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Evaluation of Off-Type Grasses in Interspecific Hybrid Bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Putting Greens

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Evaluation of Off-Type Grasses in Interspecific Hybrid Bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Putting Greens

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DEDICATION

To my parents, George and Jennifer. Thank you for providing the support and for your patience during my entire college career. I hope my hard work has made you proud.

To my brother, Luke. Thank you for always being there when you are most needed.

To my grandparents. Thank you for your guidance and sharing your love for agriculture.

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ABSTRACT

The economic impact of the golf industry in the United States (U.S.) in 2011 was estimated to be $176.8 billion. Interspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] are some of the most widely utilized grasses on golf courses throughout tropical, subtropical, and temperate climates. In 2007, bermudagrass was grown on 80% of putting green acreage in the southern U.S. 'Tifgreen' and 'Tifdwarf' were two of the first widely established cultivars on putting greens, but their genetic instability led to the occurrence of phenotypically different off-type (OT) grasses. Several OT grasses were selected and released as cultivars such as 'Champion', 'MiniVerde', and 'TifEagle'. These cultivars can also be genetically unstable and OT grasses can occur in putting greens. The objectives of this research were to genetically and phenotypically characterize OT grasses and evaluate their responses to nitrogen (N) and trinexapac-ethyl (TE) applications. Off-type and desirable bermudagrass samples were collected from Champion, MiniVerde, and TifEagle golf course putting greens in 2013 and 2014. Grasses were genetically evaluated using genotyping-by-sequencing (GBS), which determined that 11% were genetically divergent from standard cultivars. Off-types were phenotypically characterized using morphology and samples clustered into three distinct morphological groups that varied in internode length and leaf length. The response of OT grasses and cultivars to six N and eight TE treatments was evaluated by measuring clippings 7, 14, 21, and 28 days after initial treatment (DAIT). The least three N rates decreased weekly clipping production 18 to 29% [percent], whereas the greatest three rates sustained growth. We observed that peak growth regulation occurred 21 DAIT for the majority of TE rates tested where clipping weights decreased 18 to 35% from 7 to 21 DAIT. We also observed a period of increased clipping production 18 to 47% from 21 to 28 DAIT for all grasses tested. It is important to

maintain consistent growth among phenotypically different grasses in order to minimize any competitive growth advantage an OT grass may possess over a desirable cultivar in a golf course putting green.

TABLE OF CONTENTS

INTRODUCTION..... 1
CHAPTER I: The Genetic and Phenotypic Variability of Interspecific Hybrid Bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Used on Golf Course Putting Greens 4
Abstract..... 5
History of Bermudagrass Development for Putting Greens..... 7
The Genetic Instability of Commercial Cultivars Leading to Off-Types..... 13
Genetic Diversity Among Bermudagrass Cultivars Used on Putting Greens..... 20
Genetic Analysis of Off-Types..... 22
Advances in Molecular Marker Technology for Evaluating Bermudagrasses..... 24
Future Insights on the Management of Off-type Grasses..... 25
CHAPTER II: Genotypic and Phenotypic Evaluation of Off-Type Grasses in Hybrid Bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Putting Greens using Genotyping-by-Sequencing and Morphological Characterization..... 29
Abstract..... 30
Introduction..... 32
Materials and Methods..... 34
Results and Discussion..... 39
Conclusions..... 43
CHAPTER III: Response of Ultradwarf Hybrid Bermudagrass Cultivars [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] and Off-Type Grasses to Applications of Nitrogen and Trinexapac-Ethyl..... 45
Abstract..... 46
Introduction..... 47
Materials and Methods..... 49
Results and Discussion..... 53
Conclusions..... 57
CONCLUSIONS..... 58
FUTURE RESEARCH..... 60
REFERENCES..... 61
APPENDIX 75
Tables..... 76
Figures..... 82
VITA..... 93

LIST OF TABLES

Table 2.1. Plant material used in genetic and phenotypic evaluation of off-type grasses in ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting greens. Selections included 62 desirable (DS) and off-type (OT) bermudagrasses sampled from golf course putting greens in TN, MS, AR, FL, AL, GA, and SC. Six standard (ST) hybrid bermudagrass cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), Tifgreen (TG1-6), and Tifway (TW1-6)] and two progenitor species [(*C. dactylon* (TA1-3 and TB1-3) and *C. transvaalensis* (DA1-3 and DB1-3)] were included in the analysis for comparison. Ploidy level was confirmed using flow cytometry…… 77

Table 2.2. Number of nucleotide variants with different genotypes (homozygous for the reference allele, homozygous for the alternate allele, or heterozygous) between each pair of triploid hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] cultivars Champion, MiniVerde, Tifdwarf, TifEagle, Tifgreen, and Tifway. Variants with differing heterozygosity between or within the two subgenomes (i.e. 0/0/1 or 0/1/1) are not included……..... 79

Table 3.1. Analysis of variance (ANOVA) of the linear-mixed model fit on clipping weights (mg cm^{-2}) that were natural logarithm transformed due to non-constant variance for both nitrogen (N) and trinexapac-ethyl (TE) rate response experiments. The linear-mixed model and ANOVA were performed with R (v3.3.2; R Core Team; Vienna, Austria). ……... 80

Table 3.2. Pairwise comparison t-tests with pooled standard deviation and Bonferroni p-value adjustment on natural logarithm transformed clipping weights [ln(mg cm^{-2})] on the significant main effect of grass sample in the trinexapac-ethyl rate response experiment in Knoxville, TN. Clippings were harvested 7, 14, 21, and 28 days after treatment and then reported as the natural log of mg cm^{-2}. Commercial cultivars Champion, MiniVerde, and TifEagle were included in the experiment with three off-type selections from golf course putting greens. …… 81

LIST OF FIGURES

Figure A. Off-type grasses (lighter in color and noted by red circle) present in an ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting green. The difference in turfgrass color between desirable and off-type grasses disrupts aesthetic uniformity of the putting green. Photo is courtesy of Mr. Rodney Lingle. Figure was generated using Keynote (v6.6.2)…….. 83

Figure B. Close-up of an off-type grass patch (noted by red circle) present in an ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting green. The difference in growth rate between the desirable and off-type grasses has the potential to disrupt the functional uniformity of putting greens with off-type infestations. Figure was generated using Keynote (v6.6.2). …… 84

Figure 1.1. Current understanding of the lineage among accessions of interspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] used on golf course putting greens. The cultivars represented by blue, yellow, and purple colors are those with lineage explicitly reported either in scientific or patent literature. The cultivars represented by orange are those that the true lineage is unknown or are not explicitly reported by scientific or patent literature. …… 85

Figure 2.1. Venn diagram showing the number of total and shared nucleotide variants from genotyping-by-sequencing for *Cynodon transvaalensis*, *C. dactylon*, and *C. dactylon* x *C. transvaalensis.* Ploidy levels were confirmed using flow cytometry and are noted in parentheses. Figure was generated using Keynote (v.6.6.2)…… 86

Figure 2.2. Multidimensional scaling plot (MDS) of nucleotide variants from 47 desirable and off-type bermudagrasses sampled from golf course putting greens (S1-47), six hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), Tifgreen (TG1-6), and Tifway (TW1-6)], and two progenitor species [*C. dactylon* (TA1-3, TB1-3) and *C. transvaalensis* (DA1-3, DB1-3)]. Samples S19, S28, S30, S32, and S44 were not included due to lack of read depth. Nucleotide variants were generated using Freebayes, the MDS plot was calculated in Plink, and plotted in R. The asterisk on the box indicates the zoomed region in Figure 2.3..…….. 87

Figure 2.3. Multidimensional scaling plot (MDS) of nucleotide variants from desirable and off-type bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] sampled from golf course putting greens (S1-47) and cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), and Tifgreen (TG1-6)]. Samples in this MDS plot have been determined genetically similar due to the clustering in the noted box with an asterisk in Figure 2.2. Desirable and off-type bermudagrasses that were analyzed by genotyping-by-sequencing, but did not cluster in this region included the following samples: S4, S16, S31, S33, and S45. Samples S19, S28, S30, S32, and S44 were not included due to lack of read depth. Nucleotide variants were generated using Freebayes, the MDS plot was calculated in Plink, and plotted in R…… 88

Figure 2.4. Cluster means and standard deviations for internode length, leaf length, leaf length:width ratio, stolon diameter, and leaf width measurements. Morphological parameters were assessed using methods similar to Roche and Loch (2005). Measurements were made on 52 off-type and desirable hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] samples harvested from golf course putting greens in the southeastern United States. Cluster means and standard deviations were generated from the K-means algorithm in SAS Enterprise 6.1 and graphed using Prism 6.0 for Mac. Statistical differences were determined using standard deviations…….. 89

Figure 2.5. Photographs of bermudagrass samples representative of each morphological cluster in Figure 2.4. Cluster analysis was performed using a K-means algorithm in SAS Enterprise Guide (Version 6.1, SAS Institute, Cary, NC, USA) with cluster means and standard deviations graphed in Prism (Prism 6 for Mac OS X; GraphPad Software, Inc.) to determine statistical differences. Grasses in cluster one had significantly longer internode lengths than those within clusters two and three. Grasses in cluster three had significantly longer leaves than those in clusters one and two. Figure was generated using Keynote (v6.6.2)……. 90

Figure 3.1. Mean clipping yield of nitrogen rates 0, 6, 12, 18, 24, and 48 kg N ha^{-1} wk^{-1} at 7, 14, 21, and 28 days after initial N treatment. Mean clipping yields were pooled across three commercial bermudagrass cultivars Champion, MiniVerde, and TifEagle and three off-type grasses. The clipping weights (mg cm^{-2}) were natural logarithm transformed due to non-constant variance and then plotted as ln(mg cm^{-2}) in Prism software (Prism 6.0 for Mac OSX. GraphPad Software. La Jolla, CA). Days after initial treatment marked with an asterisk (*) had N rates that were significantly different as determined by pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment in R (v3.3.2; R Core Team; Vienna, Austria)…… 91

Figure 3.2. Mean clipping yield of trinexapac-ethyl (TE) rates 0, 1.6, 3.3, 6.6, 13.1, 26.3, 52.6, or 105.2 g a.i. ha^{-1} at 7, 14, 21, and 28 days after initial N treatment. Mean clipping yields were pooled across three commercial bermudagrass cultivars Champion, MiniVerde, and TifEagle and three off-type grasses. The clipping weights (mg cm^{-2}) were natural logarithm transformed due to non-constant variance and then plotted as ln(mg cm^{-2}) in Prism software (Prism 6.0 for Mac OSX. GraphPad Software. La Jolla, CA). Days after initial treatment marked with an asterisk (*) had TE rates that were significantly different as determined by pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment in R (v3.3.2; R Core Team; Vienna, Austria)…… 92

INTRODUCTION

The economic impact of the golf industry in the United States (U.S.) in 2011 was estimated to be $176.8 billion with a contribution of approximately 1.98 million jobs (SRI International; http://wearegolf.org/economy/impact). Interspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] are widely established throughout tropical, subtropical, and temperate climates (Beard 2002). Growth and stress tolerance characteristics of hybrid bermudagrass make it a highly desirable turfgrass for sports surfaces; particularly golf (Beard 2002). In 2007, hybrid bermudagrass was grown on 32% of the total golf course acreage in the U.S., and 80% of putting green acreage in the southern agronomic region (Lyman et al. 2007).

The use of sterile, triploid interspecific hybrid bermudagrasses on putting greens began with the development of 'Tiffine' (Hein 1953). A later interspecific hybrid, 'Tifgreen', improved putting quality because it could be maintained at lower mowing heights while sustaining optimum leaf density and canopy coverage (Burton 1964; Hein 1961). Shortly after its commercial release, off-type (OT) grasses began appearing in established putting greens (Burton 1966a; Burton and Elsner 1965). Off-type grasses are defined as those with differences in morphology and performance when compared to the surrounding desirable cultivar (Caetano-Anollés 1998; Caetano-Anollés et al. 1997).

