suppress a leaf spot epidemic and hasten UDBG recovery.

The trial area for each cultivar was divided into 68 plots measuring 0.9 by 1.8 m. Damage caused by RLSS during the 2015 growing season was visually assessed on 9 May 2016, by plot, and ranked from greatest to least damaged, then grouped into four categories. Plots with no RLSS damage or extensive damage were eliminated. For each cultivar, only 28 of the original 68 plots assessed were used for the study. Treatments were randomly assigned to a plot in each of the four groupings creating a randomized complete block design with four replications per treatment. The same plots were used for each year of the study.

Treatment structure was a 2 x 3 factorial with two sources of N: AMS and urea; and three N rates: 4.9, 9.8 and 14.7 kg N ha^{-1} wk^{-1}, respectively. A control, which received no N, was also included. In 2016, N treatments were initiated on 9 June and applied weekly until 29 Sept. In 2017, N treatments began on 01 June and continued weekly until 28 September. Fertilizer grade AMS (21-0-0) and urea (46-0-0) were dissolved in water then applied in a spray volume of 855 L ha^{-1} using a CO_2-powered sprayer fitted with XR8002VS flat-fan nozzles (Spraying Systems Company, Wheaton, IL) operated at 207 kPa. Immediately following application, 3 mm of water was applied using overhead irrigation to rinse N treatments off turf foliage.

A representative soil sample from every plot was collected on 09 May and 16 Nov. 2016 and 18 May and 14 Nov. 2017 by removing eight to ten soil cores per plot. Each core measured 19 mm in diameter and was taken to a depth of 76 mm. Soil samples were submitted to the Clemson University Agricultural Service Laboratory where nutrients were extracted using Mehlich-1 extractant (Mehlich, 1953). Based on 18 May 2017 soil test results, $CaCO_3$ was applied on 14 June 2017 in the form of Verde-Cal Enhanced Calcitic Lime (Aqua-Aid Solutions, Rocky Mount, NC) to individual plots at recommended rates to raise soil pH to a target of 6.0.

Data collection and analysis

Rhizoctonia leaf and sheath spot severity was evaluated monthly when symptoms were present. Each plot was divided into eight subsections, measuring 0.2 m^2 each, by overlaying a 0.9 by 1.8 m grid. The percent area of RLSS symptomatic turf in each subsection was visually estimated then summed and divided by eight to produce one value per plot. Turf quality was visually assessed on a monthly basis using a 1-9 scale, with 1 being dead turf, 6 representing acceptable turf and 9 excellent turf. Turf quality visual assessments encompassed, turf color, density and uniformity, as well as RLSS severity.

Rhizoctonia leaf and sheath spot severity data were subjected to analysis of variance using a mixed model in JMP 14.3.0 (SAS Institute Inc., Cary, NC). The main effects, N rate, N source, cultivar, evaluation date and interactions of each were treated as fixed effects. Block and all interaction terms including block, were treated as random effects. Evaluation date was significant; therefore, each evaluation date was analyzed separately. Data for the experimental control (no N) was not included in the analysis.

Results

In 2016 and 2017, RLSS severity was rated in August and September (data not shown), but symptoms were not severe or distinct, which made evaluation difficult. Disease symptom severity increased and was distinct in October and November of both years. Turf quality visual ratings (data not shown) in 2016 were initially positively correlated with N rate for both N sources as expected. However, as RLSS symptoms developed, turf quality reflected disease severity for the remainder of the study.

***Rhizoctonia* leaf and sheath spot severity**

Cultivar and interactions including cultivar were not significant at either date in 2016 (Table 1). Consequently, data for each cultivar was combined for analyses (Table 2.1). In October, RLSS severity was low, averaging 13% in turf treated with AMS versus 2% in plots receiving urea (Figure 2.1). Although RLSS severity was over two times greater in UDBG treated with AMS at 9.8 and 14.7 kg N ha^{-1} wk^{-1}

compared to 4.9 kg N ha^{-1} wk^{-1}, N rate and N source x N rate were not significant (Table 2.1). Rhizoctonia leaf and sheath spot severity in November was similar to October, but N source x N rate was significant (Table 2.1). As rate of AMS increased, disease severity increased; whereas, disease severity was unchanged across increasing rates of urea (Figure 2.1). In November, RLSS severity in UDBG treated with urea at 14.7 kg N ha^{-1} wk^{-1} was 88% less than UDBG treated with the same rate of AMS.

Rhizoctonia leaf and sheath spot severity was greater in 2017 than 2016, but more variable. In October 2017, N source x cultivar was significant (Table 2.1); therefore, RLSS severity is presented by cultivar (Figure 2.2). No factor was significant in TifEagle UDBG for the October evaluation due to substantial variation (Figure 2.2). Conversely, N source was significant (P = <0.001) in MiniVerde UDBG, where RLSS severity decreased by 90% when urea was applied versus AMS (Figure 2.2). In November, cultivar was not significant, nor were any interaction terms with cultivar (Table 2.1). Consistent with November 2016, N source was the only significant term in November 2017. When averaged across rate, mean RLSS severity for UDBG treated with AMS and urea was 28 and 7%, respectively (Figure 2.3).

Soil pH and exchangeable cation concentration

Prior to the initiation of the study, the mean soil pH of both areas was 5.7. In November 2016, following the application of AMS at the highest rate, 14.7 kg N ha^{-1} wk^{-1}, the pH decreased to 5.3 and 5.2 for MiniVerde and TifEagle UDBG, respectively (Tables 2.2 and 2.3). In contrast, soil pH of MiniVerde UDBG receiving 14.7 kg N ha^{-1} wk^{-1} urea, was 5.7 and 5.8 for TifEagle. Applications of AMS at 4.9 and 9.8 kg N ha^{-1} wk^{-1} lowered soil pH by 0 to 0.3 units in 2016, while applications of urea at equal N rates induced little change. In November 2017, at the end of the study, pH for MiniVerde and TifEagle UDBG fertilized with AMS at 14.7 kg N ha^{-1} wk^{-1} stabilized at 5.7 and 5.4, likely due to $CaCO_3$ that was applied in June 2017 to raise individual plot pH to a target of 6.0. Similarly, UDBG treated with AMS at the lower rates stabilized, with a maximum decrease of 0.1 units from the 2017 spring soil test. As was the case in 2016, soil pH of UDBG receiving applications of urea in 2017 was unchanged or increased slightly.

Exchangeable Ca concentration decreased with increasing rates of AMS in 2016 (Table 2.2). From May to November, the concentration of Ca in soil of the MiniVerde UDBG study decreased by 12, 27, and 35% and 19, 32 and 44% in the soil of the TifEagle UDBG study as the rate of AMS increased from 4.9 to 9.8 and 14.7 kg N ha^{-1} wk^{-1}, respectively. Conversely, the rate of urea was not correlated with an increase or decrease in exchangeable soil Ca. In 2017, Ca concentration remained constant or increased for all treatments in both cultivars, likely a result of $CaCO_3$ which was applied in June 2017.

Similar to Ca, a reduction of exchangeable soil Mg occurred in 2016 when AMS was applied to either cultivar; however, there was not a robust rate response (Tables 2.2 and 2.3). The mean decrease across AMS rates was 15 (38%) and 20 mg kg^{-1} (48%) for the MiniVerde and TifEagle studies, respectively. Exchangeable soil Mg decreased in 2016 when UDBG received urea treatments as well, but when averaged across N rates, that reduction was 6 and 11 mg kg^{-1} for MiniVerde and TifEagle UDBG, respectively. In 2017, soil Mg concentration decreased by 4 mg kg^{-1} in MiniVerde UDBG and 3 mg kg^{-1} in TifEagle UDBG in response to AMS applications, when averaged across N rate. In contrast, urea applications had little effect Mg soil concentration in either cultivar in 2017 (Tables 2.2 and 2.3). For comparison, exchangeable Mg concentration decreased in control plots by 6 and 2 mg kg^{-1} in 2016 and 2017, when the combined mean of both cultivars was calculated.

Exchangeable K was unaffected by N source or N rate. Soil K concentration of N treatments followed a similar pattern as the control (no N) treatment over the four sampling dates (Tables 2.2 and 2.3).

Discussion

Multiple studies have demonstrated that lowering soil or rhizosphere pH, by utilizing an ammonium-based N source, can have a suppressive effect on turfgrass soilborne diseases, such as spring dead spot and summer patch (Dernoeden et al., 1991; Thompson et al., 1995). Additionally, *in vitro* assays have shown that colony growth of *R. solani* AG2-2 LP, *O. herpotricha* and *O. korrae* is suppressed

by increasing concentrations of AMS, due to lowering the growing media pH (Cottrill et al., 2016; Koehler & Miller, 2017). In our study, acidification of the root zone from applications of AMS increased RLSS severity, contrary to most of the previous work done with turfgrass fungal pathogens. The effect of N source was not expected. Our hypothesis was that increasing rates of N would reduce RLSS severity and that N source would have little to no affect. Contrary to our hypothesis, the application of AMS increased RLSS severity, while urea limited RLSS symptom development. One plausible explanation for increased RLSS severity with the application of AMS, and lower soil pH, is that *R. zeae* growth is negatively correlated with soil pH. Voorhees (1934), assessed mycelial growth of *R. zeae in vitro* on pH adjusted 2% potato-dextrose agar medium with pH ranging from 2.5 to 9.0. Significant growth suppression (>50%) did not occur until pH was greater than 8.6 or less than 3.9, and optimum growth occurred at pH 6.8. In the present study, soil pH ranged from 5.2 to 5.9; therefore, an effect of pH alone on *R. zeae* growth is unlikely. Based on Voorhees (1934) research, if there was any direct effect of pH on *R. zeae* in our study, the decrease in pH should have caused only a slight reduction in *R. zeae* growth.

The root zone of the putting green used in this study had minimal buffering capacity demonstrated by the decrease in soil pH caused by applications of AMS and marked increase of soil pH resulting from the application of $CaCO_3$. Although AMS rate and RLSS severity were positively correlated in November 2016, there was not a rate response at any other evaluation date. In June 2017, $CaCO_3$ was applied to individual plots to raise soil pH of each plot to a target of 6.0. It is plausible that the application of $CaCO_3$ suppressed a possible AMS rate response in the fall of 2017 and increased within treatment variability of RLSS severity in 2017, compared to the fall of 2016. The application of $Ca(OH)_2$ to acidic soils has been shown to suppress damping-off of sugar beet (*Beta vulgaris* L.) caused by *R. solani* AG2-2 IIIB (Watanabe et al., 2011). Comparing pasteurized and non-pasteurized acidic soils infested with *R. solani*, Watanabe et al. (2011), demonstrated that sugar beet seeding survival was greatest in non-pasteurized soil, when $Ca(OH)_2$ was used to raise soil pH above 5.5. The application of

$Ca(OH)_2$ to pasteurized soil did not have this same effect. The authors concluded that biological suppression of *R. solani* occurred in the soil used for the study and that suppression of *R. solani* was pH dependent. The objective of our study was to determine RLSS response to N source and rate, not directly study the effect of soil pH and biological suppression of *R. zeae;* however, it is possible that soil organisms which suppress or compete with *R. zeae* may have been inhibited as pH decreased. A detailed set of research experiments like those conducted by Watanabe et al. (2011) should be conducted to determine if biological suppression of *R. zeae* is present and determine if it is pH dependent.

We did not compare the effect of AMS versus urea applications on rhizosphere pH, only the long-term effect of N source and N rate on bulk soil pH. Smiley and Cook, (1973) and Thompson et al. (1995) showed that the effect of AMS on rhizosphere pH is more immediate and short-term than the effect on bulk soil pH. Additionally, the reduction in rhizosphere pH was often more dramatic than the decrease observed in bulk soil pH. In our study, we tested the effect of multiple applications of AMS over the course of the season. Although this may be practical for some UDBG fertility regimes, from a practitioner's viewpoint, it would also be useful to know if infrequent applications of AMS might cause an increase in RLSS severity similar to multiple applications over the course of a growing season. Understanding the relationship between RLSS severity and rhizosphere pH would aide turf managers in developing fertility programs to prevent RLSS as well as manage soil chemistry.

As expected, the application of AMS decreased soil pH, and consequently, the concentration of Ca and Mg decreased. Exchangeable Ca and Mg at the beginning of the study were sufficient for all treatments according to soil test results in May 2016. As AMS was applied, both exchangeable Ca and Mg fell from medium to low levels according to the soil test report. It is difficult to assign a minimum soil concentration for either cation that could be assumed to be biological relevant in this study,

furthermore, researching the impact of Ca and Mg independent of soil pH is difficult in a field setting. Ultimately, we cannot rule out the impact of soil Ca and Mg on RLSS severity in this study.

This study shows that N fertilization with urea rather than AMS can mitigate RLSS severity in UDBG. The reason that urea suppresses RLSS while AMS increases severity is unclear. Although the decrease in soil pH with the application of AMS seems like a logical explanation, the rationale is likely more complex. Future research should focus on testing the effects of other N sources, such as calcium nitrate and potassium nitrate (KNO_3) on RLSS severity and well as the impact of $CaCO_3$ applications in conjunction with N fertility. Additionally, the short-term impact of N source on rhizosphere pH and RLSS infection could provide meaningful insight for turfgrass managers. Finally, multiple studies have shown that turfgrass systems sustain a diverse microbial community (Beirn et al., 2017; Bigelow et al., 2002; Elliott et al., 2008; Yao et al., 2006), so it is likely that biological competition to *R. zeae* is present. Furthermore, it has been shown that soil pH is a primary regulator of soil microbial composition and diversity (Yao et al., 2006; Zhalnina et al. 2014). Consequently, understanding the impact of N source and soil pH on biological suppression of *R. zeae* could potentially lead to more comprehensive recommendations regarding N fertility and soil chemistry when RLSS is problematic in UDBG.

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Table 2.1. Analysis of variance for effect of N source and N rate on disease severity of Rhizoctonia leaf and sheath spot in ultradwarf bermudagrass.

	Date			
	2016		2017	
Source of variation	13 Oct.	16 Nov.	12 Oct.	13 Nov.
N source[a]	***	***	***	***
N rate[b]	ns†	*	ns	ns
Cultivar[c]	ns	ns	ns	ns
N rate x N source	ns	*	ns	ns
N source x cultivar	ns	ns	**	ns
N rate x cultivar	ns	ns	ns	ns
N source x N rate x cultivar	ns	ns	ns	ns

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† Not significant at the 0.05 probability level.

[a] Sources of N were $(NH_4)2SO_4$ and CH_4N_2O.

[b] Rates of N comprised of 4.9, 9.8, and 14.7 kg N ha^{-1} wk^{-1}.

[c] Cultivars of ultradwarf bermudagrass were 'P18' (MiniVerde) and 'TifEagle'.

Table 2.2. Bulk soil pH and Ca, K, and Mg concentration of 'P18' (MiniVerde) ultradwarf bermudagrass putting green soil using Mehlich-1 extraction, before and after treatment with ammonium sulfate [$(NH_4)_2SO_4$] and urea (CH_4N_2O) at various N rates. Soil samples were taken to a depth of 76 mm.

		pH				Ca			
		2016		2017		2016		2017	
N source[a]	N rate	9 May	16 Nov.	18 May	14 Nov.	9 May	16 Nov.	18 May	14 Nov.
	kg N ha^{-1} wk^{-1}					mg kg^{-1}			
	0	5.7[b] (0.1)[c]	5.7 (0.1)	5.8 (0.1)	5.8 (0.1)	236 (16)	220 (22)	192 (32)	236 (49)
$(NH_4)_2SO_4$	4.9	5.8 (0.1)	5.7 (0.1)	5.7 (0.1)	5.8 (0.1)	239 (17)	210 (14)	192 (38)	220 (55)
	9.8	5.8 (0.2)	5.5 (0.1)	5.6 (0.2)	5.5 (0.1)	228 (36)	167 (35)	149 (35)	142 (27)
	14.7	5.7 (0.1)	5.3 (0.1)	5.4 (0.1)	5.7 (0.1)	231 (21)	150 (25)	130 (38)	164 (12)
CH_4N_2O	4.9	5.8 (0.1)	5.8 (0.1)	5.8 (0.1)	5.8 (0.2)	209 (20)	215 (33)	184 (29)	215 (59)
	9.8	5.7 (0.1)	5.7 (0.1)	5.7 (0.1)	5.8 (0.2)	230 (18)	194 (18)	159 (16)	226 (40)
	14.7	5.7 (0.2)	5.7 (0.1)	5.8 (0.1)	5.9 (0.2)	218 (29)	215 (28)	186 (26)	219 (20)
		K				Mg			
		2016		2017		2016		2017	
		9 May	16 Nov.	18 May	14 Nov.	9 May	16 Nov.	18 May	14 Nov.
		mg kg^{-1}							
	0	34 (5)	28 (4)	38 (7)	35 (10)	41 (9)	36 (8)	34 (6)	34 (9)
$(NH_4)_2SO_4$	4.9	32 (7)	27 (2)	35 (10)	38 (4)	48 (9)	32 (2)	32 (8)	26 (2)
	9.8	30 (7)	27 (5)	36 (12)	33 (5)	35 (11)	22 (4)	22 (5)	19 (1)
	14.7	31 (3)	27 (3)	35 (5)	31 (8)	35 (7)	20 (3)	21 (5)	18 (2)
CH_4N_2O	4.9	40 (8)	26 (5)	35 (4)	29 (5)	41 (5)	37 (10)	34 (8)	30 (10)
	9.8	31 (6)	26 (3)	30 (2)	33 (4)	40 (5)	32 (9)	28 (7)	31 (9)
	14.7	33 (5)	27 (2)	36 (2)	37 (5)	37 (3)	32 (5)	31 (4)	30 (3)

[a] Nitrogen treatments began 9 June and ended 29 Sept. 2016. In 2017, N treatments started 1 June and continued until 28 Sept. 2017.

[b] Values are the mean of four replications.

[c] Values in parentheses are the standard deviation of each mean.

Table 2.3. Bulk soil pH and Ca, K, and Mg concentration of TifEagle ultradwarf bermudagrass putting green soil using Mehlich-1 extraction, before and after treatment with ammonium sulfate [$(NH_4)_2SO_4$] and urea (CH_4N_2O) at various N rates. Soil samples were taken to a depth of 76 mm.

		pH				Ca			
		2016		2017		2016		2017	
N source[a]	N rate	9 May	16 Nov.	18 May	14 Nov.	9 May	16 Nov.	18 May	14 Nov.
	kg N ha^{-1} wk^{-1}					mg kg^{-1}			
	0	5.6[b] (0.1)[c]	5.8 (0.2)	5.8 (0.2)	5.9 (0.1)	213 (13)	212 (28)	181 (22)	198 (35)
$(NH_4)_2SO_4$	4.9	5.6 (0.1)	5.6 (0.1)	5.6 (0.2)	5.7 (0.1)	209 (39)	170 (45)	140 (23)	177 (53)
	9.8	5.7 (0.2)	5.4 (0.1)	5.4 (0.1)	5.6 (0.1)	236 (21)	161 (31)	131 (21)	162 (37)
	14.7	5.6 (0.1)	5.2 (0)	5.4 (0.1)	5.4 (0.2)	216 (32)	122 (28)	98 (7)	128 (30)
CH_4N_2O	4.9	5.8 (0.1)	5.8 (0.1)	5.8 (0.2)	5.9 (0.1)	217 (35)	197 (34)	158 (7)	194 (43)
	9.8	5.7 (0.1)	5.8 (0.1)	5.9 (0.1)	5.8 (0.2)	249 (26)	215 (9)	170 (28)	204 (31)
	14.7	5.8 (0.1)	5.8 (0.1)	5.8 (0.1)	5.9 (0.1)	239 (12)	207 (17)	165 (26)	218 (36)
		K				Mg			
		2016		2017		2016		2017	
		9 May	16 Nov.	18 May	14 Nov.	9 May	16 Nov.	18 May	14 Nov.
		mg kg^{-1}							
	0	30 (3)	28 (5)	33 (5)	26 (5)	40 (6)	33 (7)	32 (5)	28 (3)
$(NH_4)_2SO_4$	4.9	32 (8)	29 (6)	28 (3)	27 (9)	41 (9)	25 (8)	23 (3)	21 (7)
	9.8	37 (10)	32 (5)	29 (2)	29 (7)	50 (10)	24 (5)	22 (2)	18 (4)
	14.7	31 (3)	30 (4)	30 (1)	24 (4)	37 (8)	18 (3)	17 (3)	15 (2)
CH_4N_2O	4.9	32 (7)	31 (6)	29 (2)	30 (8)	42 (10)	31 (6)	26 (2)	29 (6)
	9.8	33 (5)	30 (2)	29 (3)	28 (5)	43 (6)	34 (6)	29 (4)	30 (5)
	14.7	36 (11)	29 (4)	29 (2)	29 (5)	45 (6)	31 (4)	28 (4)	28 (4)

[a] Nitrogen treatments began 9 June and ended 29 Sept. 2016. In 2017, N treatments started 01 June and continued until 28 Sept. 2017.

[b] Values are the mean of four replications.

[c] Values in parentheses are the standard deviation of each mean.

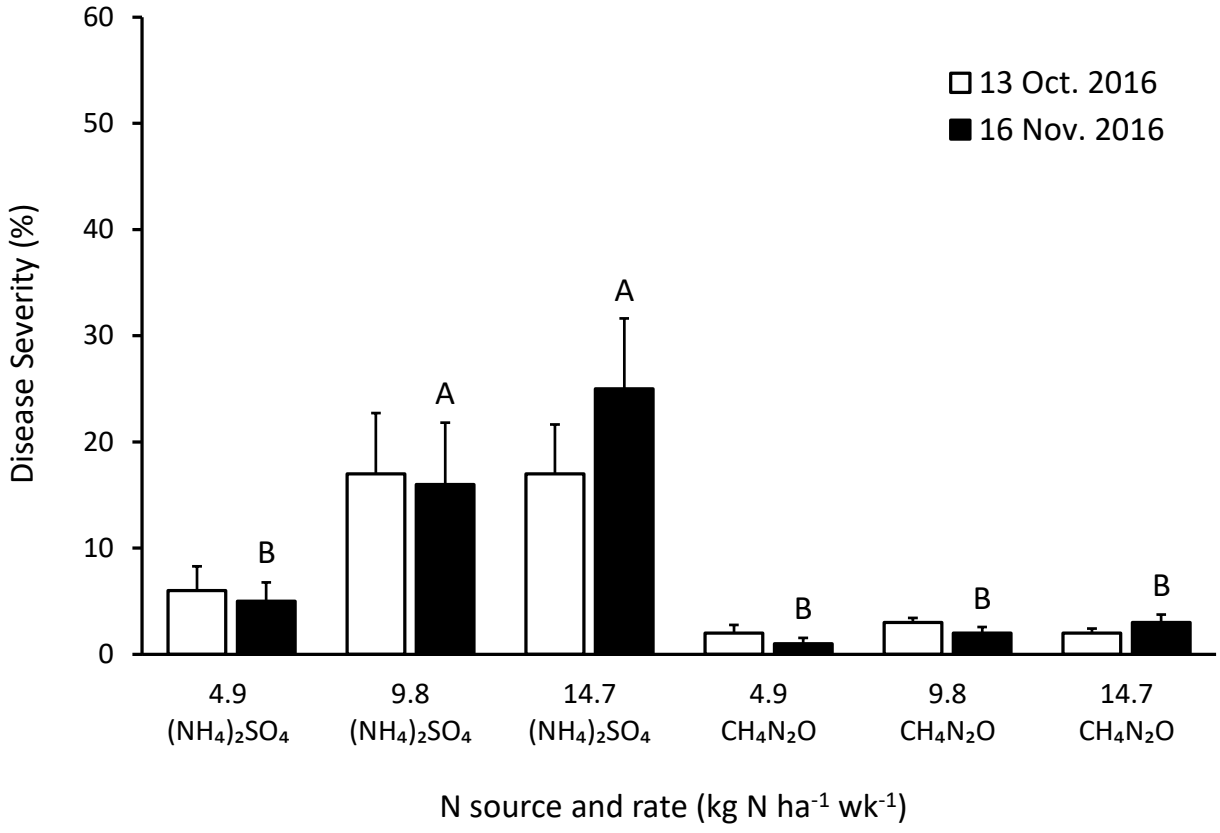

Figure 2.1. Impact of N source and N rate on Rhizoctonia leaf and sheath spot severity in ultradwarf bermudagrass visually rated on 13 Oct. and 16 Nov. 2016. Bars are means of tests conducted on 'P18' (MiniVerde) and 'TifEagle' ultradwarf bermudagrass and represent the combined mean of four replications per cultivar. Error bars are the standard error of each treatment mean. Nitrogen treatments began on 09 June and were applied weekly until 29 Sept. 2016. For the 13 Oct. 2016 assessment, N source was significant ($P < .001$); however, N rate was not ($P = .109$). For the 16 Nov. 2016 assessment, bars with the same letter are not significantly different according to Fisher's Protected LSD ($\alpha = .05$).