These distinct OT patches were presumably somatic (vegetative) mutations of 'Tifgreen' and several were selected and later registered or patented as unique cultivars including 'Tifdwarf' (Burton 1966a), 'MS-Supreme' (Krans et al. 1999), 'Floradwarf' (Dudeck and Murdoch 1998), 'Pee Dee-102' (USDA 1995), and 'TL-2' (Loch and Roche 2003b). Most of these cultivars were darker in color, had greater canopy density, and were able to withstand

lower mowing heights than Tifgreen (Burton 1965, 1966a; Burton and Elsner 1965; Dudeck and Murdoch 1998; Krans et al. 1999). The selection of new commercial cultivars from existing greens continued in the late 1980s through the early 2000s with the discovery of bermudagrasses such as 'Champion Dwarf' (hereafter referred to as 'Champion') (Brown et al. 1997), 'P-18' (hereafter referred to as 'MiniVerde') (Kaerwer and Kaerwer 2001), 'Emerald Dwarf' (Brown et al. 2009), and 'RJT' (Jones et al. 2007). 'Champion' and 'MiniVerde' were selected from somatic mutations in established 'Tifdwarf' plantings (Brown et al. 1997; Kaerwer and Kaerwer 2001), whereas another 'TifEagle' bermudagrass was a putative mutant from radiation-induced 'Tifgreen' or 'Tifway II' rhizome (Hanna and Elsner 1999; Harris-Shultz et al. 2010; Zhang et al. 1999). In all cases, these hybrid "ultradwarf" bermudagrass cultivars were identified as off-types; they had more diminutive morphology than the swards from which they were selected.

'Champion', 'MiniVerde', and 'TifEagle' are currently established on many putting greens across the world (Leslie 2013). These cultivars may be genetically unstable due to their genetic origin and clonal propagation practices and the occurrence of weedy OT grasses in putting greens and production fields is prevalent (Caetano-Anollés 1998; Caetano-Anollés et al. 1997; J.T Brosnan, personal communication, 2013). These OT grasses identified by golf course superintendents have been reported to exhibit differences in turfgrass color, density, and texture compared to desirable ultradwarf bermudagrasses (Figure A). Anecdotal observations also suggest these weedy OT grasses respond differently to typical putting green management practices of nitrogen (N) and trinexapac-ethyl (TE) applications. Grasses with different responses to these management practices within a putting green can create variations in growth rate that may disrupt golf ball roll and overall performance (Figure B; E.H. Reasor, personal observation, 2017; J.T. Brosnan, personal communication, 2013). Therefore, the objectives of

this research were to genetically and phenotypically characterize OT grasses sampled from golf course putting greens and evaluate their responses to N and TE applications.

CHAPTER I

The Genetic and Phenotypic Variability of Interspecific Hybrid Bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Used on Golf Course Putting Greens

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The publishing of this article involved an extensive peer-review process that included two journals, three associate editors, and two rejections for publication. The peer-review process improved the quality of the article tremendously and allowed the authors to expand upon certain hypotheses. The publishing of this article involved six authors from three institutions and each author made conceptual or technical contributions relative to their area of expertise. My primary contributions to this paper include (i) reading literature (ii) writing the manuscript, (iii) revisions during review process.

Abstract

Golf course putting green surfaces in subtropical and tropical climates are typically planted with an interspecific hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] because of the superior putting quality and performance of these cultivars. 'Tifgreen' was one of the first interspecific hybrids developed for putting green use in lieu of common bermudagrass. However, off-type (OT) grasses began appearing in established 'Tifgreen' stands soon after commercial release. Off-type grasses are those with different morphology and performance when compared to the surrounding, desirable cultivar. Off-type

grasses have the potential to decrease surface uniformity, which negatively affects putting green quality. However, several unique OT grasses from 'Tifgreen' have been selected as commercial cultivars, the first being 'Tifdwarf'; then 'Floradwarf', 'MS-Supreme', 'Pee Dee-102', and 'TL-2', identified later. The cultivars Champion, MiniVerde, RJT, and Emerald Dwarf were subsequently selected as OT grasses in 'Tifdwarf'. The naturally occurring OT grasses and cultivars that have been identified within the 'Tifgreen' family have widely differing phenotypes; however, they are reported to be genetically similar, supporting the hypothesis that their occurrence is a result of somatic mutations. Genetic instability in currently available commercial cultivars is likely to lead to the continued presence of OT grasses in production nurseries and putting greens. Additional research is needed to understand the nature of genetic instability in 'Tifgreen'-derived cultivars and how to manage its consequences in order to develop new cultivars, but also strategies for eradication of OT grasses in nursery production and golf course putting greens.

History of Bermudagrass Development for Putting Greens

Early Cultivars

'Tiffine' was one of the first bermudagrass cultivars reported to be more suitable than common bermudagrass (*Cynodon dactylon* (L.) Pers.; 2n=4x=36) for use on golf course putting greens (Hein 1953). 'Tiffine' was a sterile, triploid (2n=3x=27), interspecific hybrid (*C. dactylon* x *C. transvaalensis*) between a tetraploid *C. dactylon* cv. 'Tiflawn' and a diploid (2n=2x=18) *C. transvaalensis* selection (Forbes and Burton 1963; Hein 1953). Dr. Glenn W. Burton with the United States (U.S.) Department of Agriculture–Division of Forage Crops and Diseases (later renamed to Agricultural Research Service) developed 'Tiffine' in 1949 in cooperation with the University of Georgia (UGA) at the Georgia Coastal Plain Experiment Station in Tifton, GA (Forbes and Burton 1963; Hein 1953). Hein (1953) reported that 'Tiffine' was selected based on improved color, texture, and growth habit. The cultivar was released in 1953 (Hein 1953) and was established on putting greens throughout the Southeastern U.S. until the release of 'Tifgreen' in 1956.

Dr. Glenn W. Burton also developed 'Tifgreen' bermudagrass in cooperation with UGA at the Georgia Coastal Plain Experiment Station (Hein 1961). Similar to 'Tiffine', 'Tifgreen' was a sterile, triploid, interspecific hybrid between a *C. dactylon* selection from a putting green in Charlotte, NC and a *C. transvaalensis* breeding line (Burton 1964; Forbes and Burton 1963; Hein 1961). The cross-pollination program between the two *Cynodon* spp. that yielded 'Tifgreen' was initiated in 1951. The resulting interspecific hybrids were tested until the commercial release of 'Tifgreen' in 1956. The fine texture, density, and rapid growth of 'Tifgreen' made it well-suited for golf course putting greens (Burton 1964; Hein 1961). Hein (1961) reported that 'Tifgreen' had greater sod density, weed resistance, fine texture, softness,

and color compared to common bermudagrass established from seed. 'Tifgreen' survived winters in Manhattan, KS and Beltsville, MD; however, researchers only recommended 'Tifgreen' for use in southern climates where bermudagrasses were normally grown (Burton 1964; Hein 1961). 'Tifgreen' was reported to be susceptible to sod webworm (*Crambus spp.*) damage and injury from 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide applications (Hein 1961), which could negatively affect overall quality.

Genetic instability of 'Tifgreen' gave rise to off-type (OT) grasses of variable phenotypes that appeared soon after establishment (Caetano-Anollés 1998; Caetano-Anollés et al. 1997). In many cases, these OT grasses exhibited superior characteristics and were later propagated and released as commercial cultivars. The vast majority of bermudagrass cultivars established on putting greens since 1960 are genetically related to 'Tifgreen'; therefore the development and widespread use of 'Tifgreen' formed the foundation of current bermudagrass cultivars used on putting greens today.

'Tifgreen'-derived Cultivars

'Tifdwarf' was the first OT of 'Tifgreen' to be selected, researched, and released as a commercial cultivar and has since been used on putting greens throughout subtropical and tropical climates. James Moncrief first identified 'Tifdwarf' as one of two vegetative mutations in mature 'Tifgreen' putting greens in Georgia and South Carolina (Burton 1966a; Burton and Elsner 1965; O'Brien 2012). Burton (1964) reported that the mutation from which 'Tifdwarf' was selected might have been present in the first 'Tifgreen' planting stock before it was distributed for experimentation. 'Tifdwarf' was reported to have the same number of chromosomes as 'Tifgreen', but its phenotype/genotype allowed it to outperform 'Tifgreen' on golf course putting greens (Burton 1965, 1966a; Burton and Elsner 1965). 'Tifdwarf' has a

lower growth habit than 'Tifgreen', which facilitated mowing at heights of 4.76 mm (Burton 1965, 1966a; Burton and Elsner 1965). Burton (1965) reported that 'Tifdwarf' required less frequent mowing and topdressing than 'Tifgreen', which resulted in reduced maintenance expenses. Additionally, 'Tifdwarf' had softer leaves, fewer seed heads, darker green color, and slightly greater winter hardiness than 'Tifgreen' (Burton 1965, 1966a; Burton and Elsner 1965). The genetic instability of 'Tifdwarf' was similar to 'Tifgreen' (Burton 1965, 1966a; Caetano-Anollés et al. 1997; Caetano-Anollés 1998); therefore, widespread use of 'Tifdwarf', like 'Tifgreen', facilitated the selection of OT grasses that were later released as commercial cultivars.

'Pee Dee-102' was selected from a mutation in an early planting of 'Tifgreen' at the Pee Dee Experimental Station (Florence, SC). The South Carolina Agricultural Experiment Station (Clemson, SC) released 'Pee Dee-102' in 1968, and the South Carolina Foundation Seed Association (Clemson, SC) managed the foundation stock. 'Pee Dee-102' was reported to have smaller leaves and shorter internodes than Tifgreen, which provided an improved putting green (USDA 1995).

The Florida Agricultural Experiment Station registered 'Floradwarf' bermudagrass as a commercial cultivar after its release in 1995 (Dudeck and Murdoch 1998). It was selected in 1988 as an OT plant on a golf course located in Hawaii and was thought to be a mutation of 'Tifgreen'. There are contrasting reports regarding the phenotypic characteristics of 'Floradwarf' and 'Tifdwarf'. Dudeck and Murdoch (1998) reported that 'Floradwarf' has greater density than 'Tifdwarf' due to shorter stolons, internode length, and leaf length; however, Roche and Loch (2005) reported that 'Floradwarf' and 'Tifdwarf' have similar internode length, stolon diameter, leaf length, and leaf width. Thatch development occurs relatively fast in 'Floradwarf'

putting greens, necessitating timely vertical mowing and topdressing (Dudeck 1995; Dudeck and Murdoch 1998). Dudeck and Murdoch (1998) also state that winter overseeding with perennial ryegrass (*Lolium perenne* L.) in 'Floradwarf' greens is hindered due to high canopy density, but roughstalk bluegrass (*Poa trivialis* L.) can successfully be established. 'Floradwarf' is susceptible to dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett), tropical sod webworms (*Herpetogramma phaeopteralis* Guenée), mole crickets (*Scapteriscus* spp.), and sting nematodes (*Belonolaimus longicaudatus* Steiner) (Dudeck and Murdoch 1998).

'MS-Supreme' is an improved interspecific hybrid bermudagrass selected in 1991 from a 'Tifgreen' putting green originally planted in 1964 at Gulf Shores Golf Club (Golf Shores, AL) and was released by the Mississippi Agricultural and Forestry Experiment Station in 1997. 'MS-Supreme' was selected for high density, fine texture, prostrate growth habit, and tolerance to low mowing heights. Due to the morphology and growth habit of 'MS-Supreme', management requires an intensive cultivation program for thatch control (Krans et al. 1999). Krans et al. (1999) reported that internode length and stolon diameter of 'MS-Supreme' were shorter than 'Tifgreen', but not 'Tifdwarf'. In order to ensure high quality sod, the foundation stock of 'MS-Supreme' was maintained by the Mississippi Agricultural and Forestry Experiment Station (Krans et al. 1999). 'MS-Supreme' is also registered in Australia under the Australian Plant Breeders' Rights Registration application number 2002/305 (Loch and Roche 2003a).

'TL-2', also known as 'Novatek', was selected as a mutant of 'Tifgreen' in 1996 at Novotel Palm Cove in Cairns, Queensland (Loch and Roche 2003b). Loch and Roche (2003b) identified 'TL-2' due to its dark green color, finer-texture, and greater density when compared to other selections from 'Tifgreen' tested at that time. Roche and Loch (2005) later reported 'TL-2' to have similar stolon internode length, leaf length, and leaf width compared to 'Tifdwarf'.

Tropical Lawns Pty Ltd tested mutant selections and then released 'TL-2' in 2003 under the Australian Plant Breeders' Rights Registration name 'TL-2' (Loch and Roche 2003b; Roche and Loch 2005).

'Tifdwarf'-derived Cultivars

'Champion' was selected in 1987 as an OT present in a 'Tifdwarf' putting green originally established in 1969 in Walker County, TX (Brown et al. 1997). The original selection of 'Champion' was propagated in greenhouse pots from a single sprig in Bay City, TX. These plants were used to plant larger trays and then to establish the first 'Champion' production field. 'Champion' has been described as having slower vertical growth in conjunction with lateral growth similar to other *Cynodon* spp. (Brown et al. 1997). Compared to 'Tifdwarf', 'Champion' has higher shoot density and narrower leaves (Brown et al. 1997).

'MiniVerde' was a bermudagrass selected based on its fine texture, high canopy density, rapid growth rate, and uniform green color. First identified in 1992, 'MiniVerde' was an OT obtained from a putative 'Tifdwarf' line grown in a greenhouse owned by H&H Seed Company in Yuma, AZ. 'MiniVerde' was reported to exhibit darker color, higher quality, and greater density, as well as a shorter root structure than 'Tifdwarf' (Kaerwer and Kaerwer 2001).

'Champion' and 'MiniVerde' are considered "ultradwarf" bermudagrasses along with 'Floradwarf'. The term "ultradwarf" was first coined in 1995 by Dr. Philip Busey from the University of Florida to describe bermudagrass putting green cultivars with significantly more diminutive morphology than 'Tifdwarf' (P. Busey, personal communication, 2016). The term ultradwarf is now widely used in the turfgrass industry to label such cultivars.

'Emerald Dwarf' was a selection made in 1992 from a 'Tifdwarf' putting green established in the 1970s. 'Emerald Dwarf' was reported to produce longer roots and more

rhizomes than ‘Tifgreen’ or ‘Tifdwarf’, which resulted in higher quality, color, and coverage during transition periods (Brown et al. 2009).

‘RJT’, also known as ‘Jones Dwarf’, was selected from the regrowth of a sod production field that was previously established to ‘Tifdwarf’ in 1996 (Jones et al. 2007). The selection was based on fine texture, low nutrient requirements, and reduced thatch production compared to the surrounding ‘Tifdwarf’ (Jones et al. 2007).

Other Cultivars

‘TifEagle’ was an ultradwarf bermudagrass selected in 1990 for its high quality, fine-texture, and ability to tolerate low mowing heights common on golf course putting greens. Following testing as TW-72, ‘TifEagle’ was released by the USDA-ARS and the UGA Coastal Plain Experimental Station in 1997. ‘TifEagle’ was one of 48 putative mutants resulting from the irradiation of ‘Tifway II’ with 70 grays (7000 rads) of cobalt-60 gamma radiation (Hanna and Elsner 1999). While ‘TifEagle’ was reported to be derived from ‘Tifway II’ (Hanna and Elsner 1999); Harris-Shultz et al. (2010) and Zhang et al. (1999) both suggested that ‘TifEagle’ may have been derived from ‘Tifgreen’ (or a ‘Tifgreen’ related plant) due to the high dissimilarity coefficients reported between ‘TifEagle’ and ‘Tifway II’ using amplified fragment length polymorphism (AFLP) methodology. Findings of Capo-chichi et al. (2005) and Chen et al. (2009) further support this assertion in that both research teams reported a high degree of genetic similarity between ‘TifEagle’ and ‘Tifgreen’. ‘TifEagle’ is a vegetatively propagated cultivar reported to produce higher quality putting greens than ‘Tifdwarf’ when mowed daily at 4 mm or less. When compared to ‘Tifdwarf’, ‘TifEagle’ produced fewer seedheads, had a higher tolerance to tawny mole cricket (*Scapteriscus vicinus*), but produced more thatch (Hanna and Elsner 1999). Hanna and Elsner (1999) reported that ‘TifEagle’ had shorter and narrower leaves

than ‘Tifdwarf’ and produced more stolons. Since its commercial introduction, ‘TifEagle’ has been distributed under sublicensing agreements that require inspections of growing locations to limit off-types and to provide incentive for qualified producers to promote the use of ‘TifEagle’ (Hanna and Elsner 1999).

In addition to the above-described cultivars, other OT grasses of unknown parentage, presumably related to ‘Tifgreen’, have been selected from bermudagrass greens and marketed as cultivars with characteristics superior to ‘Tifgreen’ and ‘Tifdwarf.’ ‘C-1’ is an OT bermudagrass selected in 1987 from what was known as “Cotton Creek Dwarf” at Cotton Creek Golf Course (Gulf Shores, AL) (Chapman 2016). ‘C-7’ (also know as ‘Sunday’) was an ultradwarf cultivar selected in 2007 from a ‘C-1’ putting green also at Cotton Creek Golf Course. ‘C-7’ was reported to have similar internode length to ‘Tifdwarf’, but longer leaves (Chapman 2016). Other bermudagrass selections marketed on a more regional basis include ‘Quality Dwarf’, ‘Jensen Dwarf’, ‘Classic Dwarf’, ‘Australian 328’, and ‘Aussie Green’ (D. Roberts and J.E. Elsner, personal communications, 2015). The understanding of the lineage among accessions of hybrid bermudagrasses used on golf course putting greens is presented in Figure 1.1. Many bermudagrass cultivars first identified as OT grasses in established swards of ‘Tifgreen’ and ‘Tifdwarf’ have been commercialized. These grasses had different morphology, color, and performance when compared to the parent cultivar in which they were first identified.

The Genetic Instability of Commercial Cultivars Leading to Off-Types

Bermudagrass cultivars such as Tifdwarf, Floradwarf, MS-Supreme, Champion, and MiniVerde were selected from established swards of ‘Tifgreen’ or ‘Tifdwarf’ (Burton and Elsner 1965; Brown et al. 1997; Dudeck and Murdoch 1998; Krans et al. 1999; Kaerwer and Kaerwer 2001). They were identified as OT grasses in putting greens because of differences in

morphology and performance (Caetano-Anollés 1998; Caetano-Anollés et al. 1997). The presence of OT grasses spurred research exploring the genetic stability of 'Tifgreen' and 'Tifgreen'-derived cultivars.

DNA amplification fingerprinting (DAF) is a method that uses arbitrary oligonucleotide primers to detect polymorphisms among closely related organisms (Caetano-Anollés and Bassam 1993; Caetano-Anollés et al. 1995). DNA amplification fingerprinting and arbitrary signatures from amplification profiles (ASAP) were used to assess the genetic stability of both 'Tifgreen' and 'Tifdwarf'. Caetano-Anollés (1998) analyzed eleven 'Tifgreen' and eight 'Tifdwarf' authenticated accessions collected from the foundation field and plots maintained by university research programs. According to this study, 'Tifgreen' and 'Tifdwarf' were genetically unstable due to 211 out of 619 DAF polymorphic loci (from 15 mini-hairpin primers) identifying differences in all but one of the 'Tifgreen'/'Tifdwarf' accessions (Caetano-Anollés 1998). Compared to a previous study (Caetano-Anollés et al. 1997), differences were not evident between nine different 'Tifway' accessions using 273 DAF loci. Based on these findings, Caetano-Anollés (1998) concluded that 'Tifway' was 18 times more genetically stable than 'Tifgreen' and 'Tifdwarf'.

A possible explanation for the high genetic instability and OT occurrence in 'Tifgreen' and 'Tifdwarf' is aneuploidy. Aneuploidy is an abnormal number of chromosomes not due to a difference in the number of complete sets of chromosomes, which is called euploidy (Duesberg and Rasnick 2000). 'Tifgreen' bermudagrass is a sterile, triploid, interspecific hybrid, but it would be possible for aneuploidy within this cultivar to originate through mitosis and vegetative (asexual) reproduction or during meiosis of the original cross between *C. dactylon* and *C. transvaalensis*.

Vegetative reproduction of 'Tifgreen' and 'Tifdwarf' from stolons and rhizomes provides greater opportunities for point mutations to accumulate at higher rates than grasses that reproduce sexually (Caetano-Anollés 1999; Harris-Shultz et al. 2011). Subsequent cultivars selected from somatic mutations of 'Tifgreen' and 'Tifdwarf' (i.e., 'MiniVerde' and 'Champion') are proposed to possess the same level of genetic instability reported by Caetano-Anollés (1998) in 'Tifgreen' and 'Tifdwarf'. This is theorized because aneuploidy in interspecific triploid hybrids is not a terminal condition and can be exhibited in subsequent generations (Henry et al. 2005). Duesberg and Rasnick (2000) documented that aneuploidy is a source of genetic instability because the somatic mutations that affect phenotypic characteristics evolve spontaneously.

Meiotic irregularity has also been postulated to result in some superior phenotypic changes in certain accessions of interspecific hybrid bermudagrasses in the past (Forbes and Burton 1963; Henry et al. 2005). Forbes and Burton (1963) stated that the perennial growth type and vegetative reproduction associated with bermudagrass could reduce meiotic regularity, which could lead to aneuploidy (Henry et al. 2005). Additionally, triploid species can produce viable aneuploids (mostly trisomics) that have severe effects on phenotypic traits (Birchler et al. 2001; Bridges 1922; Henry et al. 2005). Blakeslee (1922) reported that a triploid *Datura* species produced 12 trisomics and each one exhibited a different phenotype. Similar results have also been reported in tomato (*Solanum lycopersicum* L.; Lesley 1928), corn (*Zea mays* L.; McClintock 1929), and tobacco (*Nicotiana tabacum* L.; Clausen and Cameron 1944).

Parental lineage may explain why aneuploidy could be exhibited in 'Tifgreen' and not 'Tifway'. Despite the fact that both cultivars are interspecific triploid hybrids of *C. dactylon* and *C. transvaalensis* (Burton 1966b; Hein 1961), different accessions and breeding lines were used

to make the crosses that produced 'Tifgreen' and 'Tifway'. Burton (1966b) reported that the male parent of 'Tifway' was a *C. dactylon* (L.) Pers. selection having 36 chromosomes and the female parent was *C. transvaalensis* Burtt-Davy selection with 18 chromosomes. The species that were the male and female parents of 'Tifgreen' are not specified in the literature.

Lack of information regarding the parental lines used to produce 'Tifgreen' is significant in that there are contrasting reports regarding the base chromosome number of bermudagrass. The majority of research suggests that the base chromosome number is nine (Advulow 1931; Bowden and Senn 1962; Brown 1950; Burton 1947; Clayton and Harlan 1970; Darlington and Wylie 1956; Forbes and Burton 1963; Harlan and de Wet 1969; Rita et al. 2012); however, there have been reports that some bermudagrass accessions may possess several fragmented chromosomes (Burton 1947; Hurcombe 1948). Other findings suggest that bermudagrass has a base chromosome number of ten (Hunter 1943; Hurcombe 1947; Rochecouste 1962; Shibata 1957; Tateoka 1954). Forbes and Burton (1963) surmised that these contrasting accounts were the result of counting fragments as whole chromosomes. Additionally, de Silva and Snaydon (1995) suggested that variation in chromosome number may be due to growing environment. Given the contrasting reports of the base chromosome number in bermudagrass and the meiotic irregularity of the *Cynodon* spp., the chromosome fragments observed by Burton (1947) and Hurcombe (1948) may have been whole chromosomes. In this scenario, some triploid bermudagrass interspecific hybrids could be aneuploid and subject to genetic instability.