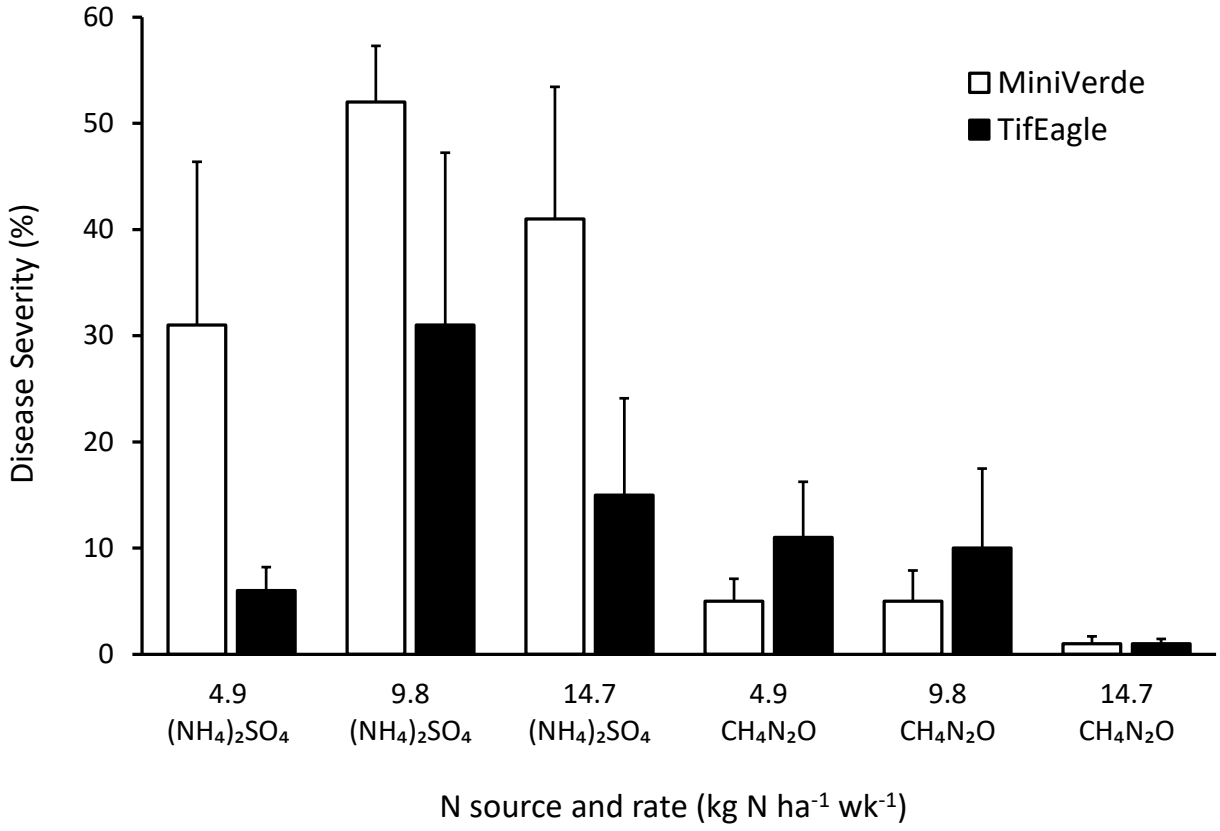

Figure 2.2. Impact of N source and N rate on Rhizoctonia leaf and sheath spot severity in ultradwarf bermudagrass visually rated on 12 Oct. 2017. Bars are means of tests conducted on 'P18' (MiniVerde) and 'TifEagle' ultradwarf bermudagrass and represent the mean of four replications for each cultivar. Error bars are the standard error of each treatment mean for each cultivar. Nitrogen treatments began on 1 June and were applied weekly until 28 Sept. 2017. For TifEagle ultradwarf bermudagrass, N source was not significant (P = .176) nor was N rate (P = .288). Nitrogen source was significant (P <.001) and N rate was not (P = .288) for MiniVerde ultradwarf bermudagrass.

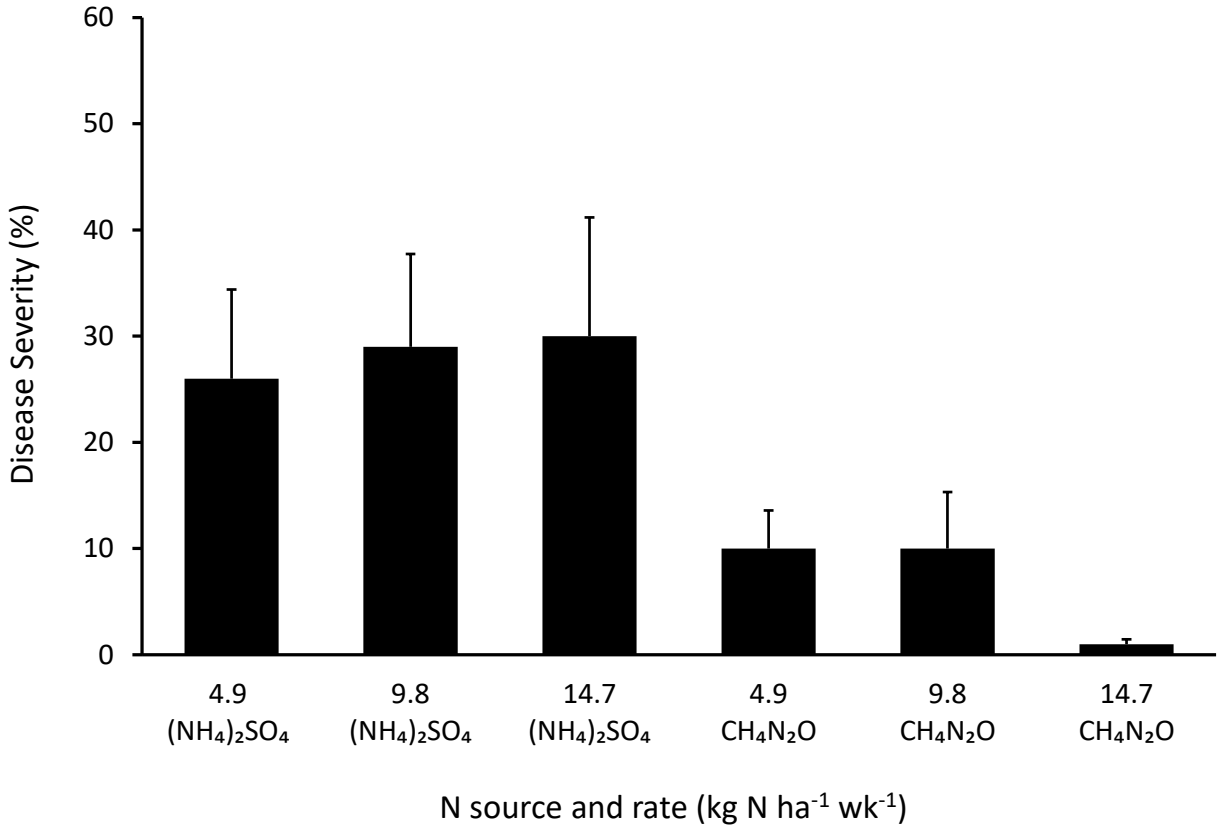

Figure 2.3. Impact of N source and N rate on Rhizoctonia leaf and sheath spot severity in ultradwarf bermudagrass visually rated on 13 Nov. 2017. Bars are means of tests conducted on 'P18' (MiniVerde) and 'TifEagle' ultradwarf bermudagrass and represent the combined mean of four replications per cultivar. Error bars are the standard error of each treatment mean. Nitrogen treatments began on 1 June and were applied weekly until 28 Sept. 2017. Nitrogen source was significant (P = <.001). Nitrogen rate was not (P = .864).

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CHAPTER THREE

ISOLATION FREQUENCY OF *WAITEA ZEAE* IN AN ULTRADWARF BERMUDAGRASS PUTTING GREEN

Abstract

Mini-ring is caused by *Waitea zeae* (Voorhees) J.A. Crouch & Cubeta. In ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)], symptoms are typically 10 to 40 cm diameter frog-eye patches, i.e., turfgrass in the center of the patch appears unaffected, and is bordered by a ring of blighted turfgrass that is tan to orange in color. In the transition zone of the United States, symptoms typically develop in late-summer and persist until UDBG dormancy. Management of mini-ring after symptom expression has proven difficult. To investigate when *W. zeae* is present in UDBG, cores were harvested from stands of 'MiniVerde' and 'TifEagle' UDBG on a putting green located at the Clemson University Pee Dee Research and Education Center in Florence, SC on approximately the 15th day of every month from June to October in 2016 and 2017. Cores were placed in moist chambers for 5 to 7 d and then leaf tissue was placed on a semi-selective medium and observed for characteristic growth of *W. zeae*. Putting green soil temperature was recorded hourly with in-ground data loggers, and air temperature was collected by an on-site weather station. *Waitea zeae* was readily isolated in July, August, and September but less frequently in June and October. Soil temperatures during months when *W. zeae* was most frequently isolated coincided with temperatures previously reported to support optimal growth of *W. zeae*. These results demonstrate management of mini-ring in UDBG putting greens should begin during summer, because *W. zeae* is present before mini-ring symptoms develop.

Introduction

In the 1990s, fine-textured cultivars of interspecific bermudagrass hybrids [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] were introduced able to withstand mowing heights at or below 3.2 mm (Hanna & Elsner, 1999; McCarty, 2018; Reasor et al., 2018; Reasor et al., 2016). These cultivars produced dense turfgrass stands ideal for putting green use, similar to creeping bentgrass (*Agrostis stolonifera* L.), and more desirable than previously used bermudagrass hybrids. Collectively, these fine-textured hybrid bermudagrass cultivars are often referred to as ultradwarf bermudagrass (UDBG) (McCarty, 2018; Reasor et al., 2016). In the late-1990s and 2000s, conversion of creeping bentgrass golf course putting greens to UDBG became common in the southern United States where air temperatures and humidity routinely exceed the ideal range of creeping bentgrass (Inguagiato & Martin, 2015; Kerns et al., 2017). In addition, existing bermudagrass putting greens were converted to UDBG, because these fine-textured cultivars produce a better playing surface and could be maintained at lower mowing heights than previous ones (Inguagiato & Martin, 2015).

A significant conversion of existing putting green surfaces to UDBG occurred within a relatively short period of time and across a broad geographic area with a wide-range of climatic conditions. Little was known about the short- and long-term management challenges across this diverse geography. Although morphological characteristics and the innate adaptability of UDBG cultivars for putting green turfgrass had been well-studied within breeding programs, performance of these cultivars under intense cultivation to produce high-quality putting surfaces, was not well understood (Hanna, 1996; Hanna & Elsner, 1999; Inguagiato & Martin, 2015). Potential fungal pathogens of UDBG and distribution of these pathogens within the geography where UDBG was being grown were largely unknown, specifically in regions where creeping bentgrass had previously been the predominate turfgrass grown on putting green surfaces.

Reports of mini-ring in the transition zone and tropical climates of the United States increased as putting greens were converted to UDBG and intensively managed to provide high-performing putting surfaces (Elliott, 1999; Kerns et al., 2017; Martin, 2009). The pathogen that causes mini-ring, *Waitea zeae,* is a pathogen of warm- and cool-season turfgrasses, although much of the research has focused on epidemiology and management in cool-season turfgrass (Burpee & Martin, 1992; Inguagiato & Martin, 2015; Martin et al., 1983; Martin & Lucas, 1983; Martin & Lucas, 1984). In warm-season turfgrass, Kammerer et al. (2011) isolated *W. zeae* from seashore paspalum (*Paspalum vaginatum* Swartz) and Haygood & Martin (1990) demonstrated St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze], and centipedegrass [*Eremochloa ophiuroides* (Munro) Hack], developed leaf lesions and stem rot when infected with *W. zeae.* Research with the *W. zea*e–bermudagrass host-pathogen system is limited. Martin & Lucas (1983) demonstrated pathogenicity of *W. zeae* on common bermudagrass [*Cynodon dactylon* (L.) Pers var. *dactylon*], and Elliott (1999) demonstrated pathogenicity on hybrid bermudagrass. Kerns et al. (2017) was first to publish studies focused on *W. zeae* as a pathogen in UDBG putting greens; although, the presence of *W. zeae* and description of symptoms produced in UDBG putting greens had been documented previously in trade publications (Inguagiato & Martin, 2015; Martin, 2009).

In UDBG putting greens, *W. zeae* causes leaf blight and dieback, discrete leaf lesions do not develop (Martin, 2009). On the putting green surface, symptoms appear as frog-eye patches 10 to 40 cm in diameter; the leading edge of the patch is bleached or straw-to-yellow in color, with a green center, where the turfgrass appears unaffected (Inguagiato & Martin, 2015; Kerns et al., 2017; Martin, 2009). Leaf and sheath spot (formerly *Rhizoctonia* leaf and sheath spot) is used to describe the disease in scientific publication; however, based on symptomology in UDBG putting greens, turfgrass practitioners commonly referred to the disease as mini-ring (Dant & Martin, 2023; Inguagiato & Martin, 2015). Faint, bronze- or orange-colored complete or partial rings may be present to the trained eye

prior to the onset of frog-eye symptoms (Inguagiato & Martin, 2015; Martin, 2009). In the transition zone, mini-ring symptoms first appear in late-summer and become more apparent as UDBG growth slows in September and October (Inguagiato & Martin, 2015; Kerns et al., 2017; Martin, 2009). Symptoms remain visible until UDBG dormancy and, in severe cases, a depressed ring forms on the putting green surface where UDBG does not recover (Inguagiato & Martin, 2015; Kerns et al., 2017; Martin, 2009).

In culture, *W. zeae* mycelium grows readily between 25 to 35°C, with growth being greatest between 30 to 32°C (Burpee & Martin, 1992; Elliott, 1999; Kammerer et al., 2011; Kerns et al., 2017; Voorhees, 1934). For this reason, mini-ring is considered a mid-summer disease becoming most prolific when day-time air temperatures are 30°C or above. In the transition zone, mini-ring symptoms are typically most severe in mid-September and October after summer air temperatures have peaked and are beginning to cool. Fungicide performance is variable once mini-ring symptoms appear and symptom severity can continue to increase into late-fall, even with fungicide use (Dant, unpublished data, 2022; Martin, 2009; Martin & Park, 2010; Inguagiato & Martin, 2015). Past research conducted by Martin et al. (1984), demonstrated that *W .zeae* isolates from tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] were sensitive to the fungicides chlorothalonil, iprodione, and triadimefon, which belong to the chloronitrile, dicarboxamide, and demethylation inhibitor (DMI) fungicide groups, respectively. More recently, Kerns et al. (2017), demonstrated that isolates of *W. zeae* from UDBG were sensitive to several demethylation inhibitor (DMI) fungicides and two succinate dehydrogenase inhibitor (SDHI) fungicides and were moderately sensitive to one quinone outside inhibitor (QoI) fungicide. Although these fungicides are often used in disease management programs for UDBG, it is unclear when exactly fungicides should be applied to maximum effectiveness. The objective of this research was to determine when *W. zeae* isolation frequency was the greatest in UDBG putting greens located in the

transition zone so to better understand when mini-ring management programs, including fungicide applications, should be initiated.

Materials and methods

This experiment was conducted in 2016 and 2017 on a hybrid bermudagrass research putting green composed with stands of 'MiniVerde' and 'TifEagle' UDBG cultivars. In the test site, mini-ring symptoms had become more numerous over the previous two seasons and *W. zeae* had been isolated from this putting green previously. The putting green was constructed in 2002 using a rootzone mix of 85% sand, 15% peat and the pH of the soil was 5.7 when the trial was initiated. Irrigation was applied to prevent drought stress and mowing occurred five times per week at a 3.2 mm height of cut. An 18-3-16 (N-P-K) fertilizer was used to apply 73.3 kg N ha^{-1} during the study in 2016 and 58.9 kg N ha^{-1} in 2017. Dollar spot (*Clarireedia* spp.) was controlled by applying thiophanate-methyl at 4.6 kg a.i. ha^{-1}, as needed.

To distribute sampling of UDBG on the research putting green, 24 plots measuring 1.2 by 1.2 m were laid out in two rows of 12 plots each. On approximately the 15th day of each month from June to October, two cores were harvested from each plot. One core was taken from what appeared to be mini-ring symptoms and a second core was taken from asymptomatic turfgrass; therefore, a total of 48 cores were collected each month. Cores included plant tissue, thatch, roots, and soil and were 3.8 cm in diameter and collected to a depth of 6.4 cm. Each year, care was taken not to resample an area sampled in previous months. By mistake, 460 g a.i. ha^{-1} azoxystrobin and 287 g a.i. ha^{-1} difenoconazole (Briskway, Syngenta Crop Protection, LLC., Greensboro, NC) were applied to the research putting green on 12 Oct. 2017 to suppress a leaf spot (*Bipolaris* spp.) epidemic. Turfgrass cores were still harvested in mid-October 2017, but *W. zeae* was only isolated from two cores; therefore, data from this sample time event were treated as missing.

Each core was placed in individual moist chambers for 5 to 7 d and stored in the dark at room temperature (approximately 22-25°C). Moist chambers were 1.2 L polypropylene food storage containers (Rubbermaid®, Newell Brands, Inc, Atlanta, GA) measuring 11 by 11 cm at the base. A moist paper towel was placed in the bottom of each container, and each container was covered with a tight-fitting lid with one 4-mm hole to allow for air exchange.

After the 5 to 7 d incubation period, four tillers from each core were placed on sterile semi-selective medium in a 9-cm Petri plate. Culture medium contained 1 L deionized water and 15 g of agar (BD Bacto, Becton, Dickinson and Company, Franklin Lakes, NJ) and was autoclaved for 20 min at 121°C, 1.5 MPa pressure before adding 0.2 g streptomycin sulfate, 0.2 g of ampicillin, and 0.05 g thiophanate-methyl (3336 F, Nufarm Americas, Inc., Alsip, IL). Tillers were selected based on the presence of mycelium that appeared to be similar to *W. zeae* on the leaf surface. Tillers were rinsed briefly in deionized water, blotted dry on a paper towel before placing on medium. Previous experience indicated surface disinfestation of leaf tissue was not required to successfully isolate *W. zeae*.

Isolation plates were placed in crisper containers, then kept at room temperature (approximately 22-25°C) under ambient light. For 2 to 10 d, isolation plates were assessed multiple times for the presence of *W. zeae* mycelium growing from leaf tissue. To confirm identification of *W. zeae*, hyphae were transferred to sterile one-quarter-strength potato dextrose agar (PDA) (10 g L^{-1} PDA, 11.2 g L^{-1} agar) (BD Difco, Becton, Dickinson and Company, Franklin Lakes, NJ) including the same antibiotics and fungicide added to water agar. Morphological identification of *W. zeae* was based on colony appearance—including right-angle branching of mycelium, and the formation of characteristic sclerotia (Kerns et al., 2017; Martin & Lucas, 1983; Voorhees, 1934). If *W. zeae* grew out of one or more tillers, the experimental unit was scored one, if no *W. zeae* was detected from any tiller, the experimental unit was scored zero.

Isolation frequency counts were analyzed in JMP ver. 16.0.1 (SAS Institute Inc., Cary, NC) using a generalized linear model with binomial distribution and logistic (logit) link function. An initial analysis of frequency counts using a model containing only core collection from symptomatic versus asymptomatic UDBG indicated that isolation frequency between symptomatic and asymptotic UDBG was not different (P = .442). Therefore, the model for final analysis contained only sampling date, which was significant (P= .001); and single-degree-of-freedom contrasts were used to compare sampling event pooled across years, month of sampling events between years, and month of sampling event within a year.

Soil temperature was recorded every hour by installing HOBO UA-001-64 Pendent data loggers (Onset Computer Corporation, Bourne, MA) in the putting green rootzone. Four data loggers each were placed at a soil depths of 5 and 10 cm. Data loggers were installed on 10 June 2016 and 18 May in 2017 and remained in the rootzone for the duration of each study year. The mean of four values, one value from each of four data loggers at each depth, was used to produce a mean, minimum, and maximum daily soil temperature at each depth. Soil temperatures at 5 and 10 cm were similar; consequently, soil temperature values were averaged across both depths to create a daily mean, minimum, and maximum soil temperature. Air temperature was recorded four times per hour by an onsite weather station (Campbell Scientific, Inc., Logan, UT). Similar to soil temperature, a daily mean, minimum, and maximum temperature were calculated.

Results

Waitea zeae isolation frequency

Waitea zeae isolation frequencies in June, August, and September was higher in 2017 compared to 2016. (Table 3.1 and Figure 3.1). However, the probability of isolating *W. zeae* in July 2016 and July 2017, were not significantly different indicating a year × month interaction (Table 3.1). The interaction of year × month required the comparison of isolation frequency by month be conducted within each year.

In 2016, isolation frequency of *W. zeae* was greatest in August and September (Figure 3.1). The probability of isolating *W. zeae* in August and September was similar and significantly higher than in June but not different than in July or October (Table 3.2). Similar to 2016, the probability of *W. zeae* isolation in 2017 was greatest in August and September (Figure 3.1). Frequencies of isolation in August and September 2017 was similar and significantly greater than June (Table 3.2 and Figure 3.1). Isolation frequency in August 2017 was also significantly higher than July; however, the probability of isolating *W. zeae* in July and September were statistically similar (Table 3.2).

Air and soil temperature

Previous in vitro research demonstrated *W. zeae* mycelium grows readily between 25 and 35°C and declines below 20°C or above 35°C (Burpee and Martin, 1992; Elliott, 1999; Kammerer et al., 2011; Kerns et al., 2017; Voorhees, 1934). Between 01 June and 30 Oct. 2016, the daily mean air temperature was in the optimal growth range for *W. zeae* (25 to 35°C), 90 d or 59% of the days air temperatures were recorded (Figure 3.2). Of those 90 d, 21, 31, 30, 8 and 0 d occurred in June, July, August, September, and October, respectively . In 2017, the daily mean air temperatures were within the optimal range 12, 26, 18, 5 and 4 d in June, July, August, September, and October, respectively, or 42% of days between 1 June and 31 Oct. (Figure 3.3). In both years, the mean daily temperature never exceeded 35°C. Maximum daily air temperatures were greater than 35°C 10 d in 2016 but only 4 d in 2017 (Figures 3.2 and 3.3). In both years, the minimum daily air temperatures were below 25°C on all but 2 d (Figures 3.2 and 3.3).

Soil temperatures were recorded a total of 144 d from 10 June to 30 Oct. 2016. Mean daily soil temperatures were within the optimal growth range for *W. zeae* 76% of those days (Figure 3.4). In June, July, August, September, and October, mean daily soil temperatures were within the optimal range on 21, 31, 31, 27, and 0 d, respectively. Minimum daily soil temperature was never greater than 30°C; however, it was greater than 25°C 14, 30, 31, 5, and 0 d in June, July, August, September, and October,

respectively (Figure 3.4). In June, July, August, and September, maximum daily soil temperatures were between 25 and 35°C every day except one in July when it rose to 35.1°C (Figure 3.4). Only 6 d in October 2016 had maximum daily soil temperatures within the optimal growth range for *W. zeae.*

In 2017, mean daily soil temperatures were within the optimal range 75% of the time (Figure 3.5). Mean daily soil temperature was within the optimal range *for W. zeae* mycelium growth every day in July and 27, 29, 21, and 6 d, in June, August, September, and October, respectively. Minimum daily soil temperatures in 2017 followed a similar pattern as in 2016 and never exceeded 30°C (Figure 3.5). In September, no days had minimum daily soil temperatures within the optimal range and October had only 3 d while June had 10 d. July and August had 28 and 23 d, respectively, with minimum daily temperatures within the optimal range for *W. zeae* growth. Maximum daily soil temperature was between 25 and 35°C every day in June, July, and August (Figure 3.5). Maximum daily soil temperatures were within the optimal growth range 28 d in September, and 14 d in October.

Discussion

Cores were collected from both symptomatic and asymptomatic UDBG to control for human bias. If cores were harvested randomly, potential existed for an unconscious bias to select cores from turfgrass perceived to be displaying mini-ring symptoms, which would over-estimate the probability of isolating *W. zeae*. The probability of isolating *W. zeae* from symptomatic versus asymptomatic UDBG was similar in this study. This is counterintuitive, as one would expect isolation from symptomatic turfgrass to be greater than asymptomatic turfgrass. As stated previously, mini-ring symptoms do not develop until late-summer, therefore, much of this experiment was conducted prior to the appearance of obvious mini-ring symptoms. Furthermore, once mini-ring symptoms were visibly present, leaf tissue had begun to senesce and other saprophytic fungi were often found colonizing the leaf tissue.

Waitea zeae isolation frequency was greater in 2017 compared to 2016 in every month. The reason for the increase in 2017 is not known, but it could be due to a combination of two factors. First,

leaf tissue placed on media for isolation was not selected at random; rather, leaf tissue selection was based on the presence of what appeared to be *W. zeae* mycelium on the leaf surface. It is possible the selection of leaf tissue infected with *W. zeae* became more proficient with time. Second, based on observations of mini-ring outbreaks on UDBG putting greens, symptoms become more severe and widespread over multiple years. It is reasonable to assume *W. zeae* was occupying a greater portion of the putting green in 2017 compared to 2016—i.e., population densities increased with time.

As documented by other turfgrass pathologists and from personal observation, mini-ring symptoms in the transition zone can appear in late-July, but typically do not become distinct and cause widespread UDBG decline until late-August or into early-fall (Dant, unpublished data, 2022; Kerns et al., 2017; Martin, 2009; Martin & Park, 2010). Isolation frequency in June 2016 was low (2.1%); however, frequency increased substantially to 17% in June 2017. This indicates *W. zeae* is likely present in UDBG putting greens when mini-ring symptoms are not visible. This may further explain why *W. zeae* was not isolated from symptomatic UDBG more frequently than from asymptomatic UDBG. In June 2016, the mean daily soil temperatures were within the range of optimal growth for *W. zeae* every day and, in June 2017, it fell below the optimal growth range only 3 d. *Waitea zeae* isolation was not performed from this UDBG putting green nor was soil temperature data recorded in the month of May. *Waitea zeae* isolation frequency in May, when soil temperatures are typically lower, may have led to a better understanding of how soil temperature impacts *W. zeae* development in UDBG putting greens.

Koehler and Shew (2017) collected isolates from tall fescue leaf tissue in North Carolina exhibiting brown patch symptoms for a 12-wk period starting in May, ending in July. The researchers were able to isolate *W. zeae* in June and July. Martin, et al. (1983) conducted an experiment to characterize and understand horizontal distribution of *Rhizoctonia* species present in a tall fescue home lawn located in North Carolina. In June of two years, they collected organic debris from turfgrass cores taken to a depth of approximately 5 cm. In their study, *W. zeae* accounted for ~47% of *Rhizoctonia*-like

fungi isolated from organic debris. Neither of these studies reported air or soil temperature, but both studies are consistent with this study, confirming the presence of *W. zeae* in turfgrass stands in June.

In 2016, *W. zeae* isolation occurrence did not differ among July, August, and September, although it was nearly twice greater in August and September compared to July. Mean soil temperatures in July and August of 2016 were above 25°C and for 27 d in September; therefore, it is reasonable isolation frequencies were greatest during these months. Furthermore, these months are typically when mini-ring symptoms appear and cause UDBG decline. *Waitea zeae* isolation frequencies and soil temperatures in July, August, and September 2017 followed a similar pattern as 2016. Isolation frequency was greatest in August 2017 at 46%, and 35% in September almost twice greater than in July.

Mini-ring symptoms in UDBG are often present in October; however, in 2016, isolation frequency was low (6%) in our study. Unfortunately, data from October 2017 was not usable, so results from 2016 could not be confirmed. Mean soil temperatures were lower in October compared to any other month data was collected, with 0 d in 2016 and only 6 d in 2017 being greater than 25°C; therefore, it is logical isolation during these times would be less than previous months. *Waitea zeae* isolation frequencies were relatively high in August and September, then declined in October. This appears to indicate when mini-ring symptoms appear in late-fall or symptom severity increases late in the season, it is likely due to the inability of UDBG to grow and recover from *W. zeae* infection.

Optimal growth of warm-season turfgrasses, including bermudagrass, occurs at air temperatures near 30°C, with growth occurring from approximately 26°C to 38°C (Beard, 1973; Esmaili & Salehi, 2012; McCarty, 2018). During this study, the maximum daily air temperatures in 2016 were above 30°C on 24 d in June, all of July and August, and only on 14 d in September and 1 d in October. Air temperatures in 2017 were slightly cooler in mid-summer and slightly warmer in fall compared to 2016 but followed the same general pattern in both years. Mean daily air temperatures in both years were rarely above 30°C and dropped below the optimal growth range for bermudagrass on 24 and 28 d in September of 2016

and 2017, respectively. Mean daily air temperatures were below the optimal growth range for bermudagrass every day in October 2016 and 30 d in October 2017.

McCarty (2018) suggests optimal root growth for warm-season turfgrass occurs between 24 and 29°C when soil temperature is taken at a 10-cm depth. In this study, soil temperatures were recorded at 5 and 10 cm, and then daily soil temperature values calculated using the mean of those two data points. Daily mean soil temperatures in 2016 were within the optimal range for bermudagrass root growth most of June, increased above 29°C for much of July and August, and then decreased to temperatures within the optimal growth range every day in September. In October 2016, daily mean soil temperature was within the optimal range for 2 d and then fell below 24°C for the remainder the month. Daily mean soil temperature in 2017 followed a similar trend as 2016; however, soil temperatures in July and August were not as high. There were 6 d in September where daily mean soil temperatures were below 24°C and October had 6 d within the optimal range for bermudagrass root growth.

Bermudagrass is intolerant of low light conductions (Beard, 1973; Christians, 2004; McCarty, 2018). McCarty (2018) states warm-season turfgrasses require a 13 h solar radiation day length for optimal growth and Bunnell and McCarty (2004) found 'TifEagle' UDBG putting greens require an average daily light integral of 32.6 mol m^{-2} d^{-1}. Bunnell and McCarty (2004) calculated a minimum of 8 h of full sunlight per day was required for UDBG to maintain acceptable turfgrass quality. In Florence, SC (34°17'14"N lat.; 79°44'09"W long.), where this mini-ring study was conducted, solar day length is greater than 13 h in June, July and in all but three days in August. Mean solar day length for September is 11 h and 57 min and for October is 11 h and 17 min (NOAA Global Monitoring Laboratory). Bunnell and McCarty (2004) conducted their study from June to August when sunlight intensity was greatest. Daily light integral values were not collected in the present study but daily light integral values in September and October would likely fall below the 32.6 mol m^{-2} d^{-1} threshold suggested by Bunnell and McCarty (2004) because of decreasing day length and sunlight intensity.

Growth of UDBG and *W. zeae* occurs within the same temperature range of approximately 25 to 35°C, with optimal temperature for both host and pathogen being near 30°C. In this host-pathogen relationship, it appears UDBG is able to overcome *W. zeae* infection during summer when temperature and light intensity are optimal for plant growth. As growing conditions for UDBG become suboptimal in late-summer, mini-ring symptoms are expressed. Martin and Park (2010) noted mini-ring symptoms often appear after UDBG is mechanically injured by aggressive agronomic practices, furthering the argument the host and pathogen coexist much of the growing season, but symptoms do not become visible until UDBG growth is compromised. The climatic data collected in this study, along with decreasing light intensity discussed previously, suggest the growing conditions for UDBG are optimal in June, July, and most of August but decline in September and October for a large portion of the southern United States. Similar to other soilborne fungi causing disease in UDBG—e.g., spring dead spot (causal agent = *Ophiosphaerella* spp.) and take-all root rot [causal agent = *Gaeumannomyces* spp.; *G. graminis* (Sacc.) Arx & Olivier var. graminis, *G. graminicola* Hern.-Restr. & Crous; *Candidacolonium cynodontis* P.L. Vines & M. Tomaso-Peterson; and *Magnaporthiopsis cynodontis* P.L. Vines & Tom.-Pet.]—management of *W. zeae* should begin before symptom appearance (Stevens et al., 2022; Tredway et al., 2009).

It is unclear if *W. zeae* first infects turfgrass roots or above-ground plant parts, but it is likely all plant parts are susceptible to some degree during disease progression. In turfgrass culture, *W. zeae* is primarily isolated from above-ground plant tissue (Chang & Lee, 2016; Haygood & Martin, 1989; Kerns et al., 2017; Koehler & Shew, 2017; Martin 1983). Voorhees (1934), who first described *W. zeae*, isolated it from ears of corn (*Zea mays* L.), but suggested the fungus overwinters in organic matter and soil as mycelium and sclerotia. Warcup and Talbot (1962) discovered the teleomorph of *W. zeae* (*W. circinata* Warcup & Talbot) and used soil along with root tissue to collect isolates for further investigation. Others also recovered *W. zeae* from organic matter and soil. Martin and Lucas (1983) isolated *W. zeae* from organic debris collected from a tall fescue turfgrass stand and Windham (1985)

isolated *W. zeae* from soil collected where a tall fescue lawn and soybean (*Glycine max* L.) field were established.

In this study, isolation frequency was greatest in months when soil temperature was consistently above 25°C, consistent with previous research showing optimal growth of *W. zeae in vitro* occurs between 25 and 35°C (Burpee & Martin, 1992; Elliott, 1999; Kammerer et al., 2011; Kerns et al., 2017; Voorhees, 1934. Kerns et al. (2017) noted temperature by itself does not predict UDBG infection, but soil temperature could be used by turfgrass managers as an indicator to initiate *W. zeae* management programs, including the application of fungicides.

In a 2-year study conducted in the months of June through September of 2016 and 2017, and October of 2016, *W. zeae* was isolated from an UDBG putting green in all months but was isolated most frequently in the months of July, August, and September. In addition, soil temperatures in the UDBG putting green used for this study were within the optimal range for growth of *W. zeae* from June through most of September. Therefore, this suggests preventative management programs for mini-ring should begin prior to symptom development in June, before *W. zeae* becomes most active. Future research should be conducted to determine if *W. zeae* infects bermudagrass at temperatures coinciding with optimum mycelium growth *in vitro* and if preventative management programs, specifically fungicide applications, can be timed using soil temperature as a predicative tool.

Table 3.1. Chi-square statistic and *P* values associated with single-degree-of-freedom contrasts comparing the probability of isolating *Waitea zeae* from an ultradwarf bermudagrass putting green in 2016 and 2017.

Contrast	χ^2 value	$P > \chi^2$
June 2016 vs June 2017	4.29	**.038**[a]
July 2016 vs July 2017	2.27	.131
August 2016 vs August 2017	11.53	**.001**
September 2016 vs September 2017	4.46	**.035**
October 2016 vs October 2017	--[b]	--[b]

[a] Bold *P* values are significant ($P \geq .05$).

[b] Missing data.

Table 3.2. Matrix of *P* values from single-degree-of-freedom contrasts comparing *Waitea zeae* isolation frequency from an ultradwarf putting green within year by month for 2016 and 2017. For each month, isolation of *W. zeae* was attempted from 48 ultradwarf bermudagrass cores. The putting green was located at the Clemson University Pee Dee Research and Education Center in Florence, SC.

	2016					2017				
Month	June	July	August	September	October	June	July	August	September	October
	$P > \chi^2$									
June	--	--	--	--	--	--	--	--	--	--
July	.155	--	--	--	--	.789	--	--	--	--
August	**.019**[a]	.334	--	--	--	**.002**	**.004**	--	--	--
September	**.038**	.213	.779	--	--	**.035**	.065	.298	--	--
October	.330	.694	.193	.103	--	--[b]	--[b]	--[b]	--[b]	--

[a] Bold *P* values are significant ($p \geq .05$).

[b] Missing data.

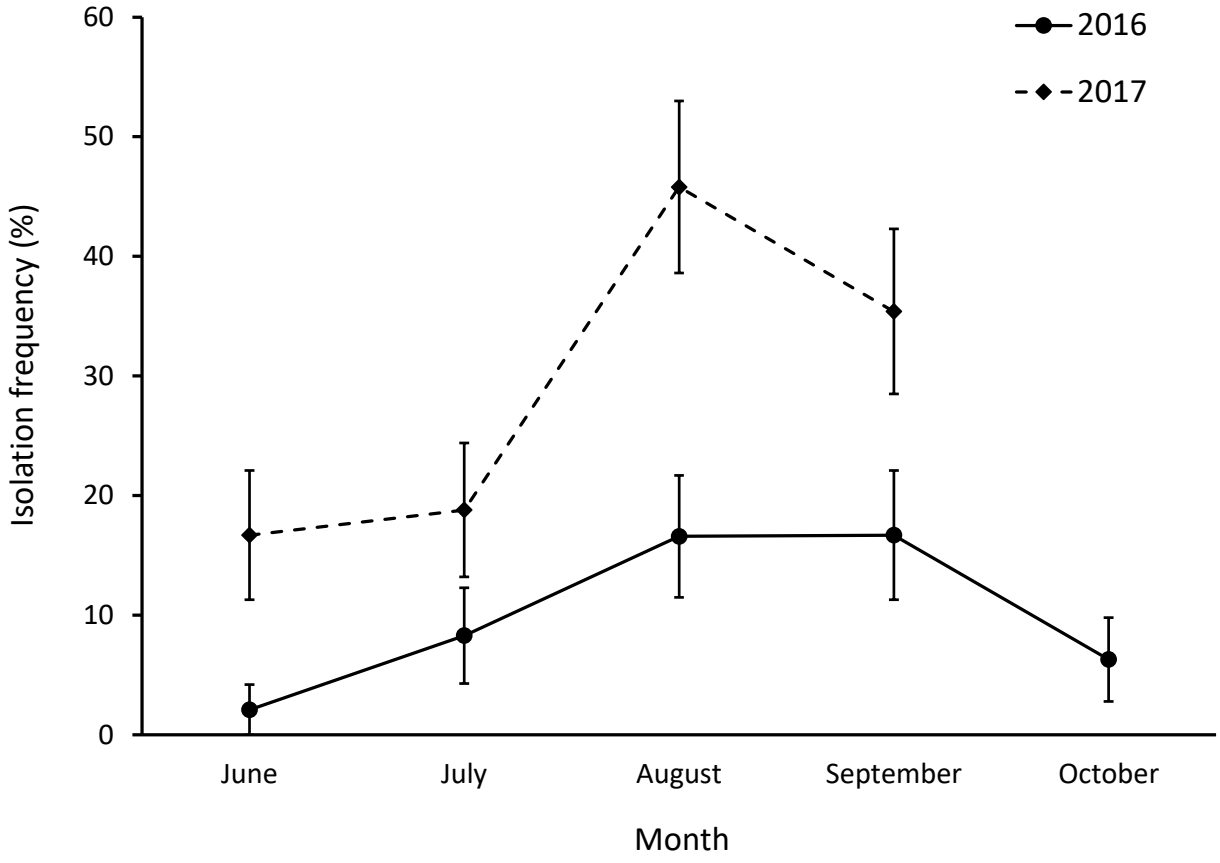

Figure 3.1. Mean monthly frequency of isolating *Waitea zeae* from 48 ultradwarf bermudagrass cores collected from a putting green in 2016 and 2017. The putting green was located at the Clemon University Pee Dee Research and Education Center in Florence, SC. For each month, isolation of *W. zeae* was attempted from 48 ultradwarf bermudagrass cores. Error bars are the standard error for each frequency. Data for October

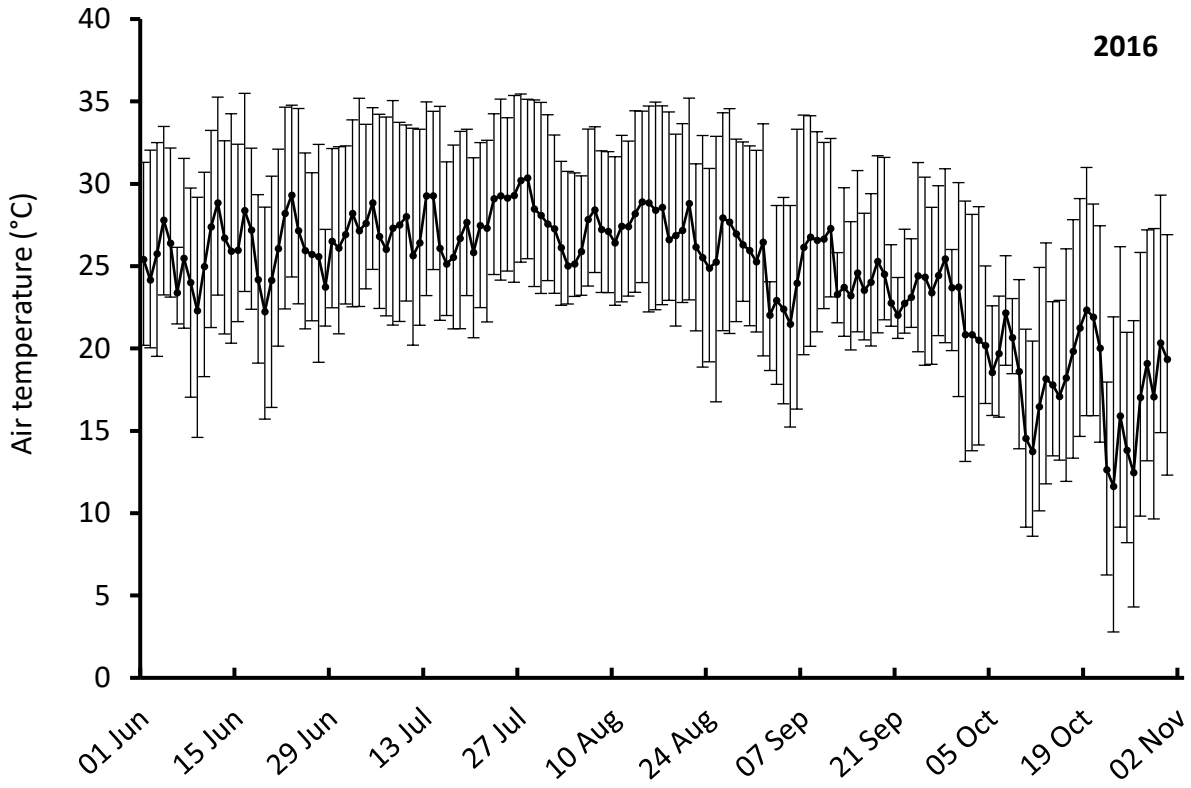

Figure 3.2. Air temperature recorded at the Clemson University Pee Dee Research and Education Center in Florence, SC from 01 June to 31 Oct. 2016 by an onsite weather station. Air temperature was recorded four times per hour. Each data point represents the daily mean temperature, which was calculated from all values recorded for that day. Error bars denote the minimum and maximum temperature for each day.