The repeated use of pesticides and plant growth regulators (PGR) could potentially influence aneuploidy (Karp 1994; Capo-chichi et al. 2005; Gadeva and Dimitrov 2008). Capo-chichi et al. (2005) reported that chronic exposure of 'Champion' bermudagrass in greenhouse culture to the dinitroaniline herbicides, pendimethalin and oryzalin, induced the formation of

four OT grasses. Three of the four OT grasses were triploid and morphologically similar to 'Tifgreen'; however, one off-type was aneuploid with several morphological traits measuring larger than 'Tifgreen' (Capo-chichi et al. 2005). Capo-chichi et al. (2005) suggested that this OT may have originated from common bermudagrass; however, this was not confirmed. Gadeva and Dimitrov (2008) reported that exposure of *Crepis capillaris* L. to high concentrations of the fungicide iprodione and insecticide propargite led to a strong presence of lagging chromosomes and anti-microtubule activity, which resulted in aneuploidy. Karp (1994) stated that high concentrations of the synthetic auxin, 2,4-D, increased chromosome instability in tissue culture. Choice and concentration of a particular pesticide or PGR can influence chromosome variations in regenerated plants, which is important because it can lead to modifications of phenotype (Karp 1994). Research regarding pesticides and PGRs as direct mutagens is inconsistent. Moreover, effects of pesticides on aneuploidy have primarily been observed in tissue culture and use of these specific pesticides in bermudagrass production nurseries and putting greens may be limited.

Aneuploidy can also result from meristem chimeric tissues (Zonneveld and Pollack 2012). Chimeras possess at least two genetically distinct kinds of tissue side-by-side, which is the result of spontaneous mutation accumulations and cell layer rearrangements (Harris-Shultz et al. 2011; Skirvin and Norton 2015; Zonneveld and Pollack 2012). Zonneveld and Pollack (2012) suggested the vegetative propagation of meristem chimeras could lead to aneuploidy in plants. Marcotrigiano (2000) reported that meristem damage can reveal mutations of inner layer cells that were previously isolated to a single cell layer, a phenomenon that has been documented in *Hosta* cultivars (Zonneveld and Pollack 2012). The researchers stated that aneuploidy in the outermost meristem layer was the major contributor to phenotypic differences among *Hosta*

cultivars and as a result, aneuploidy is a source of genetic and morphological diversity within the genus (Zonneveld and Pollack 2012).

Due to their arrangement of genetically distinct tissues, chimeras can only be successfully propagated by asexual techniques that use preformed buds and avoid adventitious buds (Skirvin and Norton 2015). Harris-Shultz et al. (2011) suggested that 'Tifdwarf' and 'TifEagle' are chimeras. Vegetative production procedures (i.e., sod nurseries) and routine low mowing of 'Tifgreen' or 'Tifgreen'-derived cultivars on putting greens have the potential to cause meristem damage, which could expose putative *de novo* mutations once isolated to a single layer (Harris-Shultz et al. 2011). These practices also have the potential to successfully propagate chimeric tissues. It should be noted that putative *de novo* mutations leading to OT grasses are likely to be more common in production nurseries than putting greens; therefore, mowing practices associated with putting greens are theoretically only a small factor causing genetic instability and OT occurrence of 'Tifgreen' or the 'Tifgreen'-derived cultivar family (J.E. Elsner, unpublished observations, 2015).

Aneuploidy in *Luzula luzuloides* has been documented in tissue culture (Madej and Kuta 2001). Madej and Kuta (2001) explained that mitotic abnormalities were the main cause of the aneuploidy observed in *L. luzuloides*, but chromosome fusion and fission were also causes. Although true aneuploidy was not reported, Goldman et al. (2004) observed phenotypic and chromosome number variations among 'TifEagle' plants in tissue culture. Only 14% of the plants regenerated from a single embroygenic tissue were morphologically similar to 'TifEagle' and only 67% remained triploid (Goldman et al. 2004). The remaining plants were hexaploid with dark green color, wider leaves, and taller (Goldman et al. 2004). Lu et al. (2006) reported similar findings in follow-up studies regenerating 'TifEagle' in tissue culture. The researchers

suggested that genotype explained the observed phenotypic variation, but the increase in ploidy was likely an effect of plants regenerating from a single embroygenic tissue (Goldman et al., 2004). Production nurseries mass-produce vegetative material to establish bermudagrass cultivars on golf courses and then allow plants to regenerate from vegetative propagules remaining in the nursery after harvest (e.g., rhizomes). Unless production nurseries are periodically rotated or re-established, the process of harvesting and regeneration can occur repeatedly over time potentially introducing variation in phenotype and chromosome number of these cultivars (Harris-Shultz et al. 2011).

Aneuploidy has been reported in a wide range of plant species, including bermudagrass. Gould (1966) reported B-chromosomes, or accessory chromosomes, in two out of three *C. dactylon* selections. De Silva and Snaydon (1995) documented that 15% of plants within a sample population of *C. dactylon* were aneuploid. Arumuganthan et al. (1999) reported that 'Tifgreen' has 0.24 pg/2C more nuclear DNA than 'Tifway'. Greater DNA content would support the assertion that 'Tifgreen' contained an extra chromosome and is therefore aneuploid. There is evidence to support the possibility that aneuploidy contributes to the genetic instability observed with bermudagrass cultivars derived from 'Tifgreen'. However, extensive cytogenetic research on 'Tifgreen'-derived bermudagrass cultivars is needed to support this idea. Regardless of the origin, genetic instability within the 'Tifgreen' family has led to the presence of OT grasses in both production nurseries and putting greens. This has spurred molecular genetics research aimed at exploring the origins and genetic diversity of OT grasses occurring in 'Tifgreen'-derived putting greens and stolon production nurseries.

Genetic Diversity Among Bermudagrass Cultivars Used on Putting Greens

Molecular genetics research in turfgrass is difficult due to the high ploidy levels and complex genomes associated with turfgrass species (Fei 2008); however, diversity among triploid bermudagrass cultivars has been researched. The genetic variation of 'Tifgreen' and 'Tifdwarf' were compared using DAF with arbitrary octamer primers. Dendrograms were generated from an unweighted pair group cluster analysis using arithmetic means (UPGMA) and phylogenetic analysis using parsimony (PAUP). DNA amplification fingerprinting revealed differences between 'Tifgreen' and 'Tifdwarf' with five polymorphisms present among three primer sequences; however, the UPGMA and PAUP analyses demonstrated that the two cultivars were very closely related (Caetano-Anollés et al. 1995). Farsani et al. (2012) were able to use inter-simple sequence repeat markers and a UPGMA clustering method to place 'Tifgreen' and 'Tifdwarf' into separate subgroups under the same cluster. These studies confirm that 'Tifgreen' and 'Tifdwarf' are genetically similar despite having differences in phenotype.

Amplified fragment length polymorphisms have also been used to examine the genetic diversity among bermudagrass cultivars and selections throughout the southern U.S. (Capo-chichi et al. 2005; Chen et al. 2009; Zhang et al. 1999). An UPGMA dendrogram created from dissimilarity coefficients clustered 'Tifgreen', 'Tifdwarf', 'TifEagle', 'Floradwarf', 'Champion', and 'MS-Supreme' together (Capo-chichi et al. 2005). Zhang et al. (1999) reported a relative genetic dissimilarity coefficient range of 0.08 to 0.33 among 'Tifgreen', 'Tifdwarf', 'TifEagle', and 'Floradwarf', which grouped these cultivars into the same cluster. Chen et al. (2009) reported similar results with 'Champion', 'Tifgreen', 'Tifdwarf', and 'TifEagle' belonging to the same UPGMA cluster group due to more than 90% genetic similarity among one another. The results of these three studies using AFLP markers are similar to the results of Caetano-Anollés et

al. (1995) and Farsani et al. (2012), suggesting that these bermudagrass cultivars are genetically similar and cannot be fully distinguished from one another.

Expressed sequence tags-derived simple sequence repeat (EST-SSR) markers have also been used to examine relationships among 'Tifgreen', 'Tifdwarf', 'TifEagle', 'Floradwarf', 'Champion', and 'MiniVerde'. Identical alleles were found for the six cultivars indicating that they were all derived from 'Tifgreen' and could not be differentiated from one another (Harris-Shultz et al. 2010). Wang et al. (2010) reported similar results to Harris-Shultz et al. (2010) using simple sequence repeat (SSR) markers, which grouped 'Tifgreen', 'Tifdwarf', 'TifEagle', 'Floradwarf', 'MS-Supreme', 'Champion', and 'MiniVerde' into a single mutation family. The SSR markers used by Wang et al. (2010) identified 22 cultivars derived via traditional breeding; however, mutation-derived cultivars (TifEagle, Floradwarf, MS-Supreme, Champion, and MiniVerde) were genetically indistinguishable from each other. Kamps et al. (2011) also failed to differentiate 'Tifgreen', 'Tifdwarf', 'Champion', 'Floradwarf', or 'MS-Supreme' using SSR markers.

While some previously described SSR markers were not able to identify 'TifEagle' from its relatives, a single amplicon from a primer (Chase 109) has been used to identify 'TifEagle' from 'Tifgreen' and 'Tifgreen'-derived cultivars (Harris-Shultz et al. 2011; Kamps et al. 2011). Harris-Shultz et al. (2011) reported that the polymorphic fragment amplified by the Chase 109 primer was approximately 142 base pairs larger than the fragment length reported by Kamps et al. (2011). Kamps et al. (2011) suggested that microsatellite instability in plant tissues may be affected by irradiation, similar to mammalian tumors (Haines et al. 2010), potentially explaining why 'TifEagle' is distinguishable from 'Tifgreen'-derived cultivars using the Chase 109 primer. This hypothesis is logical considering that 'TifEagle' has been reported to be a mutant derived

from an irradiated 'Tifway II' rhizome (Hanna and Elsner 1999). Simple sequence repeat markers were also reported to identify polymorphic fragments unique to 'Tifdwarf', 'TifEagle', and 'MiniVerde' (Harris-Shultz et al. 2011). The SSR markers used to distinguish 'MiniVerde' generated the same polymorphic fragment in shoot and root tissues; however, the markers producing polymorphic fragments specific to 'TifEagle' and 'Tifdwarf' only occurred in shoot tissue. Researchers have also identified a mutating locus of increasing polymorphic fragment length among three 'Tifdwarf' accessions using SSR markers (Harris-Shultz et al. 2011). Certified 'Tifdwarf' collected from Georgia showed one additional allele when compared with 'Tifgreen', 'Champion', and 'MiniVerde', which suggested this mutation may be unique to that location. 'Champion' and 'MiniVerde' did not contain the additional 'Tifdwarf' allele; therefore, the mutation producing the additional allele occurred after the mutations that led to the development of those improved cultivars (Harris-Schultz et al. 2011).

Despite having variable morphology and performance, molecular techniques have not clearly distinguished every ultradwarf bermudagrass from one another, or from the cultivars from which they were derived. The ability to identify unique ultradwarf bermudagrass cultivars would facilitate the production of genetically pure planting material, although this purity verification must be performed frequently because the same pedigree stock production process that led to OT grasses will be used again. Therefore, if utilized correctly, the ability to identify unique ultradwarf bermudagrass cultivars would improve the uniformity of golf course putting greens.

Genetic Analysis of Off-Types

Phenotype assessments can identify and characterize OT grasses, but genetic and molecular techniques help explain whether these grasses are mutations or contaminations of

registered cultivars (Caetano-Anollés 1998; Caetano-Anollés et al. 1997; Harris-Shultz et al. 2010). Caetano-Anollés (1998) used DAF and ASAP to explore the genetic diversity and origin of 16 OT grasses present in established 'Tifgreen' and 'Tifdwarf' putting greens on golf courses in the southern U.S., Hawaii, and Guam. Unweighted pair group cluster analysis and principal coordinate analysis revealed that eight OT grasses were genetically distinct, but similar to 'Tifgreen', meaning they were most likely the result of somatic mutations. The remaining eight OT grasses yielded genetic distances that were greater than or equal to the differences among the 'Tifgreen' accessions, suggesting they were the result of sod contamination, which is similar to previous reports in 'Tifway' (Caetano-Anollés 1997, 1998). The researchers concluded that the presence of OT grasses in the field were the result of both contaminations as well as somatic mutations (Caetano-Anollés 1998).

Similarly to Caetano-Anollés (1998), Harris-Shultz et al. (2010) used EST-SSR makers to identify OT grasses selected from 'Tifdwarf' and 'MiniVerde'. The EST-SSR markers were successful in identifying whether OT grasses were genetically similar to 'Tifgreen' (i.e., somatic mutation) or to other cultivars not readily used on golf course putting greens (i.e., contamination) (Harris-Shultz et al. 2010).