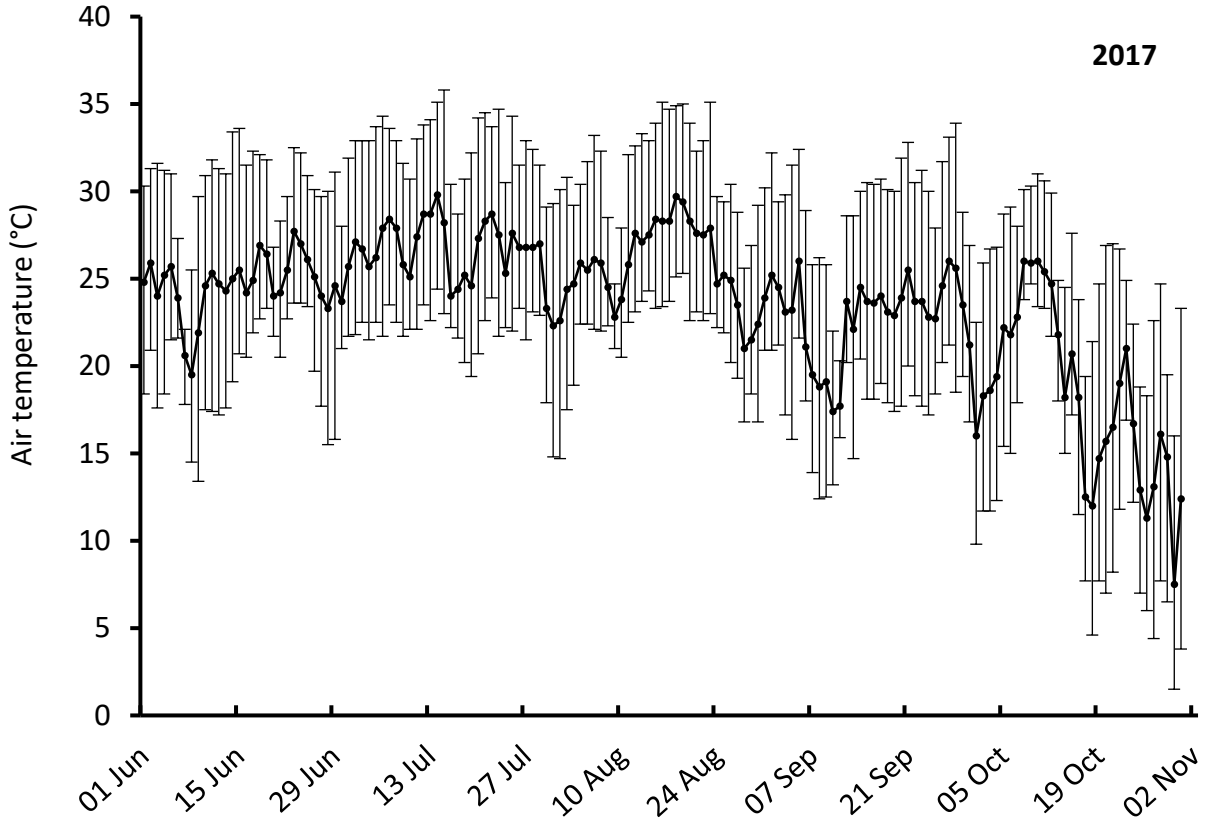

Figure 3.3. Air temperature recorded at the Clemson University Pee Dee Research and Education Center in Florence, SC from 01 June to 31 Oct. 2017 by an onsite weather station. Air temperature was recorded four times per hour. Each data point represents the daily mean temperature, which was calculated from all values recorded for that day. Error bars denote the minimum and maximum temperature for each day

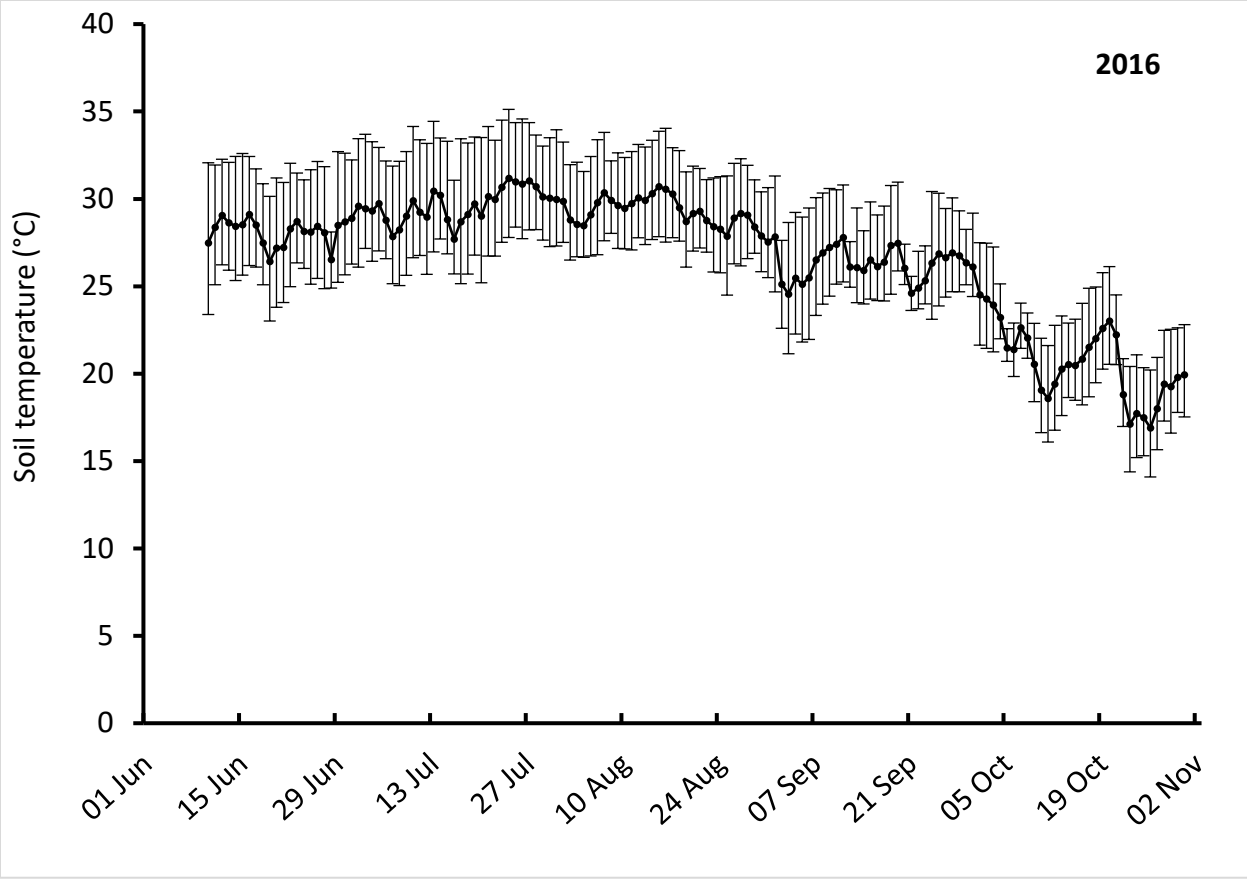

Figure 3.4. Soil temperature of an ultradwarf bermudagrass putting green located at Clemson University Pee Dee Research and Education Center in Florence, SC from 10 June to 31 Oct 2016. HOBO UA-001-64 Pendent data loggers (Onset Computer Corporation, Bourne, MA) were used to record the soil temperature one time per hour at a depth of 5 5 and 10 cm (n = 4 for each depth). Mean, minimum, and maximum daily temperatures were calculated for each depth, then averaged to produce one mean, minimum, and maximum value per day. Error bars denote the minimum and maximum soil temperature for each day.

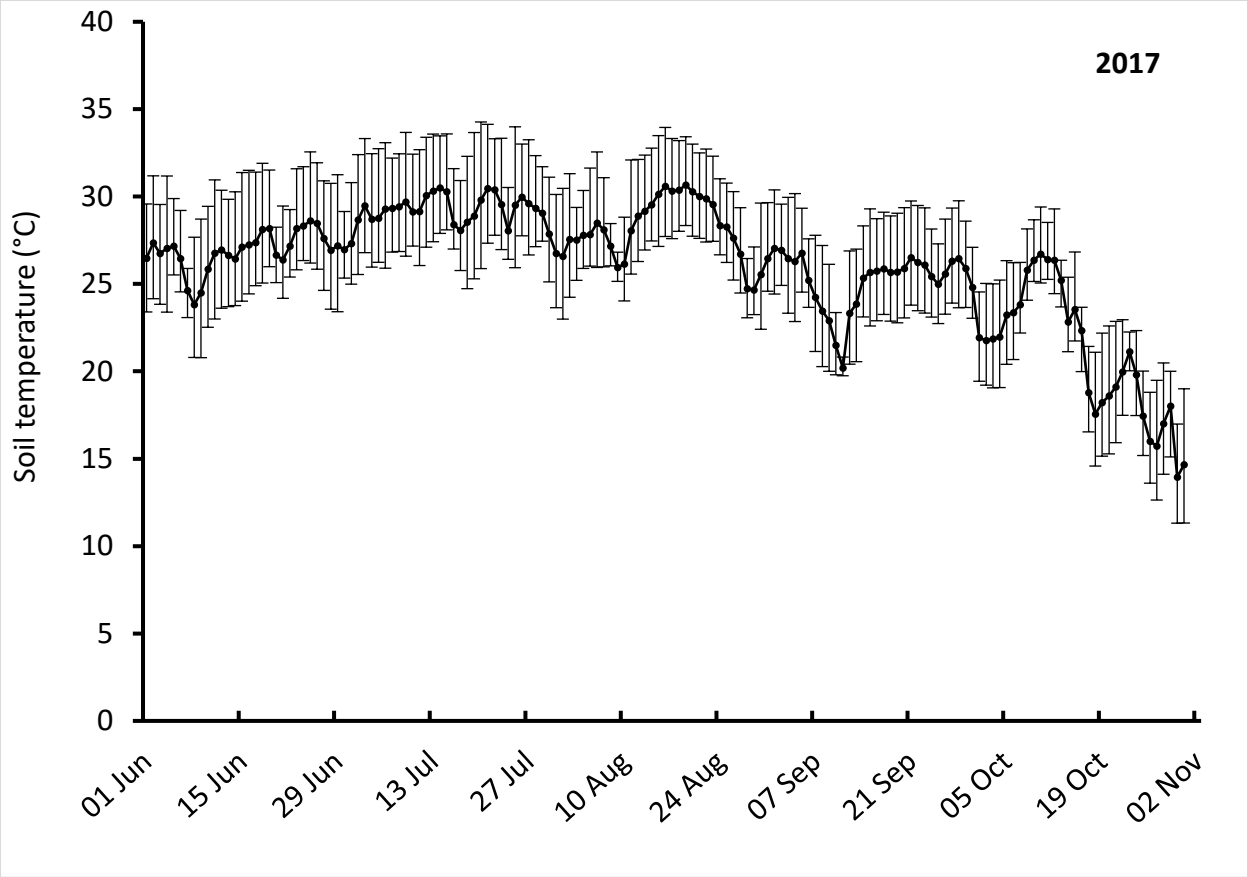

Figure 3.5. Soil temperature in an ultradwarf bermudagrass putting green located at Clemson University Pee Dee Research and Education Center in Florence, SC from 01 June to 31 Oct 2016. HOBO UA-001-64 Pendent data loggers (Onset Computer Corporation, Bourne, MA) were used to record the soil temperature one time per hour at a depth of 5 5 and 10 cm (n = 4 for each depth). Mean, minimum, and maximum daily temperatures were calculated for each depth, then averaged to produce one mean, minimum, and maximum value per day. Error bars denote the minimum and maximum soil temperature for each day.

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CHAPTER FOUR

MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF *WAITEA* SPECIES IN ULTRADWARF BERMUDAGRASS

Abstract

Waitea zeae (Voorhees) J.A. Crouch & Cubeta is the causal agent of mini-ring in ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens, a disease also refered to as leaf and sheath spot in turfgrass (Dant & Martin, 2023; Kerns et al., 2017). Although *W. zeae* is cited as the primary pathogen causing disease, *W. circinata* Warcup & P.H.B. Talbot and *W. oryzae* J.A Crough & Cubeta have also been isolated from UDBG displaying mini-ring symptoms. Molecular characterization of species of *Waitea* isolated from UDBG putting greens is limited and, although pathogenicity of several species has been demonstrated in turf, it is still not clear how these fungi infect UDBG. Species of *Waitea* were collected from UDBG putting greens displaying symptoms of mini-ring at several locations in South Carolina and North Carolina, USA. These isolates were identified using sequence data from the rDNA internal transcribed spacer region (ITS) and selected isolates were used to infest growing medium containing UDBG plants in a controlled environment bioassay. Most isolates collected from symptomatic UDBG were identified as *W. zeae*; however, *W. prodiga* J.A. Crouch & Cubeta, and *W. oryzae* were also recovered. This study demonstrates when growing medium was infested with either *W. zeae* or *W. oryzae*, infection of UDBG root, stolon, and leaf tissue can ensue. Root infection by *W. oryzae* and *W. zeae* has been reported in other crops, but to the author's knowledge infection of UDBG roots by these species have not been documented previously. Additionally, *Waitea prodiga* was isolated from mini-ring symptomatic UDBG. *Waitea prodiga* has not been reported as a potential pathogen of UDBG.

Introduction

A disease described by turf practitioners as mini-ring began appearing in ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens located in the southeastern United States in the late 1990s. Frog-eye patches with a yellow to bronze outer ring and green center, ranging in size from 10 to 40 cm generally appeared during late-summer and often remain visible through fall, into bermudagrass dormancy. Elliot (1999) was first to isolate *Waitea zeae* from hybrid bermudagrass, and Martin and Park (2010) later suggested *W. zeae* was the probable causal agent; however, Martin (2009) had previously proposed that multiple *Waitea* species may be involved in the mini-ring disease complex, including, *W. zeae* and *W. oryzae.* Kerns et al. (2017) first published a study specifically focused on mini-ring and collected *W. zeae* and *W. circinata* isolates from UDBG displaying mini-ring symptoms. Although most of their isolates were *W. zeae* (17 of 20), it remains unclear what role other members of the genus *Waitea* play in causing mini-ring in UDBG.

There are currently five species of plant-pathogenic fungi that belong to *Waitea*, a genus preserved when fungal taxa within *Agaricomycotina* were reclassified with a single scientific name (Stalpers et al., 2021). Classification of the fungi that now belong to the genus *Waitea* was previously confusing, with use of invalid naming conventions, perceived relatedness of species of *Waitea* to fungi within the genus *Rhizoctoina*, and reliance on morphological characteristics and anastomosis reactions to distinguish among species of *Waitea* (Stalpers & Andersen, 1996; Kammerer et al., 2011; Leiner & Carling, 1994; Kerns, et al., 2017; Stalpers, et al., 1998; Stalpers et al., 2021). While species of *Waitea* share some morphologically similar characteristics with species of the genus *Rhizoctonia*, Stalpers et al. (2021) recognized recent studies using molecular methods justify the need for species of *Waitea* to be placed in a separate genus. Fungi within the genus *Waitea* rarely produce sexual structures, grow quickly in culture and produce right-angle-branching hyphae 3 to 10 um in diameter, and as cultures mature, form sclerotia of varying shapes and colors large enough to be visible macroscopically (Burpee

& Martin, 1992; Kammerer et al., 2011; Stalpers et al., 2021). *Waitea* species grow optimally in culture between 25 and 35°C and at least three of five species are insensitive to benzimidazole fungicides (Burpee & Martin, 1992; Carling et al., 1990; Chang & Lee, 2016; Christensen, 1979; de la Cerda et al., 2007; Elliott, 1999; Kerns et al., 2017; Kammerer et al., 2009; Kammerer et al., 2011; Martin et al., 1984; Rios et al., 2006; Toda et al., 2005; Toda et al., 2007; Voorhees, 1934).

Martin and Lucas (1983) were first to report *W. zeae* as a pathogen of turfgrass, and demonstrated that isolates collected from tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.] were more virulent on cool-season grasses—creeping bentgrass (*Agrostis stolonifera* L.), Kentucky bluegrass, tall fescue and perennial ryegrass (*Lolium perenne* L.)—compared to warm-season grasses–common bermudagrass [*Cynodon dactylon* (L.) Pers var. *dactylon*], and centipedegrass [*Eremochloa ophiuroides* (Munro) Hack]. Haygood and Martin (1990) demonstrated that *W. zeae* caused disease in St. Augustinegrass [*Stenotaphrum secundatum* (Walter) Kuntze]; Elliott (1999) showed pathogenicity in hybrid bermudagrass and roughstalk bluegrass (*Poa trivialis* L.), and Kammerer et al. (2011), isolated *W. zeae* from seashore paspalum (*Paspalum vaginatum* Swartz). Voorhees (1934) was first to describe *W. zeae* (previously *R. zeae*) as a plant pathogen when he isolated it from rotting ears of field corn (*Zea mays* L.) in Florida. Of the five species within the genus *Waitea*, *W. zeae* has been investigated most extensively and is considered a pathogen of several agronomic crops including barley (*Hordeum vulgare L.),* cabbage (*Brassica oleracea* var. *capitata*), carrot (*Daucus carota* L.), cotton (*Gossypium hirsutum* L.), field corn, oilseed rape (*Brassica napus* var. *oleifera*), snap bean (*Phaseolus vulgaris* L.), sorghum (*Sorghum bicolor* L.), soybean (*Glycine max* L.), sugar beet (*Beta vulgaris* L.), and wheat (*Triticum aestivum* L.) with isolates originating from every continent except Antarctica (Erper et al., 2005; Erper et al., 2006; Kuznia & Windels, 1994; Tomaso-Peterson & Trevathan, 2007; Vojvodić et al., 2021). Disease in turfgrass caused by both *W. zeae,* and to a lesser extent, *W. oryzae,* is now referred to as leaf and

sheath spot (previously Rhizoctonia leaf and sheath spot) on most turfgrass species but mini-ring on UDBG (Dant & Martin, 2023; Haygood & Martin, 1990; Kerns et al., 2017; Smiley et al., 2005).

Haygood and Martin (1990) first reported *W. oryzae* as a pathogen of turfgrass when they obtained several isolates from centipedegrass and St. Augustinegrass and induced stem rot on both turf species in growth chamber experiments. *Waitea oryzae* has received significant research attention as a pathogen of both rice and wheat due to worldwide economic and nutritional importance of both crops (Gunnell, 1986; Leiner & Carling, 1994; Mazzola et al., 1995; Ogoshi et al., 1990; Okubara et al., 2008; Oniki et al., 1985; Ryker & Gooch, 1938). *Waitea oryzae* has been isolated from warm-season turfgrass by multiple researchers; however, it is unclear if *W. oryzae* is a significant pathogen of turfgrass grown for aesthetic and recreational purposes (Burpee & Martin, 1992; Dant & Martin, 2023; Haygood & Martin, 1990; Kammerer et al., 2011).

The other three species included in the genus—*W. circinata*, *W. agrostidis*, and *W. prodiga*—have been understudied as they purportedly have a narrow host range and primarily are considered pathogens of turfgrasses (Burpee & Martin, 1992; de la Cerda et al., 2007; Kammerer et al., 2011; Kerns et al, 2017; Toda et al., 2005; Toda et al., 2007).

Brown ring patch is a disease of creeping bentgrass and annual bluegrass (*Poa annua* L.) where *W. circinata* causes yellow to brown colored rings ranging from 10 to 50 cm in diameter (Toda et al.; 2005; de la Cerda et al., 2007). *Waitea circinata* is not considered a significant pathogen of warm-season turfgrasses. Toda et al. (2007) collected several isolates of an unidentified species of *Waitea* from creeping bentgrass and Kentucky bluegrass (*Poa pratensis* L.) in Japan causing "Waitea reddish-brown patch disease". Stalpers et al. (2021) later classified the isolates described by Toda et al (2007) as *W. agrostidis* J.A. Crouch & Cubeta. *Waitea agrostidis* has not been reported as a significant pathogen of warm-season turf or turfgrass outside of Japan (Stalpers et al., 2021; Toda et al., 2007). Kammerer et al. (2011) first isolated and described *W. prodiga* J.A. Crouch & Cubeta as a pathogen of seashore

paspalum, proposed naming the disease basal leaf blight and demonstrated pathogenicity in seashore paspalum, common bermudagrass, and roughstalk bluegrass in growth chamber experiments. Dant and Martin (2023) suggested disease caused by *W. prodiga* in turfgrass be referred to as leaf and sheath spot because symptoms are similar to those caused by *W. oryzae* and *W. zeae*.

It is unclear if species of *Waitea* other than *W. zeae,* play a role in the development of mini-ring. While Martin and Park (2010) proposed *W. zeae* caused mini-ring, and Kerns et al. (2017) later isolated primarily *W. zeae* when they sampled symptomatic UDBG putting greens, their isolate collection originated from a limited number of putting greens and included a low number of *W. circinata* isolates. Therefore, the first objective of this study was to collect and characterize isolates of *Waitea* species from multiple UDBG putting greens located in both North and South Carolina.

Pathogenicity of all species of *Waitea* has been studied in turfgrass; however, most of these studies demonstrated pathogenicity by inoculating above-ground plant tissue and evaluating foliar blighting or lesions (Chang & Lee, 2016; Chen et al. 2011; de la Cerda et al., 2007; Elliott, 1999, Haygood & Martin, 1990; Kammerer et al., 2011; Martin & Lucas, 1983, Martin & Lucas, 1984; Toda et al., 2005; Toda et al., 2007). Leaves from UDBG putting greens with typical mini-ring symptoms does not have discrete foliar lesion and do not appear water-soaked and blighted as expected from infection by a foliar plant pathogen, rather leaf tissue simply dies back. Consequently, the second objective of this study was to determine if species of *Waitea* infect UDBG roots and stolons, which may ultimately cause the typical above-ground symptoms associated with mini-ring.

Materials and methods

Isolate collection

Isolates used in this study were obtained by isolation from leaf tissue or by baiting turfgrass cores (Table 4.1). Turfgrass cores, 11 cm in diameter, were harvested from UDBG displaying mini-ring symptoms. Symptomatic leaves were briefly rinsed in deionized water or 70% ethanol, rinsed twice in

deionized water and blotted dry on a paper towel. Leaves were then placed in 9-cm-diameter Perti plates containing either one-quarter-strength potato dextrose agar [PDA; 10 g L^{-1} PDA (BD Difco, Becton, Dickinson and Company, Franklin Lakes, NJ)] and 11.2 g L^{-1} agar (BD Bacto, Becton, Dickinson and Company) or water agar [15 g L^{-1} agar (BD Bacto, Becton, Dickinson and Company)] both media were prepared with deionized water and were amended with 0.2 g L^{-1} streptomycin sulfate, 0.2 g L^{-1} ampicillin and 0.05 g L^{-1} thiophanate-methyl (3336 F, Nufarm Americas, Inc., Alsip, IL), respectively. Based on previous experience, extensive surface disinfestation was not required to successfully isolate species of *Waitea* from UDBG tissue (Dant, unpublished data, 2022). Plated leaves were stored in a deep plastic box (23 cm wide by 30 cm long by 9 cm) placed on a lab bench at room temperature (approximately 22-25°C) for observation.

A method originally described by Windham & Lucas (1987) was modified to bait species of *Waitea* from UDBG cores. Baits were cut from 3-mm-square wood matchsticks or 2-mm-diamter cotton-tipped wood applicators with the striking tip of the matches and cotton swabs removed and discarded; the remaining wooden sticks were cut into 2-cm segments. Baits were covered with deionized water in a glass beaker and autoclaved (20 min at 121°C, 1.5 MPa pressure) twice—once on each of two consecutive days. After autoclaving, baits were soaked for 2 h in sterilize deionized water containing 0.2 g L^{-1} streptomycin sulfate, 0.2 g L^{-1} ampicillin, 0.05 g L^{-1} thiophanate-methyl (3336 F, Nufarm Americas, Inc.) and 0.1 g L^{-1} mefenoxam (Subdue Maxx, Syngenta Crop Protection, LLC., Greensboro, NC). Turfgrass cores, as described previously, were baited by inserting approximately 12 baits into the turfgrass canopy until the top of the bait was level with the soil surface. Baited cores were placed in plastic bags to maintain high humidity and placed at room temperature for approximately 72 h, and then baits were removed and placed on water agar amended as described previously plus 0.1 g L^{-1} of mefenoxam (Subdue Maxx, Syngenta Crop Protection, LLC.).