Arbitrary primed polymorphic DNA was also used to examine the genetic relationship between 'Tifdwarf' and a single OT. The amplified products of 'Tifdwarf' and the corresponding OT sample resulted in a 23% difference between the two selections, which suggested that these grasses were genetically similar despite having variable morphology (Ho et al. 1997). The amount of genetic similarity reported by Ho et al. (1997) in combination with the results of Caetano-Anollés (1998) and Harris-Shultz et al. (2010), suggest the OT studied by Ho et al. (1997) was a somatic mutation of 'Tifdwarf'.

OT grasses resulting from somatic mutations of 'Tifgreen' or any 'Tifgreen'-derived cultivar cannot currently be distinguished from that mutation family by molecular techniques alone; therefore, these OT grasses cannot be directly linked to parent cultivars such as 'Champion', 'MiniVerde', and 'TifEagle' that are mutant selections from within the 'Tifgreen' family as well. New molecular techniques such as genotyping-by-sequencing (GBS) have the potential to relate OT grasses to their parent cultivars within the 'Tifgreen' mutation family because OT grasses with multiple mutational generations have a decreased certainty of heritage. Information of this nature would further assist in explanation of the origin of OT grasses in 'Tifgreen'-derived cultivar nurseries and putting greens.

Advances in Molecular Marker Technology for Evaluating Bermudagrasses

Single nucleotide polymorphisms (SNPs) are mutations that occur between the genomes of related organisms and are commonly used as molecular markers for genetic research (Fiedler et al. 2015; Mammadov et al. 2012; Vignal et al. 2002; Wang et al. 1998; Yang et al. 2010). Genotyping-by-sequencing described by Elshire et al. (2011) can produce thousands of SNPs, which may be more capable of elucidating differences among bermudagrass cultivars within the 'Tifgreen' mutation family (Elshire et al. 2011; Poland et al. 2012; Poland and Rife 2012). Fiedler et al. (2015) and Poland and Rife (2012) suggested that GBS offers the potential to identify sets of closely linked loci that contribute to phenotypic variation. The ability to connect phenotype to genotype is of great value to researchers in order to gain a better understanding of the development and progression of bermudagrass cultivars used on golf course putting greens. The connection of phenotype to genotype also has the potential to benefit the development of new cultivars through conventional breeding techniques.

Elshire et al. (2011) stated that GBS may identify important regions of an organism's genome that are inaccessible to other molecular marker techniques. For example, Fiedler et al. (2015) used GBS to identify markers in many regions of the switchgrass (*Panicum virgatum*) genome not previously identified by SSR makers. These previously inaccessible areas of a genome are possibly regions of non-coding DNA (Elshire et al. 2011). Elshire et al. (2011) suggested these non-coding, regulatory regions control the expression of plant genes responsible for agronomically important phenotypic traits. The ability of GBS to identify these regions of DNA could help researchers develop molecular markers able to identify genetically similar bermudagrass cultivars and OT grasses in the Tifgreen family.

The GBS approach is also beneficial because a reference genome can be developed from only the genomic areas utilized in the procedure (Elshire et al. 2011). This would benefit researchers studying bermudagrass because a fully sequenced reference genome has not been published. Poland and Rife (2012) suggest that a well-defined reference genome in combination with GBS data makes the development of genetic maps exceptionally straightforward.

Future Insights on the Management of Off-type Grasses

Phenotypic variability of bermudagrass cultivars on putting greens began to be recognized soon after the release of 'Tifgreen' in 1956 and continues to be problematic in ultradwarf greens today. The broad term to describe matrix cultivar variability is "contaminated greens" which includes plants of unrelated OT grasses from green surrounds, fairways, and production nurseries as well as OT grasses related to the matrix cultivar established on the putting green. Off-type grasses related to the matrix cultivar occur as somatic mutations in both production nurseries and putting greens. When putting greens are established with 'Tifgreen', 'Tifdwarf', or cultivars with similar morphology, contamination can result from planting stolons

infested with matrix cultivar OT grasses as well as from *de novo* mutations occurring within the putting green. After several years of putting green management, these greens can typically result in significant contamination even if they were initially established with morphologically uniform planting material (J.E. Elsner, unpublished observations, 2015). In contrast, ultradwarf bermudagrass greens have the potential to maintain morphological uniform for many years even though production nurseries have similar mutation frequencies as 'Tifgreen' and 'Tifdwarf' nurseries (J.E. Elsner, unpublished observations, 2015). It has been estimated that the frequency of somatic mutations in ultradwarf production nurseries exceeds three phenotypically different OT grasses per hectare per year (Harris-Shultz et. al.2010, Caetano-Anollés 1998, Ho et al. 1997, J. E. Elsner, unpublished observations, 2015). Maintaining genetic purity in a production nursery is challenging because field conditions that allow for profitable production often contrast with management practices that facilitate the identification of OT grasses through regular inspection. Variation in mowing height, fertility, and irrigation are management tools used to enhance OT identification.

Off-type grasses must be eradicated from the desirable cultivar before they can expand and be spread across the nursery through cultivation or harvesting procedures. The difficulty in rouging and eradicating off-types in nursery production is likely due to the phenotypic similarities between OT grasses and commercial cultivars under commonly used nursery management practices. In the event that OT grasses escape detection and are widely spread during the establishment of new golf greens, the perceived rate and impact of mutation is much higher than on greens planted with morphologically uniform sprigs, which can slowly accumulate somatic mutants over years and decades (J.E. Elsner, unpublished observation, 2015).

Several cultivars are now currently off patent and the proprietary protection offered by a U.S. Plant Patent is no longer present. These off patent cultivars have the potential to move into the public domain, presenting more difficulties with respect to keeping pedigree stock material off-type free. Use of a cultivar at more production sites makes off-type rouging more difficult. Additionally, lack of patent protection may reduce the sale price and profit potential; therefore, reducing economic incentive to remove OT grasses from planting stock.

Some OT bermudagrasses within 'Tifgreen' putting greens (O'Brien 2012) have exhibited larger internode and leaf lengths, as well as higher canopy height and greater turfgrass cover than commercially available bermudagrass cultivars used on putting greens (unpublished data). Off-type grasses with more aggressive, upright growth than commercial cultivars can negatively affect functional and aesthetic putting green quality. Anecdotal observations suggest management practices such as mowing frequency and height, fertilization, and chemical applications may be optimized to reduce negative effects of competitive OT grasses on putting quality. However, research is needed to define agronomic and OT management strategies and their economic feasibility for golf course putting greens to reduce the negative effects of OT grasses created from planting contaminated stolons.

Bermudagrass putting greens cover approximately 3,642 hectares across the U.S. (Lyman et al. 2007) with 70 to 80 conversions to ultradwarf bermudagrass occurring each year (Leslie 2013). 'Tifgreen'-derived cultivars are the mainstay of the warm-season golf course putting green market. They are planted worldwide in subtropical and tropical; however, genetic instability can result it phenotypically different OT grasses in putting greens that present significant challenges for golf course superintendents. Interdisciplinary research will be needed to better understand the genetic diversity and instability of bermudagrasses used on putting

greens, management strategies to reduce the deleterious effects that OT grasses pose on putting green quality and their economic feasibility of management practices as compared to putting green replacement.

CHAPTER II

Genotypic and Phenotypic Evaluation of Off-Type Grasses in Hybrid Bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] Putting Greens using Genotyping-by-Sequencing and Morphological Characterization

A version of this chapter was submitted to the Springer Transfer Desk service on February 22, 2017 by Eric H. Reasor, James T. Brosnan, Margaret E. Staton, Thomas Lane, Robert N. Trigiano, Phillip A. Wadl, Joann A. Conner, and Brian M. Schwartz.

A version of this chapter was originally submitted to The Journal of Theoretical and Applied Genetics and then Planta. The Springer Transfer Desk service is currently reviewing the manuscript to find the best journal for the research that is within the Springer journal system. My primary contributions to this paper include (i) reading literature, (ii) design and conducting experiments, (iii) analyzing and interpreting non-bioinformatics data, and (iv) writing the manuscript.

Abstract

Interspecific hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] is one of the most widely used grasses on golf courses, with cultivars derived from 'Tifgreen' or 'Tifdwarf' particularly used for putting greens in warmer climates. Many bermudagrass cultivars established for putting greens can be genetically unstable and lead to the occurrence of weedy off-type (OT) grasses that vary in phenotype. Beginning in 2013, OT and desirable hybrid bermudagrass samples were collected from golf course putting greens throughout the southeastern United States and genetically and phenotypically catalogued using genotyping-by-sequencing (GBS) and morphological characterization. Genotyping-by-sequencing determined that 11% of OT and desirable samples from putting greens were genetically divergent from standard cultivars such as Champion, MiniVerde, Tifdwarf, TifEagle, and Tifgreen. In addition, GBS was unable to genetically distinguish all standard cultivars from one another likely due to their genetic origin and clonal propagation practices; however, over

90,000 potentially informative nucleotide variants were identified among the triploid hybrid cultivars. GBS was able to differentiate triploid hybrids from diploid (*C. transvaalensis*) and tetraploid (*C. dactylon*) progenitor species and separated triploid hybrids of the 'Tifgreen'-cultivar family from those of different lineage (i.e., 'Tifway'). Although few genetic differences were found in this research, samples harvested from golf course putting greens had variable morphology and were clustered into three distinct phenotypic groups. The majority of OT grasses in hybrid bermudagrass putting greens are genetically similar with variable morphological traits leading to the potential to compromise surface functionality and aesthetics.

Introduction

Interspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] are some of the most widely utilized grasses on golf courses throughout tropical, subtropical, and temperate climates (Beard 2002). 'Tifgreen' was one of the first interspecific hybrids developed for putting green use (Burton 1964; Hein 1961). Soon after its commercial release, 'Tifdwarf' was selected from a somatic mutation in a 'Tifgreen' establishment (Burton 1966; Burton and Elsner 1965). Despite being genetically unstable (Caetano-Anollés et al. 1997; Caetano-Anollés 1998), 'Tifgreen' and 'Tifdwarf' were widely used on putting greens for 30 years until superior mutations were released as new "ultradwarf" cultivars (Reasor et al. 2016). 'Champion' and 'MiniVerde' were selected from somatic mutations in established 'Tifdwarf' plantings (Brown et al. 1997; Kaerwer and Kaerwer 2001), whereas 'TifEagle' was a putative mutant from radiation-induced 'Tifgreen' or 'Tifway II' rhizome (Hanna and Elsner 1999; Harris-Shultz et al. 2010; Zhang et al. 1999). In all cases, these hybrid ultradwarf bermudagrass cultivars were identified as off-type (OT) grasses; they had more diminutive morphology than the swards from which they were selected.

Several molecular marker methods have been used to explore genotypic differences among OT grasses and hybrid bermudagrass cultivars. DNA amplification fingerprinting (DAF) and signatures from amplification profiles identified contaminant OT grasses not related to the 'Tifgreen' family, but could not distinguish mutant OT grasses within the 'Tifgreen' family (Caetano-Anollés 1998). Amplified fragment length polymorphisms (AFLPs) studies determined the genetic diversity among several 'Tifgreen'-derived cultivars. Studies utilizing AFLPs grouped 'Tifgreen', 'Tifdwarf', 'TifEagle', and 'Champion' into the same genetic cluster despite differences in phenotypic characteristics among the grasses (Capo-chichi et al. 2005;

Chen et al. 2009; Zhang et al. 1999). Attempts to use various simple-sequence repeats (SSRs) to identify 'Tifgreen'-derived hybrid bermudagrass cultivars were also met with limited success (Harris-Shultz et al. 2011; Kamps et al. 2011). While SSRs identified 'TifEagle' from other 'Tifgreen'-derived cultivars and identified polymorphisms unique to 'Tifdwarf' and 'MiniVerde' (Harris-Shultz et al. 2011; Kamps et al. 2011), SSRs are not able to readily distinguish all 'Tifgreen'-derived hybrid bermudagrass cultivars from one another nor have they been able to identify OT grasses from standard cultivars used on golf course putting greens.