Mycelia originating from leaves or baits that resembled mycelium of species of *Waitea* (i.e., fast growing, single-stranded hyaline hyphae with right-angle branching glimmering slightly when refracting light) was transferred to fresh PDA containing the same amendments as described above, with or without thiophanate-methyl. Isolates were maintained on PDA with periodic transfers (approximately every 30 d) to fresh plates and stored at room-temperature in plastic boxes placed on a lab bench.

Infection of ultradwarf bermudagrass roots, stolons, and leaves by Waitea oryzae and W. zeae

Two isolates of *W. oryzae* and six isolates of *W. zeae* (Table 1) were selected to determine the ability of each isolate to infect roots, stolons, and leaves of UDBG plants. Inoculum for each isolate was prepared separately by adding two 9-mm-diameter one-quarter-strength PDA plugs per isolate to 10 g of oat seed in a 125 ml Erlenmeyer previously hydrated with 15 ml of deionized water and sterilized by autoclaving twice for 60 min—once on each of two consecutive days. Inoculated oats remained at room temperature for 14 d and were shaken regularly to prevent clumping and encourage uniform colonization of oat seeds.

Aerial rootless stolons of 'TifEagle' UDBG were placed in sterilized substrate composed of sand and heat-treated montmorillonite clay (Profile Greens Grade, Profile Products, LLC., Buffalo Grove, IL) mixed at a 4 to 1 ratio by volume. Sprigs were fertilized with a 24-8-16 (N-P-K) complete fertilizer (Miracle-Gro, The Scotts Company, LLC., Maysville, OH) to supply 12.2 kg N ha^{-1} and allowed to root for 7 d in greenhouse located in Florence, SC. Rooted UDBG sprigs with two living nodes were transplanted into 6.4 cm square pots containing the same substrate as was used during sprig establishment and fertilizing as previously described. After transplanting, UDBG plants were drenched with mefenoxam (Subdue Maxx, Syngenta Crop Protection, LLC.) at a rate of 0.19 kg a.i. ha^{-1} to prevent infection by oomycete organisms.

Seven days later, the growing substrate of each pot was infested by removing a substrate core 12 mm in diameter by 20-mm deep in two corners of each pot and placing four colonized oat seeds in

each hole; holes were then covered with substrate. Sterile oat seeds were placed in the substrate of for each non-infested control. Bermudagrass plants were watered to field capacity, then placed in a moist chamber (i.e. covered 28 by 56 cm seeding flat) within a growth cabinet set at 35/25°C day/night, with a 12 h photo period. Water was added to bring the growing substate back to field capacity 7 d after inoculation.

A randomized complete block experimental design was used with six replicates per treatment. There were nine treatments in all, eight *Waitea* species isolates and one non-infested control. The experiment was conducted twice. Before inoculation, bermudagrass plants were placed into six blocks based on visual plant quality and treatments (Waitea *species* isolate or control) were randomly assigned to one plant in each block.

Bermudagrass plants were harvested at 14 d after inoculation, and the substrate was carefully removed from plants by placing them briefly in a beaker of water. Plants were observed under a dissecting microscope (7-90×) for signs of *Waitea* species, including mycelia and sclerotia. Four leaves from each plant were excised then surface disinfested for 10 sec in 70% ethanol, rinsed twice in deionized water and blotted dry on a paper towel. All leaves were placed in a single 9-cm Petri plate containing water agar [15 g L^{-1} (BD Bacto, Becton, Dickinson and Company] modified with 0.2 g L^{-1} streptomycin sulfate and 0.2 g L^{-1} ampicillin. All roots from each node were cut and grouped by node to create two sets of roots per bermudagrass plant. Roots were surface disinfested for 30 sec, rinsed, dried, and then placed in two groups (one group per node) in a single Petri plate as previously described. The stolon between both nodes of each plant was excised and cut into two segments. Both segments where, dried, rinsed, and placed in a single Petri plate. Isolation plates were incubated in plastic boxes at room temperature.

Seven days later, isolation plates were examined for mycelium morphologically characteristic of *Waitea* species. Representative hyphae were transferred to fresh water agar for further observation

and identification. Leaves, roots, and stolons infected with *Waitea* species was scored a one (1) and leaves, roots, and stolons not infected received a score of zero (0).

The probability of isolating *Waitea* species from all plant tissue for each isolate, was analyzed in JMP 16.0.1 (SAS Institute Inc., Cary, NC) using a generalized linear model with binomial distribution and logistic (logit) link function. As expected, *Waitea* species were not recovered from control plants; therefore, these data were eliminated from the analysis. Experimental run was not significant; therefore, data from both runs were combined for final analysis. Leaves, roots and the stolon were harvested from the same UDBG plant and lacked independence. Isolation frequencies for each plant organ is presented and discussed, but isolation frequencies for sperate plant organs were not statistically analyzed.

Plant quality, a subjective visual evaluation of plant vigor and heath, was evaluated 7 and 14 d after inoculation on a 1 to 10 scale, with 1 being a healthy plant and 10 representing complete plant death.

Molecular characterization

Molecular characterization of isolates was conducted in two groups (Table 4.1), using a slightly different procedure for each group. For group one, isolates were grown in one-quarter-strength potato dextrose broth [PDB; 6 g L^{-1} PDB (BD Difco, Becton, Dickinson and Company)] prepared with deionized water and amended with 0.2 g L^{-1} streptomycin sulfate and 0.2 g L^{-1} ampicillin. For each isolate, a PDA plug (approximately 5 by 5 mm) from an actively growing colony was added to 20 ml of PDB in a sterile 50 ml centrifuge tube. Isolates were grown in PDB for 4 to 7 days at room temperature before approximately 100 mg of mycelium was removed from PDB using the using the suction force generated from an automatic pipet fitted with a 1-ml pipet tip. Mycelium was placed in a 2-ml microcentrifuge tube and macerated for 1 min with a bead beater set at 55 Hz before Genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). A NanoDrop spectrophotometer (Thermo Fisher

Scientific, Waltham, MA) was used to determine DNA concentration at 260 nm and gel electrophoresis was performed to confirm quality of extracted genomic DNA. Polymerase chain reactions (PCR) were prepared in a total volume of 20 µl, containing 10 ng of template DNA and a final concentration of 0.8 µM dNTP mix (Qiagen), 1.8 mM 5X PCR buffer (New England BioLabs, Inc., Ipswich, MA), 0.5 units *Taq* DNA polymerase (OneTaq DNA Polymerase, (New England BioLabs, Inc.), 0.2 µM of both 18S (5'-CGTAACAAGGTTTCCGTAGGTGAAC-3') and 28S (5'-GTTTCTTTTCCTCCGCTTATTAATATG-3') universal primers and nuclease-free water. Amplification of the rDNA internal transcribed spacer (ITS) region, including partial amplification of the 18S and 28S subunits was conducted in a Bio-Rad T100 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) with the initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 45 s. The final extension was 10 min at 68°C. Successful amplification and relative size of amplicon was confirmed by gel electrophoresis. Polymerase chain reaction products were cleaned using a DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA) and DNA concentration was obtained as described previously.

Polymerase chain reaction products were cloned using a NEB PCR Cloning Kit (New England BioLabs, Inc., Ipswich, MA). Transformed *Escherichia coli* cells were grown at 37°C on Luria-Bertani (LB) agar [32 g L^{-1} (PhytoTechnology Laboratories, Lenexa, KS)] containing 0.1 g L^{-1} ampicillin or carbenicillin for approximately 16 h before transferring three colonies per isolate to LB broth [15 g L^{-1} (PhytoTechnology Laboratories)], also containing 0.1 g L^{-1} ampicillin or carbenicillin. Liquid cultures were shaken in an incubator set at 37°C for approximately 24 h or until growth was sufficient for plasmid extraction. To extract plasmid DNA, 600 µl of liquid culture containing transformed *E. coli*, was lysed and cleaned using a Zyppy Plasmid Miniprep Kit (Zymo Research). Sequencing of plasmid DNA was conducted at the Clemson University Genomics institute (Clemson, SC).

Isolates in group two (Table 4.1), were grown on sterile cellophane placed on the surface of one-PDA for 14 d. For each isolate, approximately 50 mg of mycelium per isolate was scraped from the

cellophane surface into a 2 ml microcentrifuge tube containing one tungsten bead. Mycelium was macerated by two 1-min cycles at 30 Hz with a TissueLyser II (Qiagen). Genomic DNA was extracted and quantified as described for group one. Primers used for group two where the same as the primers used for group one; however, the PCR mixture was modified. Polymerase chain reactions were prepared in a total volume of 25 µl, containing 12.5 µl of Q5 High-Fidelity 2X Master (final concentration of 2.0 mM Mg++ and 800 µM dNTPs), 0.5 uM of each primer and nuclease-free water. An Eppendorf Mastercycler nexus gradient thermocycler (Eppendorf Group, Hamburg, Germany) was used for amplification with the initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 7 s, 55°C for 20 s, and 72°C for 30 s min; the final extension was 72°C for 5 min. Polymerase chain reaction products were cloned, and plasmid DNA was cleaned and lysed as described for group one isolates. Plasmid DNA was submitted to Molecular Cloning Laboratories (MCLAB, South San Francisco, CA) for sequencing.

Primer sequences were removed before forward and reverse sequences from each clone were aligned using the de novo assemble function of Geneious Prime 2020.2.5 (Dotmatics, Boston, MA). For most isolates, complete sequences ranging from 610 to 625 bp from at least two clones were used to form a consensus sequence. In some cases, only one clone per isolate produced a viable sequence; therefore, a single sequence was used to represent those isolates. Twenty-five isolates from the current study (Table 4.1) along with eighteen reference sequences from GenBank (Clark et al., 2016) (Table 4.2) were aligned, trimmed to a maximum length of 625 bp and a neighbor-joining (Saitou and Nei, 1987) phylogenetic tree was constructed using the Tamura-Nei distance model calculated using 10,000 bootstrap replicates, with a branch threshold set at 80%. Geneious Prime 2020.2.5 was used for phylogenetic tree construction and both clone and final sequence alignment was conducted utilizing Clustal Omega 1.2.2 (Sievers et al., 2011) integrated with Geneious Prime 2020.2.5.

Results

Molecular characterization

Sequences of the rDNA-ITS region from isolates ranged from 610 to 625 bp in length. Sequences of the rDNA-ITS region identified by Stalpers et al. (2021) as type sequences for *W. agrostidis*, *W. oryzae*, and *W. prodiga* were included in our molecular analysis (Table 4.1). Type sequences for *W. circinata* and *W. zeae* were not selected by Stalpers et al. (2021); however, reference sequences deposited in GenBank (Clark et al., 2016) from relevant publications were included in this study for comparison. Type sequences identified by Staplers et al. (2021) along with reference sequences were 565 to 644 bp in length. Prior to alignment, all sequences were trimmed to a maximum length of 625 bp.

Four main clades are evident in the neighbor-joining phylogenetic tree corresponding to the species *W. circinata*, *W. oryzae*, *W. zeae* and a clade comprising of both *W. prodiga* and *W. agrostidis* (Figure 4.1). Isolates obtained from UDBG in this study, grouped in the *W. oryzae*, *W. prodiga*, and *W. zeae* clades and clustered plausibly well with representative and reference sequences within each species clade. Bootstrap support for both the *W. circinata* and *W. oryzae* clade was 100%. All *W. zeae* isolates formed a large clade comprising of 28 isolates with 89% branch support, including reference isolates and those from the current study. Bootstrap support for the *W. agrostidis* and *W. prodiga* clade was 100% and was further divided into two distinct groupings based on species, with both groupings receiving high branch support.

One large and three small groupings are also evident within the *W. zeae* clade (Figure 4.1). Most isolates in the current study are contained in the large grouping, along with one reference sequence, HM597139 (Kammerer et al., 2011). Two reference sequences and three sequences from this study formed a grouping with 86% branch support. Reference sequences, AB213594 and AB213595 (Toda et al., 2007) formed a small group as did MK815577 and MK817602 (Vojvodic et al., 2021) with 82 and 100% branch support, respectively.

Infection of ultradwarf bermudagrass roots, stolons, and leaves by *Waitea oryzae* and *W. zeae*

Six *W. zeae* and two *W. oryzae isolates* were evaluated in the infection experiment, including six (Table 4.1). Four *W. zeae* isolates were recovered from UDBG—three from leaves at two locations in South Carolina and one at a location in North Carolina and one isolate was baited from a UDBG core collected in South Carolina—and two were isolated from creeping bentgrass leaves. One *W. oryzae* isolate was obtained from UDBG leaves and the other by baiting an UDBG putting green core, both of these samples were collected in South Carolina.

Mean plant quality scores for the first and second runs of this experiment were evaluated at 14 d after inoculation, when the experiment concluded, and were 2.7 ($\sigma = 2.1$) and 2.4 ($\sigma = 1.0$), respectively. Plant quality scores for UDBG control plants (the substrate was not infested) ranged from 1 to 3 for run one and 1 to 4 for run two. Plant quality ratings did not coincide with isolation frequency and there was no difference among treatments; therefore, these data are not presented. Each plant was examined under a dissecting scope (7-90×) for characteristic signs of *W. zeae* or *W. oryzae*. Although not quantified, sclerotia were present on roots, on stolons, in leaf sheaths, and near the nodes on many UDBG plants. During this inspection, we did not observe any obvious symptoms of infection, such as necrotic lesions or water-soaked plant tissues.

Waitea zeae isolates FIANND122, FIANND130, FIANND141, and *W. oryzae* isolate FIANND164 were isolated from all inoculated plants (Table 4.3). *Waitea zeae* isolate FIANND103 was isolated from 82% of inoculated plants, a lower isolation rate compared to other *W. zeae* isolates obtained from UDBG (Tables 4.3 & 4.4). *Waitea zeae* isolates originating from creeping bentgrass were isolated significantly less frequently than those from UDBG (0% for isolate FIANND147 and 33% for isolate FIANN150) (Tables 4.3 and 4.4). *Waitea oryzae* isolate FIANN164 was isolated from all inoculated plants but isolate FIANN127 was only isolated 25% of the time. Overall, however, there was no difference in isolation frequency between *W. zeae* and *W. oryzae* isolates originally obtained from UDBG (Table 4.4).

Isolation frequency from roots for *Waitea* zeae isolates FIANND122, FIANND130, and FIANND141, all isolates originally recovered from UDBG, was 100% (Table 4.3). *Waitea zeae* isolate FIANND103, was also originally isolated from UDBG but only recovered from 55% of roots (Table 4.3). *Waitea oryzae* isolates FIANND127 and FIANND164 were recovered from roots of 25 and 100% of UDBG plants (Table 4.3). *Waitea zeae* isolate FIANND150 was originally isolated from creeping bentgrass leaves and was recovered from 25% of UDBG plant roots (Table 4.3).

Waitea zeae isolates FIANND122 and FIANND130, and *W. oryzae* isolate FIANND164 were readily isolated from stolons of UDBG plants (90% or greater) (Table 4.3). *Waitea zeae* isolate FIANND103 was isolated from stolons of 64% of UDBG plants, whereas isolation frequency from stolons for isolate FIANND141 was 50%. Isolation frequencies from stolons of UDBG plants for all other isolates was 8% or less.

Waitea zeae isolates FIANND122 and FIANND130, and *W. oryzae* isolate FIANND164, which were frequently isolated from roots and stolons of UDBG plants, were also readily isolated from leaves (Table 4.3). *Waitea oryzae* isolate FIANND127 was only isolated from 8% of UDBG plant leaves. Isolation frequency from leaves for *W. zeae* isolate FIANND141 was 67%, which was higher relative to the isolation frequency from stolons for this isolate. *Waitea zeae* isolate FIANND103 infected 82% of UDBG plants; but isolation frequency from leaves was only 27%. Isolation frequency of *W. zeae* isolates FIANND147 and FIANND150, both isolates originating from creeping bentgrass leaves, was low relative to other *W. zeae* isolates.

Discussion

Pathogenicity of species of *Waitea* in turfgrass has been examined by numerous researchers prior to this study (Chang & Lee, 2016; Chen et al., 2011; de la Cerda et al., 2007; Elliott, 1999; Haygood & Martin, 1990; Kammerer et al., 2011; Martin & Lucas, 1983; Martin & Lucas, 1984; Toda et al., 2005; Toda et al., 2007). In several previous studies, inoculum was grown on grain or PDA medium and placed

in or near the turfgrass canopy, or a mycelial slurry was applied over turfgrass foliage. For example, Kammerer et al. (2011) grew *Waitea* species isolates on sterilized, moist oat seeds, and then dried the colonized oat seed prior to placing five seeds in a pre-wet turfgrass canopy. Elliott (1999) grew isolates in PDB, homogenized the liquid mycelium suspension after 4 d of growth, and then poured a mycelium slurry over pre-wet turfgrass leaves. In most of these previous studies, turfgrass plants were placed in a growth chamber or glasshouse maintained at an optimum temperature for the pathogen being studied and leaf wetness was sustained for the duration of the experiment. Furthermore, most of these studies subjectively evaluated presence of leaf blighting, leaf lesions, and sheath rot as a measure of pathogenicity to turf. Although this methodology may be appropriate for confirming pathogenicity, it may not directly reflect how infection occurs in UDBG putting greens.

In UDBG putting greens, mini-ring symptoms do not include leaf lesions or overt sheath rot (Dant and Martin, 2023; Kerns et al., 2017). Rather, declining UDBG leaves are generally chlorotic or bronze to orange in color, and symptoms are only indicative of mini-ring when viewing turfgrass from a standing position. Additionally, mycelium is not easily visible on UDBG foliage until turf has been held in a moist incubation chamber for 24 to 48 h (Dant, unpublished data, 2022). In the present study, inoculum was placed in the soil, which required the pathogen to spread to the infection court. Isolation frequency of *W. zeae* from root tissue was high for three of four isolates collected from UDBG. One of two *W. oryzae* isolates was also readily isolated from root tissue. It seems likely that root tissue of bermudagrass could be an infection court or potentially the primary infection court in UDBG putting greens. Infection of turfgrass root tissue by *Waitea* spp. has not been well studied; however, previous researchers have studied isolates recovered from soil or below-ground plant tissue (de la Cerda et al., 2007; Gürkanli et al., 2016; Kammerer et al., 2011; Oniki et al., 1985; Summer & Bell, 1982; Toda et al., 2005; Toda et al., 2007; Vojvodić et al., 2021; Warcup & Talbot, 1962; Windham & Lucas, 1987). Moreover, Vojvodić et al., (2021) demonstrated in a bioassay that *W. zeae,* originally isolated from

oilseed rape and cabbage, caused root necrosis on several dicotyledonous crops and two monocotyledonous crops, wheat and maize.

The methodology used in this study limited the ability to conclusively determine if infection started in roots of UDBG plants and then progressed stolons and leaves or if each plant organ was infected independently; however, isolation frequency for several isolates was high in both roots and stolons. Aerial UDBG stolons were used to start plants for this study and had to be covered with a thin layer of rootzone medium to encourage nodes to develop roots and leaves. This thin layer of medium may have increased potential for stolon infection. Although the study did not quantify or objectively evaluate presence of sclerotia on each plant organ, sclerotia were present on roots, stolons, and leaves as well as near the crown and on leaf sheaths of UDBG plants. This is further evidence that *W. zeae* and *W. oryzae* infect more than just UDBG leaves. Additional research should be conducted to evaluate vertical distribution of *Waitea* spp. in the rootzone, thatch layer, and turf canopy of UDBG putting greens.

Pathogenicity of isolates in this study was not proven conclusively because characteristic symptoms of mini-ring (i.e. 10 to 40 cm yellow to bronze rings) cannot be replicated in an experiment where small pots are used (Kerns, 2017). Although the inability to definitively demonstrate pathogenicity is a limitation of this experiment, symptoms on leaf tissue are not explicit even when UDBG is collected from symptomatic UDBG putting greens. Visual foliage symptoms of mini-ring typically occur on large patches of turf and not on individual leaves. Furthermore, previous studies have shown members of the genus *Waitea* are pathogens of both common and hybrid bermudagrass (Elliot, 1999; Kammerer et al., 2011; Kerns et al., 2017; Martin & Lucas, 1983; Martin & Lucas, 1984).

It is unclear why both *W. zeae* isolates obtained from creeping bentgrass foliage were not readily recovered from UDBG root tissue in this experiment. Elliot (1999) found *W. zeae* isolates originally obtained from creeping bentgrass, and a mixed stand of Kentucky bluegrass and perennial

ryegrass were equality effective as pathogens in hybrid bermudagrass as *W. zeae* isolates recovered from hybrid bermudagrass. Similarly, but with a different pathogen, Kammerer et al. (2011) demonstrated that *W. prodiga* isolated from seashore paspalum caused lower leaf blight and sheath rot on bermudagrass, roughstalk bluegrass, and seashore paspalum in controlled environment experiments. In contrast, Martin and Lucas (1983 and 1984) indicated *W. zeae* isolates from tall fescue and creeping bentgrass were more virulent on cool-season turfgrass compared to warm-season turfgrasses, common bermudagrass, and centipedegrass. Martin and Lucas (1983) noted *W. zeae* caused disease in the warm- and cool-season grasses tested; however, acknowledged growing conditions during their study were more conducive for warm-season turf and likely caused physiological stress to cool-season turfgrass, making them a more susceptible host. In the present study, only UDBG was tested as a host; therefore, it cannot be definitively stated host preference did or did not play a role in isolation frequency with creeping bentgrass isolates. Based on previous studies, host preference may influence isolate virulence (Martin & Lucas, 1983). Elliot (1999), Kammerer et al. (2011), and Martin and Lucas (1983 and 1984) inoculated only above-ground plant parts and used the presence of foliar symptoms to evaluate *Waitea* species pathogenicity and isolate virulence. It is possible the isolates from creeping bentgrass in the present study infect leaves more readily than roots or stolons; therefore, the inoculation method impacted the ability of these isolates to infect UDBG. Given the stark difference between *W. zeae* isolates recovered from creeping bentgrass versus those originating from UDBG in this study, future research should explore the affinity of *Waitea* species isolates to infect specific plant organs and the impact of host preference.

Of the two *W. oryzae* isolates included in the infection experiment, FIANND164 was recovered from roots, stolons, and leaves more frequently than FIANND127. Both isolates originated from UDBG putting greens; however, FIANND127 was baited from a turfgrass core, whereas FIANND164 was

collected from UDBG leaves. Isolate FIANND127 may not be a facultative parasite living in the soil and weakly virulent to UDBG, while FIANND164 maybe a primary pathogen of UDBG.