Morphological characterization has been considered a traditional method of studying turfgrass classification and diversity (Bonos et al. 2002; Hitchcock 1971; Kang et al. 2008; Romani et al. 2004). Morphological characteristics such as internode length, leaf length, leaf width, and stolon diameter are of particular interest for classification of bermudagrasses (Kang et al. 2008; Kenworthy et al. 2006; Romani et al. 2004) because differences in morphology can determine OT grasses from desirable cultivars (Caetano-Anollés 1998; Caetano-Anollés et al. 1997). Additionally, Roche and Loch (2005) stated that morphological characterization could provide useful information to further research for adaptation and management of different hybrid bermudagrasses. Researchers have used morphological characterizations to compare hybrid bermudagrass cultivars within the 'Tifgreen' family (Magni et al. 2014; Roche and Loch 2005); however, inconsistent responses among studies suggest that molecular techniques are also needed to accurately evaluate hybrid bermudagrass diversity (Reasor et al. 2016).

Genotyping-by-sequencing (GBS) is a high-throughput, next generation sequencing method capable of generating large numbers of single nucleotide polymorphism (SNPs) from species with high diversity (Elshire et al. 2011). Genotyping-by-sequencing offers several advantages over other molecular marker techniques including the amount of data generated and

the price per sample (Elshire et al. 2011; Fiedler et al. 2015). Moreover, GBS allows analysis of a species (e.g., hybrid bermudagrass) for which a complete reference genome sequence is not available. Fiedler et al. (2015) identified over 4,600 high-quality SNPs in switchgrass (*Panicum virgatum*) using an early draft genome assembly with only half of the assembled DNA contigs scaffolded. Poland et al. (2012) used GBS to map over 34,000 SNPs for the Oregon Wolfe Barley (*Hordeum vulgare*) reference population and 20,000 SNPs for the Synthetic W9784xOpata85 wheat (*Triticum aestivum*) reference population, both of which lacked a complete reference genome sequence. Based on the robustness of the technique and successful use in other grasses without a complete reference genome (Elshire et al. 2011; Fiedler et al. 2015; Poland et al. 2012; Poland and Rife 2012), we hypothesize that GBS may be able to identify genetic variation among OT grasses and hybrid bermudagrasses used on putting greens. Therefore, our objectives were to explore the genetic and the phenotypic variation among OT grasses sampled from hybrid bermudagrass putting greens using GBS and morphological characterization.

Materials and Methods

Genotyping-by-Sequencing

Plant Material and DNA Isolation

Desirable and OT hybrid bermudagrass samples were harvested in 2013 from putting greens on golf courses in Alabama, Arkansas, Florida, Georgia, Mississippi, South Carolina, and Tennessee (Table 2.1). The golf course superintendent at each golf course determined samples that were desirable from those that were OT. Samples were harvested with a 7.5 cm diameter tubular plugger (Turf Tec International; Tallahassee, FL, USA) and established using one three node stolon planted in a 64 cm^2 pot filled with a peat moss based growing medium (Pro-Mix BX

Mycorrhizae; Premier Horticulture, Inc.; Quakertown, PA, USA) in a greenhouse environment at the University of Tennessee (Knoxville, TN, USA; 35.5°N, -83.5°W). Plants were maintained with 24 kg N ha^{-1} wk^{-1} of a water-soluble complete fertilizer (20N-8.7P-16.6K; Southern Agriculture; Hendersonville, NC, USA), irrigated to promote active growth, and insecticides (abamectin 0.01 kg ai ha^{-1}, Avid 0.15EC, Syngenta; pymetrozine 0.35 kg ai ha^{-1}, Endeavor, Syngenta) were applied on a preventive basis.

Desirable and OT samples labeled S1 to S47 in Table 2.1 were included in GBS. These OT samples were included because their corresponding desirable sample was collected from the same putting green or the golf course nursery putting green. Hybrid bermudagrass cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), Tifgreen (TG1-6), and Tifway (TW1-6)] and two selections of progenitor species [*C. dactylon* (TA1-3 and TB1-3) and *C. transvaalensis* (DA1-3 and DB1-3)] were used as standards in the GBS analysis (Table 2.1). Only three biological replicates of the progenitor species were included due to the expense of the analysis. Plant material for these standard entries was obtained from the University of Georgia Coastal Plain Experiment Station (Tifton, GA, USA).

For all samples, plant genomic DNA was isolated from actively growing leaf tissue on a single stolon using the Qiagen DNeasy Plant Mini Kit (Qiagen; Valencia, California, USA) according to the manufacturer's protocol. DNA concentration was quantified using an intercalating dye (Quant-iT™ PicoGreen dsDNA Asasy Kit; Life Technologies; Carlsbad, CA, USA). DNA working solutions for the GBS protocol had a total volume of 30 µL and a concentration ranging from 50 to 105 ng μL^{-1}.

Ploidy Analysis

Ploidy levels were confirmed for each sample included in the GBS assay using flow cytometry (Table 2.1). Fresh leaf tissue was isolated from samples and chopped using a razor in 300 µL of LB01-lysis buffer (15 mM Tris, 2mM Na_2EDTA, 0.5 mM spermine-4HCl, 80 mM KCl, 20 mM NaCl, 0.1% v/v Triton X-100 pH 7.5 and 16 mM ß-mercaptoethanol) to release nuclei. Each unknown bermudagrass (i.e., desirable or OT grass harvested from a golf course) and standard sample was combined with *Sorghum bicolar* for a standard genome size (Price et al. 2005) comparison. Samples were passed through a 30-µm filter (CellTrics; Partec; Munster, Germany) and then 150 µL of RNase and propidium iodide solution (PI/RNase Staining Buffer, BD Biosciences, San Jose, CA, USA) was added. Samples were incubated on ice for 15 minutes and analyzed on an Accuri C6 flow cytometer (BD Biosciences; San Jose, CA, USA). Gating was set by the selection of objects that exhibited a strong correlation between the FL2 and FL3 signals using a flow rate of 14 µL per minute and a minimum cell count of 10,000. The mean FL2-A peaks from the signals were determined for *S. bicolar* and each bermudagrass sample using Accuri C6 software (BD Biosciences; San Jose, CA, USA). These mean FL2-A values were then used with *S. bicolar* genome size (1.67 pg/2C) to calculate the genome size of each bermudagrass sample (Price et al. 2005).

Genotyping-by-Sequencing Analysis

Genotyping-by-sequencing was conducted at the Cornell University Institute for Biotechnology (Ithaca, NY) using the protocol described by Elshire et al. (2011). *Ape*KI restriction enzyme was selected based on optimization trials for the GBS digestion to maximize the number of sampled genomic loci (Elshire et al. 2011). Libraries for next-generation

sequencing were constructed from DNA samples and multiplexed using Illumina HiSeq 2500 and then Illumina NextSeq 500 to increase read coverage and depth.

Bioinformatics Analysis

The combined Illumina data sets were initially analyzed with the UNEAK pipeline of the Tassel software package (Bradbury et al. 2007; Glaubitz et al. 2014). One of the limitations of the UNEAK pipeline is that its nucleotide variant calling algorithm relies on a diploid model. Bermudagrasses sequenced in our GBS analysis included diploid (2n=2x=18), triploid (2n=3x=27), and tetraploid (2n=4x=36) samples; therefore, an alternative approach was used to call variants. Sequence tags with a predicted variant were extracted from the topm.bin libraries generated during the Tag-Pair-Export phase using the UNEAK Binary-to-text-plugin; then raw reads were mapped with Bowtie2 v2.2.7 to these tags generated from the UNEAK pipeline as a pseudo-reference (Langmead and Salzberg 2012).

The haplotype-based variants caller, Freebayes v1.0.2-15, was used to call nucleotide variants for each set of samples with the same ploidy level with the correct ploidy level specified with parameter p (Garrison and Marth 2012). The sorting, indexing, and merging of alignment files was performed with the SAMtools v1.3 package (Li et al. 2009). Multidimensional scaling (MDS) plots were generated from these variants using PLINK v1.9 to illustrate the variation among samples. The bermudagrass samples S19, S28, S30, S32, and S44 were not included because they had less than one million raw-reads (Purcell et al. 2007). The individual samples for each triploid cultivar were pooled to increase the read depth for each cultivar. The read alignment files were pooled using SAMtools v1.3 package (Li et al. 2009) and then the FreeBayes method of determining variants was utilized again. The pooled data was then used in a custom Python script to determine loci that differed between at least two cultivars

(github.com/statonlab/UDBG_Informative_SNPs/blob/master/find_informative_SNPs.py). Any loci with two different genotypes (homozygous for the reference allele, homozygous for the alternate allele, or heterozygous) for at least two cultivars were flagged. Loci were not flagged if heterozygosity differed between or within the two subgenomes in triploid cultivars (i.e., 0/0/1 or 0/1/1). A final level of filtering was applied to use only individual variant calls in each cultivar with a read depth of at least 40. All raw read data has been submitted to NCBI under BioProject accession PRJNA353769.

Phenotypic Evaluation

Plant Materials

Bermudagrass samples labeled S1 to S62, except for S13, S14, S19, S20, and S43 were used in phenotypic evaluation (Table 2.1). Off-type and desirable grass samples were harvested and established using methods previously described. For phenotypic evaluation, one three-node stolon of each sample was established in four 64 cm^2 pots filled with a peat moss based growing medium (Pro-Mix BX Mycorrhizae; Premier Horticulture, Inc.; Quakertown, PA, USA). The stolon length at transplant of the 52 selections ranged from 3.4 to 11.3 cm. The plants were maintained as previously described, but regular clipping was ceased two weeks prior to evaluation.

Morphological Measurements and Statistical Analysis

Phenotypic evaluation of OT and desirable samples was conducted by measuring plant morphological characteristics via methods outlined by Roche and Loch (2005). Five parameters were assessed and included internode length and stolon diameter, leaf length and width, and the leaf length:width ratio (LWR). Measurements were made between the third and fourth node and on the outer leaf from the third node using digital calipers (Digimatic Caliper, Model No.CD-6”

CX, Mitutoyo Corporation, Kawasaki-shi, Kanagawa, Japan). The experiment was a completely randomized design with pots replicated four times and morphology measured on three stolons per pot. Morphology was assessed on 3 June 2014 and repeated again on 25 June 2014.

All morphological data describing desirable and OT hybrid bermudagrass samples were analyzed using cluster analysis in SAS Enterprise Guide (Version 6.1, SAS Institute, Cary, NC, USA). K-means clustering algorithm was used to partition the data set into a user-defined number of clusters (MacQueen 1967). Three clusters were determined based on the cubic clustering criterion and the frequency of observations in each cluster (MacQueen 1967). Cluster means and standard deviations for each morphological measurement were then graphed in Prism (Prism 6 for Mac OS X; GraphPad Software, Inc.) to determine statistical differences among cluster means.

Results and Discussion

Genotyping-by-Sequencing

The total number of variants yielded from GBS analysis included single nucleotide variants, multiple nucleotide variants, and indels. An initial 1,088,920 total variants were identified with an average read depth of 4.9 for the triploid (2n=3x=27) hybrid bermudagrass samples. Genotyping-by-sequencing identified 347,512 total variants with an average read depth of 9.5 per individual for the two diploid (2n=2x=18), *C. transvaalensis* selections. For the tetraploid (2n=4x=36), *C. dactylon* samples, 587,053 total variants were identified with an average read depth of 7.4 (Figure 2.1). Only 136,205 variants were shared among diploid, triploid, and tetraploid species; therefore, the remaining variants are fixed in at least one species (Figure 2.1).