As expected, most of the isolates recovered from UDBG putting greens exhibiting symptoms of mini-ring were *W. zeae*. Based on sequences of the rDNA-ITS region, these isolates were similar to one another and to the seven reference isolates, specifically isolates collected in the USA. Six *W. zeae* isolates collected from creeping bentgrass in South Carolina showed a strong relationship with *W. zeae* isolates collected from UDBG. Reference isolates from Toda et al. (2007) and Vojvodić et al. (2021) formed separate groups within the *W. zeae* clade. This is not unexpected as isolates from Toda et al. (2007) originated from soil and rice in Japan. Vojvodić et al. (2021) isolated *W. zeae* from cabbage and oilseed rape in Serbia; therefore, it is plausible these isolates would form a separate grouping. It is unclear why isolates FIANND137, FIANND138, and FIANND161 formed a sub-group with reference isolates from Kammerer et al. (2011) and de la Cerda et al. (2007). The three isolates from the present study were collected from UDBG putting greens at three geographically distinct locations and were baited from UDBG cores or isolated from foliage. Reference isolate HM597142 (Kammerer et al., 2011) was collected from UDBG in Florida, and the isolate from de la Cerda et al. (2007), DQ900594, was recovered from annual bluegrass in California.

Three *W. oryzae* isolates were collected from separate locations, two by baiting an UDBG putting green core and one was isolated from UDBG leaves. Martin (2009) suggested *W. oryzae* may be a causal agent of mini-ring and Kammerer et al. (2011) isolated *W. oryzae* from an UDBG putting green in South Carolina. Kammerer et al. (2011) published the rDNA-ITS sequence for their *W. oryzae* isolate, HM597137, but did not describe symptoms present when it was isolated or study pathogenicity. Therefore, to the author's knowledge, this study is the first to isolate *W. oryzae* from symptomatic UDBG, demonstrate infection of UDBG by *W. oryzae*, and confirm identification of the pathogen using rDNA-ITS sequence data. It still appears *W. zeae* is the primary pathogen causing mini-ring based on the

results from this study and those reported from Kerns et al. (2017), but *W. oryzae* should not be dismissed as a potential pathogen of UDBG.

Kammerer et al. (2011) first documented *W. prodiga* as a pathogen of turfgrass and demonstrated it caused basal leaf blight in seashore paspalum. Later Chen et al., (2011) isolated *W. prodiga* from kikuyugrass [*Cenchrus clandestinus* (Hochst. ex Chiov)] and reported yellow lesions formed on leaves and stems of inoculated kikuyugrass plants. In this study, *W. prodiga* was isolated from leaves of UDBG putting green displaying symptoms of mini-ring located in South Carolina. The isolate FIANND162 grouped with reference sequences from Chen et al. (2011) and Kammerer et al. (2011). *Waitea prodiga* has not been previously isolated from UDBG, nor reported as a potential pathogen of UDBG.

In the present study, three species of *Waitea— W. zeae, W. oryzae,* and *W. prodiga*, were recovered from UDBG putting greens with typical symptoms of mini-ring but W. *circinata* was not isolated. In contrast, Kerns et al. (2017), reported a small number of their isolates collected from symptomatic UDBG in North Carolina and Alabama were *W. circinata*. However, similar to the results from the current study, Kerns et al. (2017) also found a majority of isolates recovered from symptomatic UDBG were *W. zeae*; therefore, it seems reasonable to conclude that *W. zeae* is the predominate causal agent of mini-ring in UDBG putting greens. More research should be conducted to determine pathogenicity and virulence of all *Waitea* spp. in UDBG.

Morphological observations and morphometric data for isolates in the current study were not collected. This could be seen as a limitation; however, morphometric data within the genus *Waitea* generally are limited to diameter of hyphae and sclerotia, and nuclear number, because the teleomorph for all members of the genus are rarely observed in nature or culture (Burpee & Martin, 1992; de la Cerda, Kammerer et al., 2011; Kerns et al., 2017). All species of *Waitea* are multinucleate and although useful for distinguishing species of *Waitea* from some species of *Rhizoctoina*, this characteristic alone

cannot be used to distinguish among species of *Waitea* (de la Cerda, 2007; Burpee & Martin, 1992) Diameter of hyphae among species of the genus *Waitea* range from 3 to 10 µm, with a variance of 3 µm or more within a single species; therefore, hyphal diameter is rarely used as a discretionary characteristic (de la Cerda, 2007; Toda et al., 2005; Toda et al., 2007; Kammerer et al., 2011; Voorhees, 1934). All other morphological characteristics are largely subjective including color, shape, and size of sclerotia, and color and appearance of mature cultures (Stalpers et al., 2021). Subjective morphological features can be used to categorize members of the genus *Waitea* but vary by isolate; therefore, visual observations cannot be used to conclusively speciate members of the genus. For example, *W. oryzae* and *W. prodiga* produce irregular-shaped pink to salmon colored sclerotia, while sclerotia of *W. circinata* and *W. agrostidis* are irregular and dark-orange to dark-brown in color (de la Cerda, 2007; Kammerer et al., 2011; Stalpers et al., 2021; Toda et al., 2005; Toda et al., 2007). Sclerotia of *W. zeae* are spherical, orange to dark-red in color and, therefore, are generally visibly distinct from other members of the genus but could be confused with *W. circinata* and *W. agrostidis* if not observed carefully (Stalpers et al., 2021; Voorhees, 1934). Variance in subjective descriptions of isolate morphology necessitates molecular characterization be used to distinguish among the species of *Waitea*.

In 2021, Stalpers et al. published a paper to clarify the names of fungi within *Agaricomycotina* and selected a single scientific name for individual fungal taxa. They preserved the genus *Waite*a, with *W. circinata* as the type species. Furthermore, they established that *W. zeae* is related to but sperate from *W. circinata*, which had been suggested by many authors previously, but never formerly recognized. Additionally, Stalpers et al. (2021) selected type sequences for *W. agrostidis*, *W. oryzae*, and *W. prodiga*. Given this framework, the present study had a distinct advantage of inferring relationships among isolates using typified sequences for three of five species within the genus *Waitea* and selecting

relevant reference sequences in GenBank from previously published research where species of *Waitea* has been accurately identified (Clark et al., 2016).

In the current study, only the rDNA-ITS region was sequenced, while other researchers studying fungi in the genus *Waitea* have sequenced two or more markers including the small subunit (SSU), large subunit (LSU), RNA *polymerase II* gene (*RPB2*), and β-*tubulin* (Gürkanli et al., 2016; Vojvodić et al., 2021). In both cases, sequences of a second or third marker did not provide any further resolution among species already inferred from rDNA-ITS region sequences. Additionally, Vojvodić et al. (2021) noted a limited number of reference sequences are available for markers other than the rDNA-ITS region, which limits the use of sequences for these other markers. Schoch et al. (2012) and Xu (2016) discuss advantages and disadvantages of using rDNA-ITS sequences in detail and conclude sequences of this region are useful as a universal barcode for fungi, acknowledging limitations dependent on objectives of a given study and citing areas for improvement. For the present study, the main objective was to properly identify the species of each isolate, and rDNA-ITS sequences were effective for this purpose.

The results from the current study suggest multiple species of *Waitea* may be involved in causing the disease on UDBG putting greens commonly referred to as mini-ring, but the primary causal agent appears to be *W. zeae.* *Waitea prodiga* and *W. oryzae* also were isolated from UDBG putting greens displaying symptoms of mini-ring. Neither pathogen has been extensively studied as a pathogen in UDBG putting greens and should be the target of future research. In this study, both *W. zeae* and *W. oryzae* infected UDBG roots and stolons, which had not been reported previously. This suggests that fungicides applied to manage mini-ring should be targeted at the rootzone of UDBG putting greens and agronomic practices that promote root growth may increase UDBG recuperative potential. Additionally, future research should focus on interactions among species of *Waitea* and other organisms in the turfgrass rhizosphere–particularly that of ultradwarf bermudagrass.

Table 4.1. Twenty-six isolates of three species of W*aitea* included in this study. Isolates were recovered from ultradwarf bermudagrass or creeping bentgrass putting greens in North Carolina and South Carolina between 2013 and 2021.

Species[a]	Isolate	Host[b]	Origin	Isolation method[c]
W. oryzae	**FIANND127**[d]	**UDBG**	**Batesburg-Leesville, SC, USA**	**baited core**
	FIANND136	UDBG	Gramling, SC, USA	baited core
	FIANND164[e]	**UDBG**	**Greenville, SC, USA**	**leaves**
W. prodiga	FIANND162[e]	UDBG	Florence, SC, USA[f]	leaves
W. zeae	**FIANND103**	**UDBG**	**Chapin, SC, USA**	**leaves**
	FIANND104	UDBG	Florence, SC, USA	leaves
	FIANND122	**UDBG**	**Chapel Hill, NC, USA**	**leaves**
	FIANND124	UDBG	Chapel Hill, NC	leaves
	FIANND130	**UDBG**	**Greenville, SC, USA**	**baited**
	FIANND131	UDBG	Barnwell, SC, USA	baited core
	FIANND132	UDBG	Tarboro, NC, USA	baited core
	FIANND134	UDBG	Florence, SC, USA	baited core
	FIANND135	UDBG	Hilton Head Island, SC, USA	baited core
	FIANND137	UDBG	Myrtle Beach, SC, USA	baited core
	FIANND138	UDBG	Sumter, SC, USA	baited core
	FIANND139	UDBG	Sumter, SC, USA	baited core
	FIANND140	UDBG	Shelby, NC, USA	baited core
	FIANND141[e]	**UDBG**	**Florence, SC, USA**	**leaves**
	FIANND144	CB	Florence, SC, USA	leaves
	FIANND145	CB	Florence, SC, USA	leaves
	FIANND146	CB	Florence, SC, USA	leaves
	FIANND147	**CB**	**Florence, SC, USA**	**leaves**
	FIANND149	CB	Florence, SC, USA	leaves
	FIANND150	**CB**	**Florence, SC, USA**	**leaves**
	FIANND161[e]	UDBG	Florence, SC, USA	leaves

[a] *Waitea* species determined by analysis of rDNA-internal transcribed region sequences.

[b] CB: creeping bentgrass; UDBG: ultradwarf bermudagrass.

[c] Baited core: Isolates were collected by placing 2.5-cm-long wooden sticks into the UDBG canopy and soil, then plating baits onto selective medium for isolation. Leaves: Symptomatic UDBG leaves were placed onto selective medium.

[d] The eight isolates listed in bold font were included in the experiment to evaluate UDBG infection by W. oryzae and W. zeae.

[e] Isolates belong to group two in the molecular characterization portion of this study; all other isolates belong to group one.

[f] All isolates from Florence, SC, USA were obtained from research putting greens located at the Clemson University Pee Dee Research and Education Center.

Table 4.2. rDNA internal transcribed spacer region sequences from 18 reference isolates of five species of *Waitea* deposited in Genbank were used for comparison in this study.

Species	Isolate	Host[a]	Origin[b]	Reference
W. agrostidis	**AB213567**[c]	**CB**	**Aichi, Japan**	**Toda et al. 2007**
	AB213578	CB	Chiba, Japan	Toda et al. 2007
W. circinata	AB213581	CB	Kagawa, Japan	Toda et al. 2007
	AB213582	CB	Nagasaki, Japan	Toda et al. 2007
	FJ154894	AB	Reston, VA, USA	Kammerer et al. 2011
W. prodiga	**HM597146**	**SP**	**Ft. Myers, FL, USA**	**Kammerer et al. 2011**
	HQ850254	kikuyugrass	Oceanside, CA, USA	Chen et al. 2011
W. oryzae	AB213588	soil	Aichi, Japan	Toda et al. 2007
	AB213589	**rice**	**Toyama, Japan**	**Toda et al. 2007**
	HM597135	SP	Sarasota, FL, USA	Kammerer et al. 2011
	HM597137	UDBG	Columbia, SC, USA	Kammerer et al. 2011
W. zeae	MK817577	cabbage	Futog, Serbia	Vojvodić et al. 2021
	MK817602	oilseed rape	Rimski Šančevi, Serbia	Vojvodić et al. 2021
	AB213594	soil	Wakayama, Japan	Toda et al. 2007
	AB213595	rice	Ishikawa, Japan	Toda et al. 2007
	HM597139	SP	Sarasota, FL, USA	Kammerer et al. 2011
	HM597142	UDBG	West Palm Beach, FL, USA	Kammerer et al. 2011
	DQ900594	AB	Irvine, CA, USA	de la Cerda et al. 2007

[a] AB: annual bluegrass; CB: creeping bentgrass; SP: seashore paspalum; UDBG: ultradwarf bermudagrass.

[b] Isolates originating from Japan are listed by prefecture, Serbian isolates by city or neighborhood, and isolates from USA, by city and state.

[c] Staplers et al. (2021) selected isolates in bold font as type rDNA-ITS sequences for each *Waitea* species.

Table 4.3. Isolation frequency for isolates of *Waitea oryzae* and *W. zeae* from a 'TifEagle' ultradwarf bermudagrass in a growth chamber bioassay.

Species[a]	Isolate	Host[b]	Isolation method[c]	*n*[d]	Isolation frequency			
					Entire plant[e]	Plant organ		
						Root	Stolon	Leaf
W. zeae	FIANND103	UDBG	leaves	11	82 (9)[f]	55 (6)	64 (7)	27 (3)
	FIANND122	UDBG	leaves	10	100 (10)	100 (10)	90 (9)	100 (10)
	FIANND130	UDBG	baited	12	100 (12)	100 (12)	92 (11)	83 (10)
	FIANND141	UDBG	baited	12	100 (12)	100 (12)	50 (6)	67 (8)
	FIANND147	CB	leaves	11	0 (0)	0 (0)	0 (0)	0 (0)
	FIANND150	CB	leaves	12	33 (4)	25 (3)	0 (0)	17 (2)
W. oryzae	FIANND127	UDBG	baited core	12	25 (3)	25 (3)	8 (1)	8 (1)
	FIANND164	UDBG	leaves	12	100 (12)	100 (12)	100 (12)	92 (11)
	Non-infested check	--	--	12	0 (0)	0 (0)	0 (0)	0 (0)

[a] *Waitea* species determined by analysis of rDNA-internal transcribed region sequences.

[b] CB: creeping bentgrass; UDBG: ultradwarf bermudagrass.

[c] Baited cores: Isolates were collected by placing 2.5-cm-long wooden sticks into the UDBG canopy and soil, then plating baits onto selective medium for isolation. Leaves: Symptomatic UDBG leaves were placed onto selective medium.

[d] Combined data from two trials each with six replications per treatment. Values less than 12 indicate an ultradwarf bermudagrass plant died and was treated as missing data.

[e] Isolation frequency if the isolate was recovered from any plant organ.

[f] Values in parentheses are the number of experimental units the *W. oryzae* or *W. zeae* isolate was recovered from.

Table 4.4. Chi-square statistic and *P* values associated with single-degree-of-freedom contrasts comparing isolation rate for isolates of *Waitea oryzae* and *W. zeae* from a 'TifEagle' ultradwarf bermudagrass in a growth chamber bioassay**.**

Contrast	χ^2 value	$P > \chi^2$
Ultradwarf bermudagrass isolates vs creeping bentgrass isolates (FIANND103, FIANND122, FIANND130, FIANND141 versus FIANND147 & FIANND150)	54.87	**.001**[a]
W. oryzae vs *W. zeae* isolates (FIANND103, FIANND122, FIANND130, FIANND141 versus FIANND127 & FIANND164)	0.00	.998
Ultradwarf bermudagrass *W. zeae* isolate comparison (FIANND122, FIANND130, & FIANND141 versus FIANND103)	5.85	**.016**
W. oryzae isolate comparison (FIANND127 versus FIANND164)	18.53	**.001**

[a] Bold *p* values are significant ($P \geq .05$).

Table 4.5. Percent similarity among rDNA internal transcribed spacer region sequences from five species of *Waitea*.

Species	*W. agrostidis*	*W. circinata*	*W. prodiga*	*W. oryzae*	*W. zeae*
W. agrostidis	**99.3**[a]	86.6-88-5	97.5-98.4	82.6-85.7	88.6-92.7
W. circinata	--	**97.5-99.8**	86.1-88.5	83.6-86.4	83.9-88.7
W. prodiga	--	--	**99.7-100**	81.9-85.7	87.5-92.2
W. oryzae	--	--	--	**93.3-99.8**	83.4-89.9
W. zeae	--	--	--	--	**93.5-100**

[a] Bold values represent similarity within a *Waitea* species.

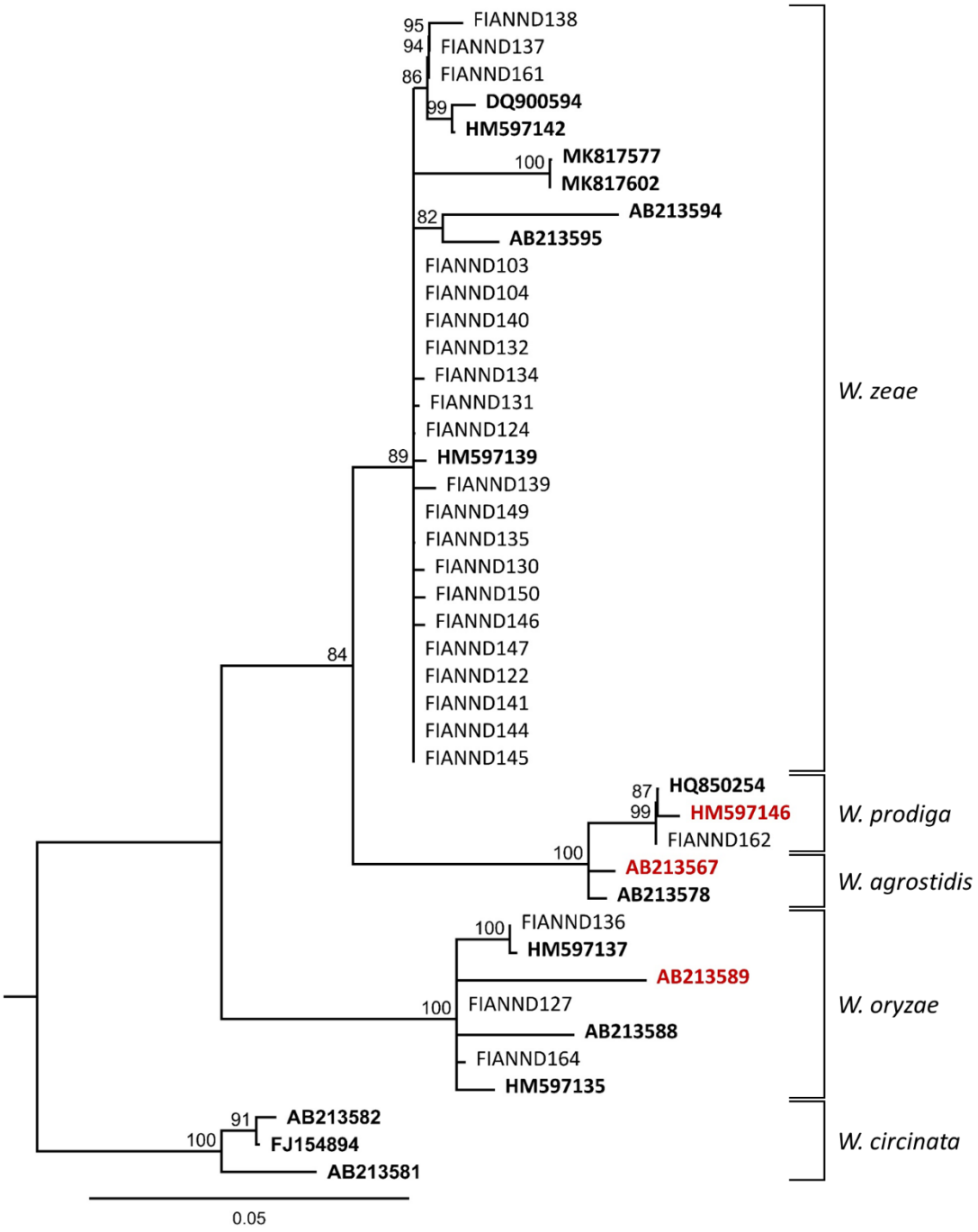

Figure 4.1. Neighbor-joining phylogenetic tree for isolates of *Waitea* species based on rDNA-internal transcribed spacer region sequences. The tree was constructed using the Tamura-Nei distance model calculated with 10,000 bootstrap replicates and a branch threshold set at 80%. Bootstrap values greater than 80% are positioned next to branch nodes. The scale bar represents 5% nucleotide sequence divergence. Isolates in bold font are reference sequences obtained from GenBank. Isolates in red font are sequences selected by Stalpers et al. (2021) as type sequences for a specific *Waitea* species.

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APPENDICES

APPENDIX A

COMPLETING KOCH'S POSTULATES FOR WAITEA ZEAE ON ULTRADWARF BERMUDAGRASS PUTTING GREENS AND IMPACT OF N SOURCE ON MINI-RING SEVERITY

Introduction

The purpose of this study was to evaluate the impact nitrogen source and rate on mini-ring development (formerly Rhizoctonia leaf and sheath spot) in ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] putting greens. A second objective was to complete Koch's postulates, which has not been done for this disease in a field setting. *Waitea zeae* (Voorhees) J.A. Crouch & Cubeta (formerly *Rhizoctonia zeae*) is the primary causal agent of mini-ring and is a pathogen of several cool- and warm-season turfgrasses. Mini-ring appears as frog-eye patches 10 to 40 cm in diameter with a tan to bronze colored outer ring. The center of the patch is most often green, and turf appears unimpacted. Mini-ring is wide-spread in the southeastern United States but also can be problematic in UDBG putting greens in other regions.

Materials and methods

Nitrogen fertilizer treatments

This study was conducted at the Clemson University Pee Dee Research and Education Center located in Florence, SC on a 'TifEagle' UDBG putting green. The putting green was constructed in 2002 using a 85:15 sand:peat (by volume) rootzone mix, designed for putting green construction. Irrigation was applied to prevent drought stress and UDBG was mowed five times per week at a height of 3.2 mm.

Treatments were two N sources, ammonium sulfate (AMS) [$(NH_4)_2SO_4$] and urea (CH_4N_2O) applied at three rates—4.9, 9.8, and 14.7 kg N ha^{-1} wk^{-1}—to create a two by three factorial treatment structure. All treatments were replicated four times and applied to 1.8- by 1.8-m plots arranged in a randomized complete block design. A control, which received no nitrogen fertilizer treatments, was included for comparison and had four replicate plots of the same size. Commercially available AMS (21-

0-0) and urea (46-0-0) were dissolved in water and applied using a CO_2-pressurized sprayer in a spray volume of 855 L ha^{-1}. Boom pressure was 207 kPa and TeeJet XR8002VS flat-fan nozzles (Spraying Systems Company, Wheaton, IL) were used to distribute the fertilizer solution uniformly over each plot. Immediately following application, 3 mm of overhead irrigation was applied to rinse fertilizer off leaf tissue and prevent phytotoxicity. Fertilizer treatments were initiated on 19 June and applied weekly until 01 Sept. 2015. In 2016, treatments were applied weekly from 09 June through 29 September.

Soil cores, 19 mm in diameter by 76 mm deep, were collected on 15 June 2015 and 14 Nov. 2017. A total of 10 to 12 soil cores were harvested diagonally across each plot and then mixed to create a representative composite sample for each plot. Soil samples were processed by the Clemson University Agricultural Service Laboratory and nutrients were extracted using Mehlich-1 extractant method (Mehlich, 1953).