The majority of OT and desirable samples harvested from golf courses were genetically similar to the hybrid bermudagrass cultivars Champion, MiniVerde, Tifdwarf, TifEagle, and Tifgreen (Figure 2.2). Of the 47 unknown samples, only five (~11%) were genetically divergent from the standard cultivars (S4, S16, S31, S33, and S45), as illustrated by the MDS plot (Figure 2.2). Figure 2.3 is an MDS plot showing the desirable and OT samples in Figure 2.2 that are located in the box marked with an asterisk (*). These samples have been determined to be genetically similar due to their proximity and clustering. Caetano-Anollés (1998) and Caetano-Anollés et al. (1997) revealed that eight of sixteen OT grasses were genetically divergent from standard cultivars using DAF, leading researchers to conclude that OT grasses that were not genetically distinct, but were the result of somatic mutations within 'Tifgreen' and 'Tifdwarf'. The inability of GBS, as well as other molecular marker techniques, to distinguish OT grasses from hybrid bermudagrass cultivars used on putting greens could be the result of aneuploidy within the 'Tifgreen'-cultivar family (B.M. Schwartz, unpublished data, 2016; Reasor et al. 2016). It is expected that some variant locations are not going to be sampled by random chance due to the sparse nature of the GBS analysis. This is a limitation of GBS because it cannot determine presence/absence or copy number variations for individual locations that are needed to determine aneuploidy (Elshire et al. 2011).

Triploid hybrid bermudagrass cultivars Champion, MiniVerde, Tifdwarf, TifEagle, and Tifgreen were genetically similar to one another; however, GBS separated these cultivars from 'Tifway' bermudagrass (Figure 2.2). Pooling individual cultivar samples yielded a higher average read depth of 31 per variant site per cultivar. Using the pooled data, 675,578 loci were identified as different between at least two cultivars. The majority of these genotype differences were only able to differentiate cultivars within the 'Tifgreen'-cultivar family from those with

different lineage (i.e., ‘Tifway’) (Table 2.2). Despite also being a triploid hybrid, ‘Tifway’ bermudagrass has been genetically distinguished from the ‘Tifgreen’-cultivar family using SSRs (Harris-Shultz et al. 2010; Kamps et al. 2011; Wang et al. 2010) and AFLPs (Chen et al. 2009; Zhang et al. 1999). Arumuganathan et al. (1999) reported that ‘Tifway’ had less nuclear DNA content (1.37±0.01 pg/2C) than ‘Tifgreen’ (1.61±0.00 pg/2C) despite having the same number of chromosomes. Furthermore, Reasor et al. (2016) hypothesized that this difference in DNA content could also be a result of aneuploidy in the ‘Tifgreen’-cultivar family of hybrid bermudagrass, which includes the hybrid ultradwarf cultivars. This difference in DNA content could also aid in genetic identification between hybrid bermudagrasses using GBS.

The pooled data from variants among triploid cultivars still encompassed individual loci with a wide range of individual read depths. Low read depth could miss heterozygotes, whereas high read depth could indicate a repetitive region instead of an individual locus. To mitigate read depth issues, a further filter was applied to identify only the most robust variants with a read depth of at least 40, but no more than 100. Filtering using these read depths yielded 93,188 nucleotide variants between at least two genotypes (Table 2.2). However, our experiment only included six biological replications of each standard cultivar from a single geographic location (Tifton, GA). Additional research and replication of this study with more samples will be needed to ascertain which variants can be used to identify standard hybrid bermudagrass cultivars, specifically hybrid ultradwarf cultivars, from one another.

The MDS plot revealed clear clustering of the diploid and tetraploid progenitor species apart from the standard hybrid bermudagrass cultivars and the majority of OT and desirable samples harvested from putting greens (Figure 2.2). In addition to the identification of ‘Tifway’ from other triploid hybrids, the ability of GBS to distinguish diploid, triploid, and tetraploid

bermudagrasses align with previous efforts to genetically identify these grasses from one another using AFLPs (Chen et al. 2009; Zhang et al. 1999) and SSRs (Harris-Shultz et al. 2010; Kamps et al. 2011; Wang et al. 2010). The two progenitor species also clustered separately from one another with the exception of DA2 due to possible contamination during DNA isolation. The clustering demonstrated that GBS was effective for distinguishing diploid, triploid, and tetraploid bermudagrasses. The ability to distinguish among bermudagrass species is likely due to the large number of unshared variants (Fig. 2.1).

Phenotypic Evaluation

The K-means cluster algorithm yielded three clusters containing fourteen, twenty-six, and twelve bermudagrass samples, respectively. Cluster one contained nine OT and five desirable samples, cluster two had twelve OT and fourteen desirable grasses, and cluster three had eight OT and four desirable samples. The cluster analysis overall expected R^2 was 0.61 with a cubic clustering criterion of -19.36. Cluster means and standard deviations for each morphological assessment are presented in Figure 2.4. Mean internode length, leaf length, and LWR were the only statistically different morphological parameters among clusters (Figure 2.4). A representative hybrid bermudagrass sample from each cluster is illustrated in Figure 2.5.

The mean internode length for grasses in cluster one (34.6 mm) was significantly longer than the mean internode length of clusters two (21.9 mm) and three (24.7 mm) (Figure 2.4). The internode length of grasses in this experiment align with Magni et al. (2014) who reported an internode length range of 15 to 34 mm on ultradwarf hybrid bermudagrass cultivars used on putting greens. However, Roche and Loch (2005) reported internode lengths of 9.4 to 12.5 mm for hybrid bermudagrasses used on putting greens. In our experiment, mean internode length for each cluster was in the uppermost half of the internode length range reported by Magni et al.

(2014) and greater than the range measured by Roche and Loch (2005). Internode lengths measured in this experiment varied greatly among desirable and OT grasses as well as grasses measured in other experiments. This is an indication of the amount of phenotypic variability that can occur in individual putting greens as well as from golf course-to-golf course and cultivar-to-cultivar. Differences in internode length within the same putting green can lead to decreased turfgrass density and reductions in putting green quality and playability (Reasor et al. 2016).

Morphological cluster three had a significantly longer mean leaf length than clusters one and two (Figure 2.4). The leaf length mean for cluster three was 29.8 mm, compared to 14.9 and 9.9 mm for clusters one and two, respectively. This relationship was also present in LWR among clusters. Similar to the internode length data, leaf length values (and subsequently LWR values) documented in our experiment were far greater than those reported by Roche and Loch (2005). Mean stolon diameter among clusters ranged from 0.7 to 0.8 mm and mean leaf width ranged from 2.0 to 2.2 mm, with no statistical differences present among clusters for either parameter. Stolon diameter and leaf width values were similar to those reported by Roche and Loch (2005), but less than those reported by Magni et al. (2014).

Conclusions

Off-type grasses reported to have phenotypic differences from that of standard hybrid bermudagrass cultivars were sampled from golf course putting greens and subjected to GBS and morphological characterization under controlled growth conditions. Genotyping-by-sequencing only distinguished five OT grasses from standard hybrid bermudagrass cultivars. In addition, GBS failed to completely distinguish standard hybrid bermudagrass cultivars from one another, including ‘Champion’, ‘MiniVerde’, ‘Tifdwarf’, ‘TifEagle’, and ‘Tifgreen’. The final bioinformatics analysis did yield 93,188 variants that offer the potential to be useful in

distinguishing standard cultivars from one another; however, additional research beyond the scope of this project would be needed to determine which ones are diagnostic. Genotyping-by-sequencing was successful in determining triploid hybrid bermudagrass cultivars from diploid and tetraploid progenitor species. Additionally, GBS was also successful in determining triploid hybrid bermudagrass cultivars with lineage to 'Tifgreen' from those not developed from 'Tifgreen' (e.g., 'Tifway'). Morphological characteristics varied among sampled grasses that allowed them to be clustered into three distinct phenotypic groups varying predominately in internode and leaf length.

It is not clear why the majority of grasses included in our experiment exhibited variable morphological characteristics while being similar in genotype. Aneuploidy could be a reason for this because GBS cannot determine chromosome number. Differential gene expression may also be a possible explanation for the genetic similarities among hybrid bermudagrass samples varying in phenotype. Multiple genes control important turfgrass traits and gene expression can be greatly influenced by environment or management practices (Fei 2008). Golf course putting greens are intensely managed surfaces subjected to daily mowing (often at heights of cut ≤ 3 mm), annual aerification and cultivation, as well as treatment with plant growth regulators and silica sand topdressing on a weekly basis. Any of these practices could up or down regulate genes associated with hybrid bermudagrass phenotypic characteristics; however, no research has been conducted on this effect. Studying changes in gene expression as a result of these maintenance practices could benefit researchers and industry practitioners through an increased understanding of how putting green management could potentially lead to the occurrence of phenotypically different off-type grasses in hybrid bermudagrass putting greens.

CHAPTER III

Response of Ultradwarf Hybrid Bermudagrass Cultivars [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] and Off-Type Grasses to Applications of Nitrogen and Trinexapac-Ethyl

Abstract

Hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] cultivars Champion, MiniVerde, and TifEagle are commonly established on golf course putting greens in warmer climates. These cultivars were selected as desirable off-type (OT) grasses due to differences in morphology and performance; however, many putting greens are infested with undesirable OT grasses. The objective of this study was to compare the response of 'Champion', 'MiniVerde', 'TifEagle' and three OT grasses (OTC1, OTC2, and OTC3) to applications of nitrogen (N) and trinexapac-ethyl (TE). Nitrogen rates included 0, 6, 12, 18, 24, or 48 kg N ha^{-1} wk^{-1} and TE rates included 0, 1.6, 3.3, 6.6, 13.1, 26.3, 52.6, or 105.2 g a.i. ha^{-1}. Clippings were harvested 7, 14, 21, and 28 days after initial treatment (DAIT). N rates of 0, 6, and 12 kg N ha^{-1} wk^{-1} decreased weekly clipping production 29, 17, and 18%, respectively. Rates greater than 18 kg N ha^{-1} wk^{-1} were required to maintain levels of acceptable growth. The majority of TE rates resulted in peak growth regulation at 21 DAIT with the largest change in percent clipping production occurring from 21 to 28 DAIT. Peak regulation at 21 DAIT yielded 26 to 39% reduction in clipping weights from clipping weights measured at 7 DAIT. OTC1 produced significantly more clippings than 'Champion', 'MiniVerde', OTC2, and OTC3 in response to TE. Adequate N, balanced TE applications, and consideration of OT grass morphology are critical for ultradwarf bermudagrass putting green management with OT grass infestations.

Introduction

Interspecific hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] is commonly established on golf course putting greens in warmer climates (Beard 2002). The majority of hybrid bermudagrass putting greens are established with the cultivars 'Champion', 'MiniVerde', and 'TifEagle' (Reasor et al. 2016) that were selected from mutations of 'Tifgreen' or 'Tifdwarf' (Brown et al. 1997; Harris-Shultz et al. 2010; Kaerwer and Kaerwer 2001; Reasor et al. 2016; Zhang et al. 1999). Genetic instability in Tifgreen, Tifdwarf, as well as Champion, MiniVerde, and TifEagle (Reasor et al. 2016) has led to the occurrence of off-type (OT) grasses in putting greens planted with these cultivars (Caetano-Anollés et al. 1997; Caetano-Anollés 1998) (Figure A). Caetano-Anollés et al. (1997) and Caetano-Anollés (1998) defined OT grasses as those with different morphology and performance when compared to the surrounding, desirable cultivar.

Nitrogen (N) fertilizer applications are routine in hybrid bermudagrass putting green management (McCullough et al. 2006a). McCarty et al. (2005) reported that hybrid bermudagrass greens are supplied with annual N rates of 390 to 1170 kg ha^{-1} due to nutrient losses through regular defoliation. However, N rates of this magnitude may produce excessive turfgrass shoot growth capable of disrupting surface uniformity and putting quality (McCullough et al. 2006a). Several research studies have measured various turfgrass responses (e.g., quality, color, rooting, and nutrient retention) on hybrid bermudagrass putting greens treated with different rates of N (Goatley et al. 1994; Hollingsworth et al. 2005; Tucker et al. 2006), with others exploring how different N rates affect shoot growth (McCullough et al. 2006b; Baldwin et al. 2009). McCullough et al. (2006b) reported a positive quadratic response in clipping yield on TifEagle treated with N rates of 96 to 384 kg ha^{-1} yr^{-1}. In contrast, Baldwin et al. (2009) reported

a positive linear response in clipping yield on Champion treated with N rates of 147 to 440 kg ha^{-1} yr^{-1}. Increasing N rates from 6 to 24 kg N ha^{-1} wk^{-1} also led to a 9 to 12% reduction in ball roll distance on TifEagle, which was attributed this reduction to increased friction created by greater shoot growth and wider leaves (McCullough et al. 2006a).