Ultradwarf bermudagrass *W. zeae* inoculation

Inoculum was prepared in a 500 ml Erlenmeyer flask by placing 375 g of oat seeds in 125 ml of deionized water and autoclaving the mixture twice— on each of two consecutive days at 30 min at 121°C, 1.5 MPa. Plugs from one-quarter-strength potato dextrose agar plates with actively growing *W. zeae* were mixed with oat seed after autoclaving. Two *W. zeae* isolates, one from an UDBG putting green located in Florence, SC and the other obtained from an UDBG putting green in Chapin, SC were grown to produce a single batch of inoculum. Inoculum was placed in a dark incubator set at 30°C for 10 to 14 d and mixed regularly to prevent clumping.

On 05 June, 30 June, and 31 July 2015, two 10.8 cm diameter by 10 cm deep UDBG cores were harvested from each plot. A 2-cm deep slit was created in the canopy of the core and then approximately 50 ml of oat seed inoculum was pressed into the slit. Inoculated cores were placed in polypropylene bags to maintain a humid environment and bags were placed in a growth chamber set at

32°C with a 12-h photoperiod for 11 to 14 d. Inoculated UDBG cores were reinstalled in the research putting green on 16 June, 14 July, and 14 Aug. 2015, respectively.

Data collection and analysis

Disease severity assessments were made monthly as mini-ring symptoms developed. To assess disease severity in 2015 and 2016, a grid with nine sub-plots, each measuring 0.6 by 0.6 m was laid over the plot and the percent area of UDBG with mini-ring symptoms was visually estimated. Nine values from each plot were summed, then divided by nine to produce one disease severity value per plot. In 2017, a 0.9 by 1.8 m grid divided into eight subsections each measuring 0.2 m^2 was place over each plot two times to assess the percent area of each plot with mini-ring symptoms. The mean of 16 disease severity values were used to create a mean disease severity value for each plot. Turf quality was also visually rated monthly on a scale 1-9 scale, with 1 being dead turfgrass, 6 acceptable turfgrass and 9 excellent turf quality. Visual turf quality assessments included turf uniformity, density, color, and mini-ring severity.

Mini-ring severity data was analyzed using analysis of variance with a mixed model in JMP ver. 16.0.1 (SAS Institute Inc., Cary, NC). Data for the control (no N treatment) was not included in the analysis. Nitrogen source, N rate, and interactions of each were treated as fixed effects. Block and interactions including block were considered random effects.

Results

Turf quality ratings were greatly impacted by mini-ring disease severity and largely reflected disease severity ratings; therefore, this data is not presented.

Overt mini-ring symptoms were not present in 2015, but blemishes in turf uniformity were evaluated on 14 October as potential early symptoms of mini-ring. Interaction of N rate and N source were not significant; however, main effects of N rate and N source were (Table A.1). Mini-ring severity was very low in all treated plots with mean disease severity of 2.0% for AMS treated plots and 3.7% for

plots treated with urea (data not presented). Disease severity decreased from 4.8 to 3.5 to 2.8% as the rate of N increased from 4.9 to 9.8 to 14.7 kg N ha^{-1} wk^{-1} (data not presented). Mini-ring severity remained low in the fall of 2016 with no plot receiving a disease severity rating greater than 5% (data not presented). However, there was a significant interaction of N source and N rate (Table A.1). Disease severity for all plots treated with urea was less than 1% (data not presented). Plots treated with AMS resulted in disease severity ratings of 0, 1.3 and 3.8%, as rate of N increased from 4.9 to 9.8 to 14.7 kg N ha^{-1} $wk^{-1,}$ respectively (data not presented). Nitrogen fertilizer treatments were suspended at the end of 2016 because disease severity was low, and the trial area was maintained similar to a golf course putting green.

In September 2017, overt mini-symptoms began to develop in the trial area and treatment differences were apparent. In 2017, mini-ring disease severity evaluations occurred on 08 Sept., 12 Oct., and 14 Nov. 2017 For all three evaluations, N rate × N source was significant (Table A.1). Mini-ring severity remained below 1% in control plots and for all urea treatments; whereas disease severity in plots treated with AMS increased with time (Figure A.1). When AMS was applied at 4.9 kg N ha^{-1} wk^{-1}, disease severity was 3.5% or less and similar to UDBG treated with all rates of urea. Mini-ring severity in ultradwarf bermudagrass turf receiving applications of AMS at 9.8 and 14.7 kg N ha^{-1} wk^{-1} was greater than turf treated with AMS at 4.9 kg N ha^{-1} wk^{-1} and all rates of urea. On 12 Oct. and 14 Nov. 2017, disease severity increased as the rate of AMS increased (Figure A.1).

Soil pH decreased for all treatments from June 2015, when treatments were initiated, to November 2017 when this study ended (Table A.2). Soil pH at the end of the study ranged from 5.2 to 5.5 and plots receiving applications of AMS at 9.8 and 14.7 kg N ha^{-1} wk^{-1} resulted in the lowest pH. Turf receiving no N treatments and turf where urea was applied at 9.8 kg N ha^{-1} wk^{-1} resulted in the greatest pH. Turf where applications of AMS was applied 9.8 and 14.7 kg N ha^{-1} wk^{-1} also had soil calcium concentrations lower than turf treated with urea or turf not receiving a N treatment (Table A.2).

Discussion

Koch's postulates were not satisfied in this study. Unfortunately, disease severity in UDBG not receiving a N treatment was less than 1% in fall of 2017 when mini-ring symptoms were evident. Although symptoms were present in turf treated with AMS, a native *W. zeae* population may have been present in this putting green prior to initiation of this study and as shown in other studies (study summarized in chapter two of this dissertation), AMS increases mini-ring severity.

Similar to the study described and summarized in chapter two of this dissertation, using AMS as a primary N source increased mini-ring severity. There was a significant rate response, where applications of AMS at 14.7 kg N ha^{-1} wk^{-1} resulted in 16 times greater disease severity than when AMS was applied at 4.9 kg N ha^{-1} wk^{-1}. In this study, the N treatments were suspended in September 2016, yet mini-ring severity in AMS treated turf remained low until fall of 2017. This demonstrates N source can have an impact on mini-ring severity well after applications are applied.

Soil pH of all plots decreased from initiation of the study to November of 2017 when the study was terminated. Plots treated with AMS at 9.8 and 14.7 kg N ha^{-1} wk^{-1} resulted in a pH of 5.2, lower than all other treatments. Calcium concentration in plots treated with AMS at 9.8 and 14.kg N ha^{-1} wk^{-1} was also lower than the other treatments included in this study. This study was not designed to evaluate the impact of soil pH or Ca concentration on mini-ring severity; however, the influence of these factors should be studied in future experiments.

Table A.1. Results of analysis of variance for effect of N source and N rate on mini-ring disease severity in a 'TifEagle' ultradwarf bermudagrass putting green.

	Date				
	2015	2016	2017		
Source of variation	20 Oct.	14 Oct.	08 Sept.	12 Oct.	13 Nov.
N source[a]	*	***	ns	*	**
N rate[b]	*	***	**	***	***
N source x N rate	ns†	***	ns	*	**

* Significant at the .05 probability level.

** Significant at the .01 probability level.

*** Significant at the .001 probability level.

† Not significant at the .05 probability level.

[a] Sources of N were $(NH_4)2SO_4$ and CH_4N_2O.

[b] Rates of N comprised of 4.9, 9.8, and 14.7 kg N ha^{-1} wk^{-1}.

Table A.2. Bulk soil pH and Ca, K, and Mg concentration of soil collected from a 'TifEagle' ultradwarf bermudagrass putting green before and after treatment with ammonium sulfate [$(NH_4)_2SO_4$] and urea (CH_4N_2O) at various N rates. Soil samples were taken to a depth of 76 mm and processed using the Mehlich-1 extraction method.

N source[a]	N rate	pH		Ca	
		16 June 2015	14 Nov. 2017	16 June 2015	14 Nov. 2017
	kg N ha^{-1} wk^{-1}			mg kg^{-1}	
$(NH_4)_2SO_4$	0	5.9[b] (0.2)[c]	5.5 (0.2)	507 (61)	328 (44)
	4.9	6.1 (0.2)	5.3 (0.1)	517 (62)	322 (34)
	9.8	6.0 (0.2)	5.2 (0.0)	493 (37)	265 (9)
	14.7	6.1 (0.2)	5.2 (0.1)	548 (98)	234 (56)
CH_4N_2O	4.9	6.1 (0.1)	5.4 (0.2)	517 (77)	319 (64)
	9.8	6.1 (0.2)	5.5 (0.3)	567 (34)	316 (43)
	14.7	6.0 (0.1)	5.3 (0.1)	496 (73)	336 (66)

		K		Mg	
		16 June 2015	14 Nov. 2017	16 June 2015	14 Nov. 2017
		mg kg^{-1}			
$(NH_4)_2SO_4$	0	54 (8)	86 (12)	57 (10)	84 (5)
	4.9	69 (6)	87 (3)	59 (14)	82 (6)
	9.8	54 (11)	89 (18)	58 (6)	77 (17)
	14.7	53 (7)	80 (8)	70 (19)	66 (6)
CH_4N_2O	4.9	60 (9)	85 (7)	61 (12)	83 (12)
	9.8	57 (6)	80 (7)	73 (19)	75 (7)
	14.7	52 (8)	78 (4)	57 (8)	76 (4)

[a] In 2015, N treatments began on 19 June and were applied until 01 Sept. 2015. Treatments began on 09 June and were applied until 29 Sept. in 2016.

[b] Values are the mean of four replications.

[c] Values in parentheses are the standard deviation of each mean.

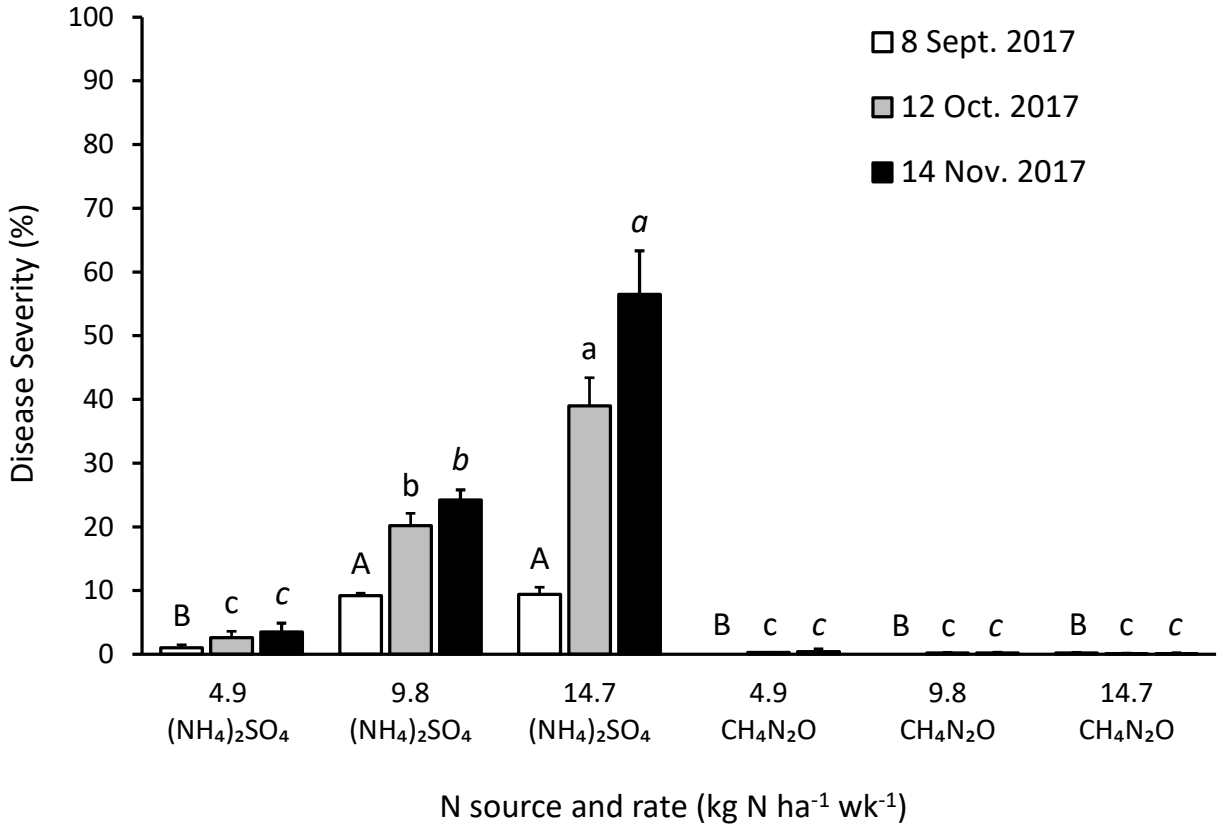

Figure A.1. Impact of N source and N rate on mini-ring severity in 'TifEagle' ultradwarf bermudagrass visually evaluated on 08 Sept., 12 Oct. and 14 Nov. 2017. Bars are treatment means of four replications; error bars are the standard error of each treatment mean. In 2015, N treatments began on 19 June and were applied weekly until 01 Sept. 2015. Treatments began on 9 June and were applied weekly until 29 Sept. in 2016. For each evaluation date, bars with the same letter are not significantly different according to Fisher's Protected LSD (α = 0.05).

APPENDIX B

MINI-RING SEVERITY IN GOLF COURSE PUTTING GREENS IS IMPACTED BY NITROGEN SOURCE

Introduction

In 2016, a study conducted and summarized in Chapter two of this dissertation, demonstrated weekly applications of ammonium sulfate (AMS) [$(NH_4)_2SO_4$] increased mini-ring severity compared to urea (CH_4N_2O). The 2016 study was conducted at the Clemson University Pee Dee Research and Education Center on a ultradwarf bermudagrass (UDBG) [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* (Burtt-Davy)] research putting green. The objective of this study was to confirm results from 2016, by conducting a trial on a golf course putting green maintained under a commercial golf course maintenance regimen.

Mini-ring, the disease also known as leaf and sheath spot (formerly *Rhizoctonia* leaf and sheath spot) is primarily caused by *Waitea zeae* (Voorhees) J.A. Crouch & Cubeta (formerly *Rhizoctonia zeae*). Golf course superintendents coined the disease name mini-ring based on symptom appearance in UDBG putting greens. The symptoms are described as frog-eye patches 10 to 40 cm in diameter with a tan to bronze colored outer ring of turf and a center containing green UDBG. Mini-ring can occur in UDBG putting greens wherever they are maintained; however, mini-ring is especially problematic on putting greens located in the southeastern United States.

Materials and methods

Fertilizer treatments

A chipping green established to 'Tifdwarf' bermudagrass, a dwarf-type, located at Cobblestone Park Golf Club in Blythewood, SC was utilized for this study. Golf course staff maintained this green similar to other putting greens on the golf course except no fungicide or nitrogen (N) fertilizer applications were made once the trial was initiated. The putting green was mowed five to seven times per week at a cutting height of approximately 3 mm

Nitrogen treatments were applied biweekly at 19.6 kg N ha^{-1} using AMS or urea as the N source. Due to limited plot space and because this trial was on a golf course putting green, control plots (turf receiving no N applications) were not included.

All N treatments were applied as described in the 2016 research trial summaries in Chapter two of this dissertation. Readily available commercial fertilizers were purchased, and AMS (21-0-0) and urea (46-0-0) were dissolved in water, and applied in a volume of 855 L ha^{-1} using a CO_2-pressurized sprayer. Boom pressure was 207 kPa and TeeJet XR8002VS flat-fan nozzles (Spraying Systems Company, Wheaton, IL) were used to distribute the fertilizer solution uniformly over each plot. Nitrogen treatments were watered-in immediately following application with 3 mm of overhead irrigation. The first treatments were applied on 02 June and continued biweekly until 21 Sept. 2017. Plots were 1.8- by 1.8-meters and arranged in a completely randomized design with each treatment replicated three times.

On 02 June and 17 Oct 2017, 10 to 12 soil cores were harvested diagonally across each plot, them mixed to create a representative composite sample for each plot. Soil cores were 19 mm in diameter and harvested to a depth of 76 mm. Soil samples were processed by the Clemson University Agricultural Service Laboratory and nutrients were extracted using the Mehlich-1 extractant method (Mehlich, 1953).

Data collection and analysis

Disease severity was assessed on 18 Sept. and 17 Oct. 2017 when mini-ring symptoms became evident. A 1.5- by 1.5-m frame, subdivided into nine 0.17 m^2 sections was placed over the center of each plot to visually assess disease severity as a percentage of the plot with mini-ring symptoms. A 0.15-m border around every edge of the frame was not rated. To produce one disease severity data point per plot, the mean value from the nine sections was calculated. Turf quality was rated monthly on a scale 1-9 scale, with 1 being dead turfgrass, 6 acceptable turfgrass and 9 excellent turf quality. Visual turf quality assessments included turf uniformity, density, color, and mini-ring severity.

Mini-ring severity data were analyzed by subjecting data to analysis of variance in JMP ver. 16.0.1 (SAS Institute Inc., Cary, NC).

Results

Visual turf quality was rated in this study and was greatly influenced by mini-ring disease severity; therefore, turf quality ratings are not presented.

On 08 September, mini-ring severity was 14% in turf treated with AMS, which was five times greater than turf treated with urea (Figure B.1). Mini-ring severity increased in October for turf treated with both AMS or urea. Bermudagrass treated with urea resulted in 8% disease severity, 2.8 times greater than in September (Figure B.1). When turf was treated with AMS, disease severity increased to 44%, a 3-fold increase compared to September.

Soil pH of turf treated with AMS and urea increased during the trial; however, pH increased 1 unit in turf treated with urea versus 0.5 units in turf treated with AMS (Table B.1). Calcium soil concentration when turf was treated with AMS was similar at trial initiation and trial termination. Conversely, when urea was applied, Ca soil concentration increased by 137 mg kg^{-1} (Table B.1). Similar to Ca, Mg soil concentration increased when urea was applied and decreased when turf received applications of AMS (Table B.1).

Discussion

The primary objective of this trial was to confirm results from a 2016 trial conducted on a research putting green demonstrating that applications of AMS increased mini-ring disease severity compared to applications of urea. This study confirms the 2016 results and indicates AMS can increase disease severity in both a research putting green and a commercial putting green.

Soil pH increased during over the duration of this study in plots treated with both N sources. This is contrary to results discussed in Chapter two of this dissertation. Due to the unexpected soil test

results, the golf course superintendent was consulted, and it was determined calcitic lime was applied on 20 Mar. and 19 June 2017, resulting in a total application of approximately 700 kg ha^{-1} of $CaCO_3$.

Table B.1. Bulk soil pH and Ca, K, and Mg concentration of soil collected from a 'Tifdwarf' hybrid bermudagrass putting green before and after treatment with ammonium sulfate [$(NH_4)_2SO_4$] and urea (CH_4N_2O). Soil samples were taken to a depth of 76 mm and processed using the Mehlich-1 extraction method. Golf course maintenance staff applied calcitic lime on 20 Mar. and 19 June 2017, resulting in a total application of approximately 700 kg ha^{-1} $CaCO_3$.

N source[a]	N rate	pH		Ca	
		2 June 2017	17 Oct. 2017	2 June 2017	17 Oct. 2017
	kg N ha^{-1}			mg kg^{-1}	
$(NH_4)_2SO_4$	19.6	5.2[b] (0.3)[c]	5.7 (0.2)	632 (130)	630 (140)
CH_4N_2O	19.6	5.3 (0.2)	6.3 (0.1)	783 (260)	920 (163)

		K		Mg	
		2 June 2017	17 Oct. 2017	2 June 2017	17 Oct. 2017
		mg kg^{-1}			
$(NH_4)_2SO_4$	19.6	93 (12)	78 (16)	48 (11)	40 (5)
CH_4N_2O	19.6	114 (24)	71 (11)	53 (13)	57 (2)

[a] Nitrogen treatments began on 02 June 2017 and were applied biweekly until 21 Sept. 2017.

[b] Values are the mean of three replications.

[c] Values in parentheses are the standard deviation of each mean.

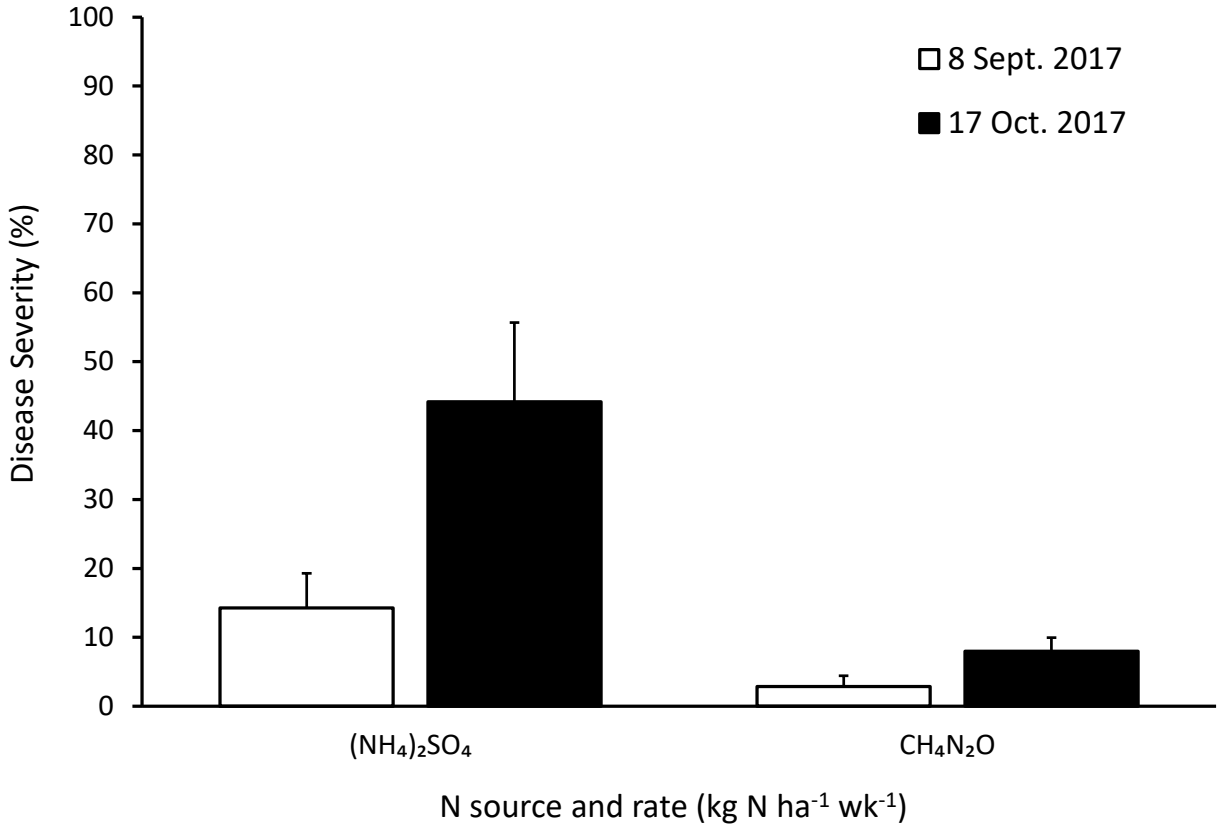

Figure B.1. Impact of two nitrogen (N) sources, ammonium sulfate [$(NH_4)_2SO_4$] and urea (CH_4N_2O), on mini-ring severity (percent area with visual symptoms) in 'Tifdwarf' bermudagrass evaluated on 08 Sept. and 17 Oct. 2017. Nitrogen treatments were initiated on 02 June 2017 and were made bi-weekly until 21 Sept. 2017 at a rate of 19.6 kg N ha^{-1}. Bars are treatment means from three replications; error bars are the standard error of each treatment mean. There was not a significant difference between nitrogen sources on 8 Sept. 2017 (*P* = .072); however, there was significant difference when the trial was assessed on and 17 Oct. 2917(*P* = .021).

APPENDIX C

REVIEW OF LEAF AND SHEATH SPOT DISEASES OF TURFGRASSES

Dant, L. A., and Martin, S. B. (2023). Leaf and sheath spot. In: L.P. Tredway, M. Tomaso-Peterson, J. P. Kerns, B. B. Clarke, (Eds.), *Compendium of turfgrass diseases* (4th ed., pp. 68-70). American Phytopathological Society.

Leaf and sheath spot is broadly used to describe symptoms in turfgrass caused by *Waitea zeae* (formerly *Rhizoctonia zeae*, syn. *W. circinata* var. *zeae*) and *W. oryzae* (formerly *R. oryzae*, syn *W. circinata* var. *oryzae*). Although *W. oryzae* has been associated with leaf and sheath spot symptoms, *W. zeae* is more commonly isolated from symptomatic turfgrass. Leaf and sheath spot commonly occurs in cool-season grasses especially during extreme heat but rarely causes widespread damage. On warm-season turfgrasses, particularly hybrid *Cynodon* spp. cultured as putting greens, this disease can be devastating. The disease is also known as 'mini-ring' as stand symptoms typically develop as small rings on *Cynodon* putting greens. *Waitea zeae* has been documented as a pathogen of nearly all economically significant warm-season turfgrasses including *Cynodon dactylon* and hybrids, *Eremochloa ophiuroides*, *Stenotaphrum secundatum, and Paspalum vaginatum.*

Symptoms and signs

Warm-season grasses

Leaf and sheath spot symptoms in *Cynodon* are distinctly different from those caused by *R. solani*, the disease commonly referred to as large patch. Initially the disease may appear as small chlorotic or tan spots ranging in size from 2 to 4 in. (5 to 10 cm) in diameter (Fig. 149). Early in the disease cycle, leaf and sheath spot can be confused with other diseases such as take-all root rot or root-knot nematode damage. As the disease progresses frog-eye patches ranging from 4 to 16 inches (10 to

30 cm) in diameter are produced (Figs. 150 and 151). The outer ring of the patch appears bronze, tan or bleached with the center generally unaffected; the disease is often referred to as “mini-ring” in reference to these symptoms. Diseased *Cynodon* leaf blades do not have lesions but are often entirely blighted. The symptoms described above are most often associated with the disease on hybrid *Cynodon* golf course putting greens, yet the disease can occur at higher cutting heights as well.

Leaf and sheath spot symptoms in *Eremochloa* and *Stenotaphrum* consist of both foliar lesions and basal sheath rot. These symptoms may be difficult to discern from lesions characteristic of large patch caused by *R. solani*. Symptoms of leaf and sheath spot in *Paspalum* are not well described, but generally present as chlorosis and necrosis of lower leaf blades in the absence of foliar lesions. In addition to *W. zeae* and *W. oryzae*, *W. prodiga* appears to play some role in the development of leaf and sheath spot in *Paspalum*.

Cool-season grasses

In cool-season turf, primarily *Agrostis stolonifera* and *Festuca arundinacea*, symptoms of leaf and sheath spot can be similar to those of brown patch (*R. solani*), and diagnosis requires laboratory isolation of the causal fungi. In *Agrostis* spp., *W. zeae* may incite rings or patches that are yellow, orange, brown, or gray in color (Fig. 152). A smoke-ring typical of brown patch does not form. In *Lolium* and *Festuca*, symptoms are indistinguishable from those caused by *R. solani*.

Signs of leaf and sheath spot are rarely visible to the unaided eye, however; white to cream-colored mycelia has been observed in cool-season turfgrass when environmental conditions are favorable. With the use of a dissecting microscope, mycelia deep in the turfgrass canopy or thatch layer are often visible. Although the disease is called leaf and sheath spot, most of the pathogen activity in *Cynodon* is observed on stolons and rhizomes (Fig. 153). Careful examination of numerous turfgrass tissues is required to make a confident diagnosis of this disease. Sclerotia are rarely found when turfgrass is grown under field conditions. When diseased *Cynodon* is incubated or grown under

controlled conductions optimal for *W. zeae*, sclerotia have been observed on leaves, sheaths, roots and are often embedded near the crown of the turfgrass plant.

Causal agents

Waitea zeae, *W. oryzae*, and *W. prodiga* are documented causes of leaf and sheath spot in turfgrasses. These fungi rarely produce asexual or sexual spores naturally. It appears that reproduction is nearly always via vegetative hyphae. Hyphae range from 3 to 10 µm in diameter, are multinucleate and form right-angle branches. Hyphal branches are commonly constricted, and septa are present near the origin of each branch. Microscopic characteristics of *W. zeae*, *W. oryzae*, and *W. prodiga* hyphae are similar; therefore, macroscopic characteristics of isolate cultures and sclerotia are often used to identify isolates to the species level (Table 4). Mycelia of *W. zeae* and *W. oryzae* tends to grow at or below the agar surface and is white to buff to salmon-colored on potato dextrose agar (PDA). In comparison, *W. prodiga* commonly forms aerial mycelium yellow to pink in color when cultured on PDA. Mature sclerotia of *W. zeae* are orange to dark-orange or dark-brown, 0.5 to 1.0 mm in diameter and appear uniformly spherical. In contrast, sclerotia of *W. oryzae* are not uniformly spherical, but somewhat angular, vary in size from less than 1 mm to greater than 3 mm and are salmon in color. Similar to the sclerotia of *W. oryzae*, mature sclerotia of *W. prodiga* vary widely in size and shape and range in color from yellow to salmon to brown.

Disease cycle

The disease cycle of leaf and sheath spot has not been extensively researched. The causal fungi likely survive unfavorable environmental conditions as sclerotia embedded in plant tissue or in the soil and mycelia within plant tissue or organic matter. Germinating sclerotia or surviving mycelia are most likely the sources of initial infection. Based on growth chamber studies, the initial infection in *Cynodon* appears to take place in the root or at the soil surface near the crown. This coincides with diagnostic

observation of diseased *Cynodon* turf, where *W. zeae* mycelium is generally present in the soil-thatch interface or deep within the plant canopy.

Epidemiology

Leaf and sheath spot in cool- and warm-season turfgrasses most often occurs at temperatures ranging from 83 to 97 °F (28 to 36 °C), with the optimum temperature between 86 and 90 °F (30 to 32 °C). This temperature range is above optimal for cool-season turf and is generally when symptoms become visible. In warm-season turfgrass, the ideal temperature for leaf and sheath spot growth is similar to the temperature range favorable for turfgrass growth; consequently, factors other than high temperatures often trigger the onset of disease symptoms. Mechanical damage from agronomic practices, low light conditions and the onset of cool temperatures appear to encourage symptom expression, especially in *Cynodon*. It is unclear if leaf wetness or humidity play a significant role in the development of leaf and sheath spot.

Management

Leaf and sheath spot control in cool-season turfgrass has not been extensively studied. It is assumed that cultural controls similar to those employed to combat brown patch, such as reducing turfgrass stress, avoiding excessive nitrogen fertilization, managing thatch, and eliminating excess moisture during favorable environmental conditions will also suppress leaf and sheath spot. Leaf and sheath spot is generally considered a minor disease in cool-season turfgrass, therefore, little is known about resistant cultivars. Limited research has shown that endophytic fungi may suppress *W. zeae* in *Festuca arundinacea*. Fungicides applied to control brown patch also appear to suppress leaf and sheath spot, with the exception of thiophanate-methyl which is not active against these pathogens.

In warm-season turf, specifically *Cynodon*, leaf and sheath spot has proven difficult to manage. Symptoms often appear when the turf is not actively growing; therefore, recovery is hampered even when the casual fungi are suppressed. For this reason, preventative strategies must be employed.

Fungicides should be applied when environmental conditions favor the causal fungi prior to the appearance of leaf and sheath spot symptoms. Balanced fertility, including adequate nitrogen to promote active growth, is required to manage leaf and sheath spot in *Cynodon*. Research has shown that ammonium sulfate increases leaf and sheath spot severity, although the mechanisms are not well understood. Alternative nitrogen sources, such as urea, should be utilized when leaf and sheath spot is problematic. Cultivation practices and abiotic stress should be minimized when turfgrass is not actively growing.

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APPENDIX D

PHOTOGRAPHS

Figure D.1. Initial mini-ring symptoms present on an ultradwarf bermudagrass putting green. This photograph was taken on 06 July 2016 in Barnwell, South Carolina.

Figure D.2. Fully developed mini-ring symptoms on an ultradwarf bermudagrass putting green located in Blythewood, South Carolina. The photograph was taken on 17 Oct. 2017.

Figure D.3. Severe mini-ring symptoms on an ultradwarf bermudagrass putting green located in Florence, South Carolina. This photograph was taken on 25 Aug. 2022.

Figure D.4. A close-up photograph of mini-ring symptoms on an ultradwarf bermudagrass putting green located in Florence, South Carolina. The approximate length of the knife in the photograph is 20 cm. This photograph was taken on 25 Aug. 2022.

Figure D.5. A photograph of the nitrogen source and nitrogen rate experiment summarized in Chapter two of this dissertation. This trial was conducted on a 'TifEagle' ultradwarf bermudagrass putting green located at the Clemson University Pee Dee Research and Education Center in Florence, SC. The photograph was taken on 13 Oct. 2016. The frame in the bottom-right corner of the picture was used to estimate the percent area of each plot with mini-ring symptoms. Plots outlined in white were treated with ammonium sulfate [$(NH_4)_2SO_4$] at 14.7 kg N ha^{-1} wk^{-1} and plots treated with urea (CH_4N_2O) at 14.7 kg N ha^{-1} wk^{-1} are outlined in black. The evaluation frame is outlining a plot treated with ammonium sulfate at 9.8 kg N ha^{-1} wk^{-1}.

Figure D.6. The trial in this photograph was conducted at Cobblestone Park Golf Club in Blythewood, South Carolina on a 'Tifdwarf' bermudagrass chipping green and is summarized in Appendix B. The photograph was taken on 17 Oct. 2017. A plot treated biweekly with ammonium sulfate [$(NH_4)_2SO_4$] at 19.6 kg N ha^{-1} is outlined in white. The plot outlined in black was treated biweekly with urea (CH_4N_2O) at 19.6 kg kg N ha^{-1}.

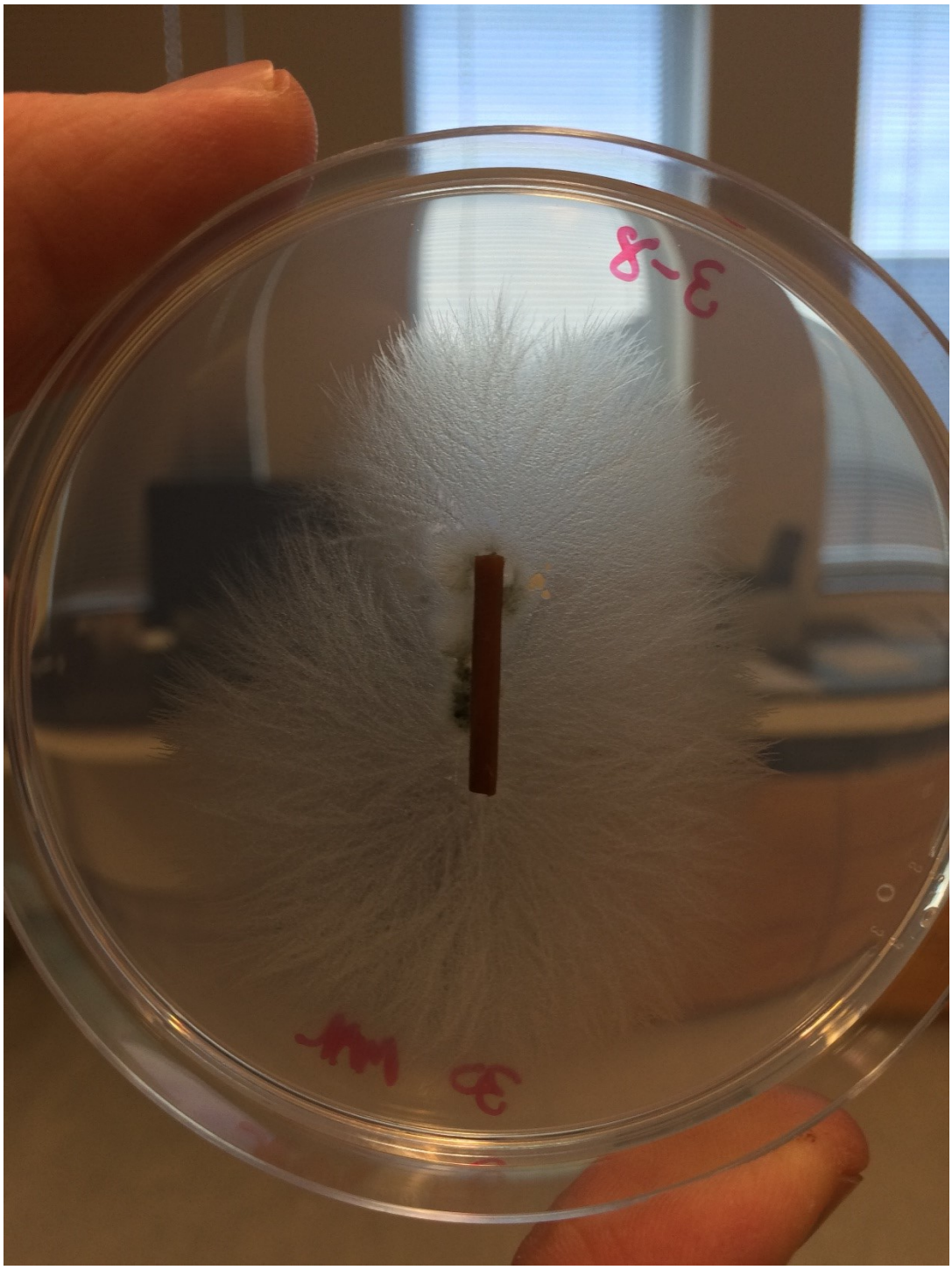

Figure D.7. *Waitea zeae* mycelium growing out of a wood match bait segment used to bait *Waitea* species from an ultradwarf bermudagrass putting green core. The methodology for baiting *Waitea* species was adapted from Windham & Lucas (1987) and described in Chapter four of this dissertation.

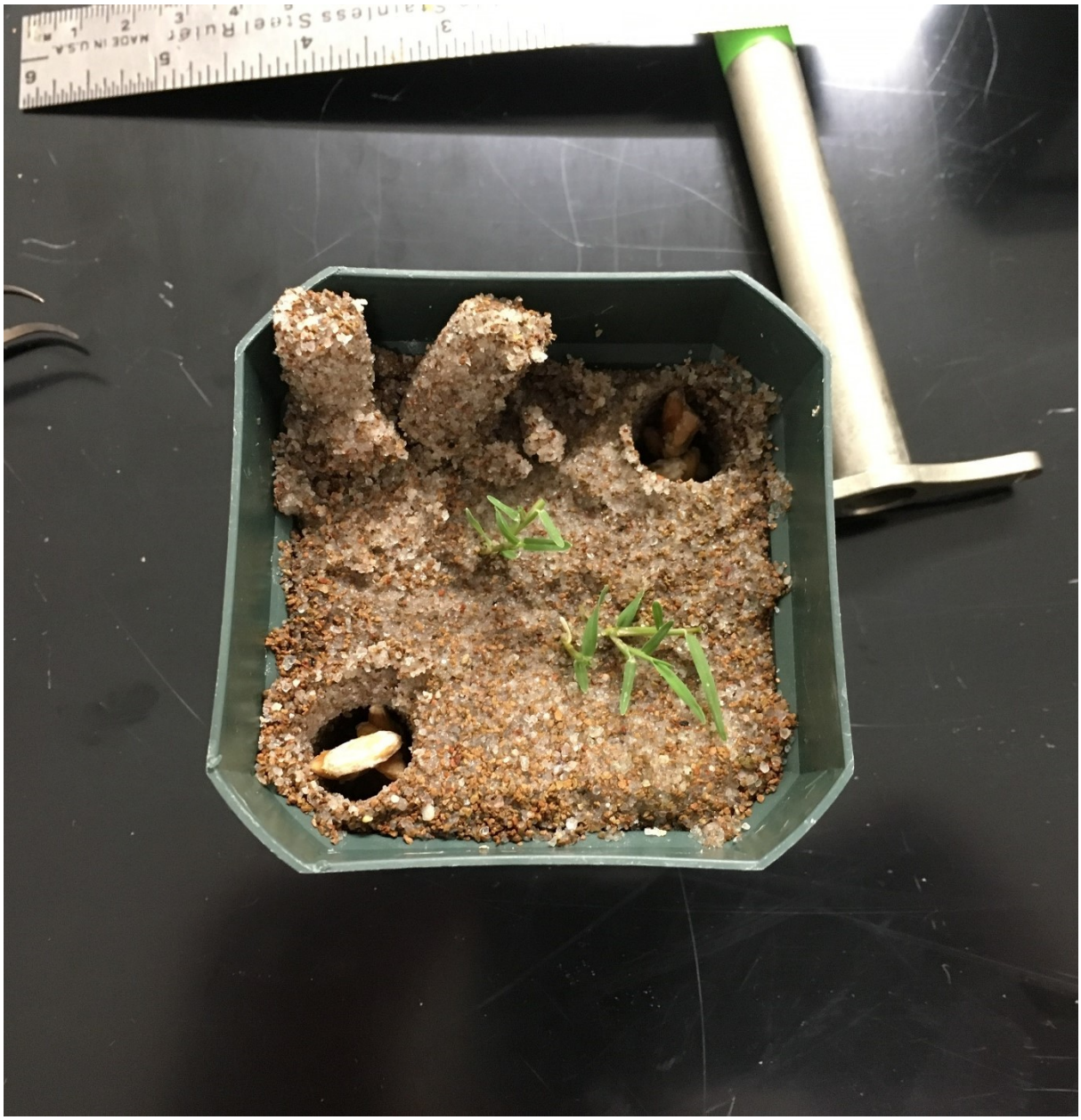

Figure D.8. This photograph illustrates how growing medium was infested with either *Waitea oryzae* or *W. zeae* for the bioassay summarized in Chapter four of this dissertation. Growing medium was infested with by placing four oat seeds colonized by *W. oryzae* or *W. zeae* in the corner of each pot. Oat seeds were then covered with growing medium.

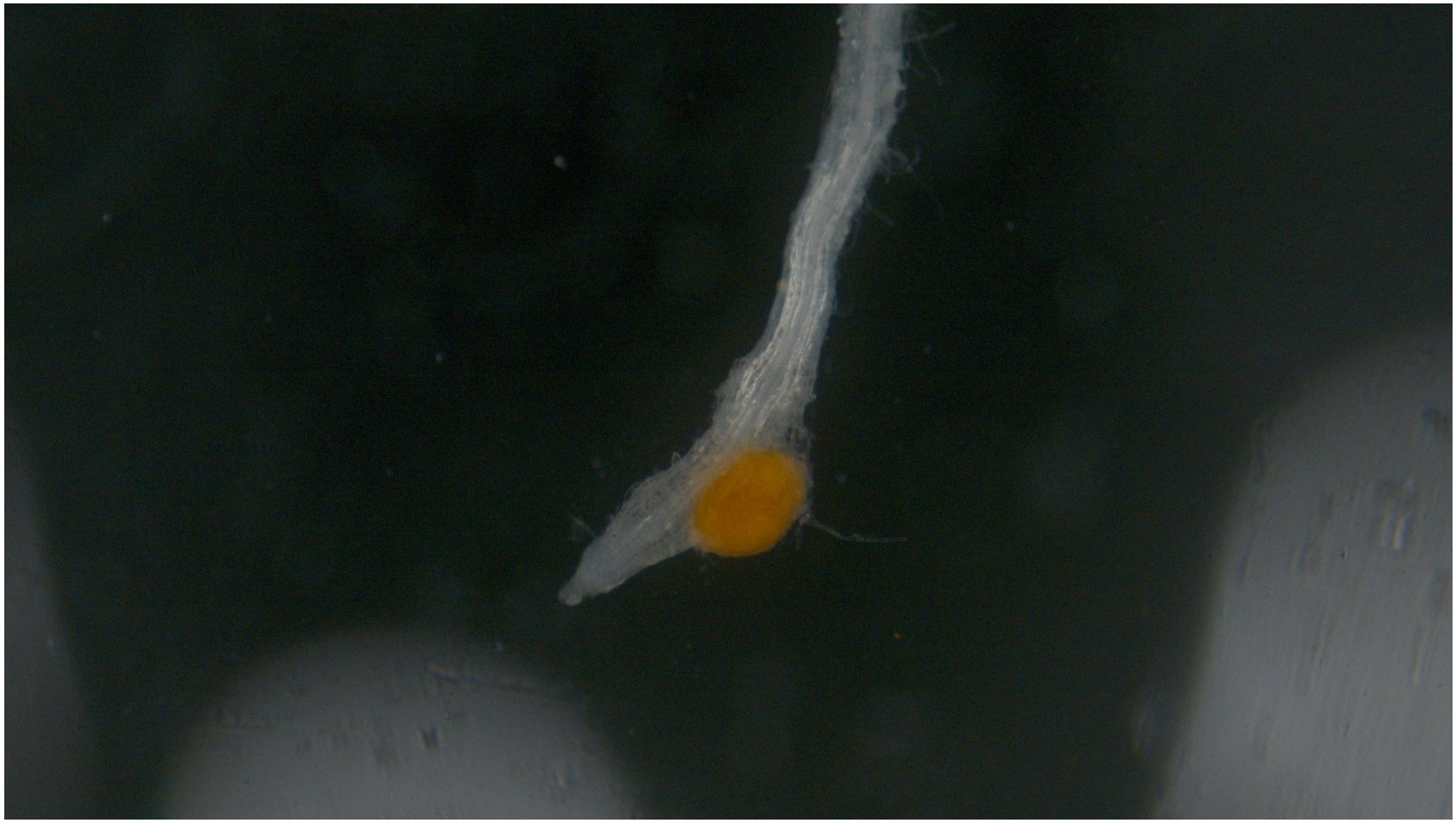

Figure D.9. A *Waitea zeae* sclerotium which formed on an ultradwarf bermudagrass root growing in medium infested with *W. zeae.* Magnification not known.