Plant growth regulator (PGR) applications are also common practice in managing hybrid bermudagrass putting greens (McCullough et al. 2006a). Trinexapac-ethyl (TE) {[4-cyclopropyl-[α]-hydroxymethylene)-3,5-dioxo-cyclohexane carboxylic acid ethyl ester]} is a frequently used PGR that is shoot absorbed to inhibit the production of active gibberellic acid (GA) by blocking the late 3β-hydroxylation conversion reaction of GA_{20} to GA_1 (Fagerness et al. 2000; Watschke and DiPaola 1995). The purpose of a PGR application is to reduce rapid shoot growth that could potentially disrupt surface smoothness and overall putting quality (McCullough et al. 2006; Fagerness et al. 2000).

Applications of TE at 0.02 kg a.i. ha^{-1} 2 wk^{-1} on Champion reduced clipping yield 48% compared to non-TE treated Champion two weeks after the final application (Baldwin et al. 2009). McCarty et al. (2011) and McCullough et al. (2006b) reported a 32 to 46% and 67% clipping yield reduction on TifEagle treated with TE at 0.0175 kg a.i. ha^{-1} 2 wk^{-1} and 0.05 kg a.i. ha^{-1} 3 wk^{-1}, respectively, compared to non-treated plots. Moreover, the 32 to 46% clipping reduction with 0.0175 kg a.i. ha^{-1} 2 wk^{-1} resulted in a five to ten cm increase in ball roll distance (McCarty et al. 2011). This increased ball roll distance as a result of TE application may be insignificant due to the average golfer only detecting differences on putting green ball roll distances that vary greater than 15 cm (Karcher et al. 2001). McCullough et al. (2006a) also reported a similar increase in ball roll distance on TifEagle treated with TE at 0.05 kg a.i. ha^{-1} 3 wk^{-1}. The aforementioned studies were conducted on a single cultivar established in field

conditions; however, McCullough et al. (2005) examined the response of six bermudagrass cultivars to applications of TE at 0.0125 kg a.i. ha^{-1} 10 d^{-1}. Applications of TE significantly reduced clipping yield of Champion, 'Floradwarf', MiniVerde, 'MS-Supreme', Tifdwarf, and TifEagle compared to non-treated plots with MiniVerde being affected the greatest (69% reduction) and TifEagle the least (46% reduction) (McCullough et al. 2005). Different responses to TE applications among morphologically different, but genetically similar, hybrid bermudagrass cultivars suggest that undesirable OT grasses may also respond differently.

Roche and Loch (2005) reported morphological and growth differences among Champion, Floradwarf, MiniVerde, MS-Supreme, Tifdwarf, and TifEagle. Off-type grasses in bermudagrass putting greens were also reported to have different morphology and growth characteristics compared to commercial cultivars (See Chapter II). Off-type grasses with different morphology and growth characteristics have the potential to disrupt putting quality (Reasor et al. 2016), but little is known about OT responses to different N and TE rates. Our hypothesis was that desirable and OT grasses respond differently to applications of N and TE. Knowledge of how OT grasses respond N and TE applications may help golf course superintendents manage infested ultradwarf putting greens. Therefore, the objective of this study was to compare the response of three commercial hybrid bermudagrass cultivars and three OT grasses to six N and eight TE rates.

Materials and Methods

Plant Materials and Maintenance

Samples of hybrid bermudagrass cultivars Champion, MiniVerde, and TifEagle were obtained from the University of Georgia Coastal Plain Experiment Station (Tifton, GA). The three OT grasses (OTC1, OTC2, and OTC3) used in this study were selected based on the results

of a cluster analysis performed on the morphology of OT and desirable bermudagrasses sampled from golf course putting greens. The cluster analysis is detailed in Chapter II, but it is summarized below.

Desirable and OT hybrid bermudagrass samples were harvested in 2013 from putting greens on golf courses in Arkansas, Alabama, Florida, Georgia, Mississippi, South Carolina, and Tennessee. The golf course superintendent at each golf course determined which samples classified as desirable from those that delineated as OT. Morphological characterization of OT and desirable samples harvested from golf course putting greens was conducted by measuring plant morphological characteristics via methods similar to Roche and Loch (2005). Five total parameters were assessed including internode length, internode diameter, leaf length, leaf width, and leaf length:width ratio (LWR). A cluster analysis performed on all morphological data yielded three distinct clusters (Figure 2.4). One OT sample from each cluster was included in this study and hereafter referred to as cluster one (OTC1), cluster two (OTC2), and cluster three (OTC3). Cluster one mean internode length was significantly longer than clusters two and three; however, cluster three had a significantly longer mean leaf length and leaf length:width than the other two clusters (Figure 2.4). Ploidy analysis also confirmed that the desirable hybrid bermudagrass cultivars and OT grasses used in this study were all triploid (2n=3x=27) (Table 2.1).

Hybrid bermudagrass cultivars and OT grasses were planted into 656 cm^3 cone-shaped containers (Stuewe and Sons, Inc.; Tangent, Oregon, USA) filled with a sand-based growing medium in a greenhouse environment at the University of Tennessee (Knoxville, TN; 35.5°N, -83.5°W). The containers had a surface area of 37 cm^2 and a single three-node sprig of each cultivar and OT grass was transplanted into the center of the container. Transplanting of the

sprigs occurred from 27 to 31 August 2015. The growing medium was mixed with 80% vol vol^{-1} of silica sand that met United States Golf Association particle size recommendations (Anonymous 2004) and 20% vol vol^{-1} of two mm sieved peat moss (Pro-Mix BX Mycorrhizae; Fafard, Inc.). Plants were maintained at a daily mowing height of one cm and treated with 24 kg N ha^{-1} wk^{-1} using a water-soluble complete fertilizer (20N-8.7P-16.6K, Southern Agriculture, Hendersonville, NC). Insecticides (abamectin, pymetrozine, cyfluthrin, and imidacloprid) were applied on a preventative basis and all plants were irrigated daily with approximately 1.3 cm of irrigation.

Nitrogen Rate Response

Three desirable hybrid bermudagrass cultivars and three OT grasses were treated with 0, 6, 12, 18, 24, or 48 kg N ha^{-1} wk^{-1} for four consecutive weeks. The experiment ran from 2 June through 30 June 2016 and was repeated from 16 August to 14 September 2016. Average greenhouse daily maximum/minimum temperatures measured 32/26°C and maximum/minimum photosynthetic photon flux densities measured 1544/0.1 μmol m^{-2} sec^{-1} during the course of the experiment. N fertilization for plant maintenance ceased 14 days prior to the initial N treatment. N treatments were supplied by dissolving urea (46-0-0 micro-prill, PCS Sales USA Inc., Northbrook, IL, USA) in distilled water and the resultant solution was then applied to the soil surface of each container using a pipette. All other plant essential nutrients were supplied using a full-strength, N deficient, Hoagland solution via a pipette (D.A. Kopsell, personal communication, 2016). Daily clipping was suspended at the time of the initial N treatment application. Growth above one cm was harvested 7, 14, 21, and 28 days after initial treatment (DAIT) and then oven dried (Model LR-271C; Greive Corporation, Round Lake, IL) at 100°C four days to quantify weekly clipping production in response to N treatment.

Trinexapac-ethyl Rate Response

Three desirable hybrid bermudagrass cultivars and three OT grasses were treated with TE (Primo MAXX, Syngenta Crop Protection, LLC., Greensboro, NC, USA) at 0, 1.6, 3.3, 6.6, 13.1, 26.3, 52.6, or 105.2 g a.i. ha^{-1} using an enclosed spray chamber (DeVries Manufacturing, Hollandale, MN) calibrated to deliver 280 L ha^{-1} at 276 kPa using an 8002EVS nozzle (TeeJet, Wheaton, IL). These rates represent 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, or 4 times the labeled rate of TE for use on ultradwarf hybrid bermudagrass putting greens (26.3 g a.i. ha^{-1}). The experiment ran from 1 June through 29 June 2016 and was repeated from 11 August to 9 September 2016. Average greenhouse daily maximum/minimum temperatures measured 32/26°C and maximum/minimum photosynthetic photon flux densities measured 1544/0.1 μmol m^{-2} sec^{-1} during the course of the experiment. All plants in this TE received regular nutrition and irrigation as previously described. Daily clipping was suspended at the time of TE application. Growth above one cm was harvested 7, 14, 21, and 28 DAIT and then oven dried (Model LR-271C; Greive Corporation, Round Lake, IL) at 100°C four days to quantify weekly clipping production.

Experimental Design and Statistical Analysis

Both rate response experiments were a completely randomized design with main effects of grass sample (i.e., cultivar/OT) and N or TE treatments in a factorial treatment arrangement. All treatments were replicated three times and both experiments were repeated in time. An additive blocking factor was included in the model to partition variability among experimental units and across runs (Oehlert, 2010). Variation due to time after treatment (TAT) was accounted for by analyzing TAT as a repeated measure (i.e., 7, 14, 21, and 28 DAIT) (Oehlert 2010). Clipping weights (mg cm^{-2}) were natural logarithm transformed due to non-constant

variance and then a linear-mixed model was fit to the transformed clipping weight data using the *nlme* package (v3.1-128) in R (v3.3.2; R Core Team; Vienna, Austria). Analysis of variance of the linear-mixed model factors was performed in R to determine significant factors affecting clipping production in response to N or TE treatment (α=0.05). Transformed clipping weight means were used in further comparative analysis to avoid back-transformation bias.

Means of significant interactions were plotted in Prism software (Prism 6.0 for Mac OSX. GraphPad Software. La Jolla, CA). Pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment were performed on means of each significant interaction and main effect in R at $\alpha = 0.05$. All data and analysis code from these experiments are publically available at the following link:

figshare.com/projects/Response_of_Bermudagrass_Off-Types_and_Cultivars_to_Nitrogen_and_Trinexapac-ethyl_Applications/19324.

Results and Discussion

Nitrogen Rate Response

The blocking factor, rate, and TAT were significant main effects in the N rate response experiment (Table 3.1). Grass sample by N rate interaction was not significant suggesting desirable and OT grasses responded similarly to N; however, the N rate by TAT interaction was significant (Table 3.1). Mean clipping weights [ln(mg cm^{-2})] for 7, 14, 21, and 28 DAIT are plotted in Figure 3.1 and pairwise comparisons yielded significant differences among N rates at 7, 14, and 28 DAIT.

Weekly clipping production decreased an average of ~29, 17, and 18% for 0, 6, and 12 kg N ha^{-1} wk^{-1}, respectively; whereas, 18 and 24 kg N ha^{-1} wk^{-1} increased clipping production by 4 and 8%, respectively. Interestingly, 48 kg N ha^{-1} wk^{-1} yielded no change in average weekly

clipping production. McCullough et al. (2006b) and Baldwin et al. (2009) both reported positive quadratic and linear responses to increasing N rates on Champion and TifEagle bermudagrass in a field setting. Conversely, the results of this greenhouse experiment suggest that rates above 18 kg N ha^{-1} wk^{-1} do not cause vast increases in clipping production; however, greater rates of N (i.e., 18, 24, 48 kg N ha^{-1} wk^{-1}) are required for sustained and consistent growth. The direct application of these results for putting green management is not clear due to the potential differences between plant culture in a greenhouse compared to the field. It is also expected that ultradwarf bermudagrasses maintained at one cm in a greenhouse would perform differently compared to plants maintained at putting green mowing heights (i.e., < 5 mm). Bush et al. (2000) reported an average of 3% increase in clipping production of common carpetgrass (*Axonopus affinis* Chase) when mowed at 3.8 cm compared to 7.6 and fertilized with 0 to 196 kg N ha^{-1} yr^{-1}. This was the first research on off-type responses to N applications; however, field research is warranted to support the results of this experiment.

Trinexapac-ethyl Rate Response

The TE rate response experiment had similar results as the N rate response experiment, with the addition of a significant grass sample main effect (Table 3.1). Grass sample by TE rate interaction was also not significant. The mean clipping weights [ln(mg cm^{-2})] for 7, 14, 21, and 28 DAIT are plotted in Figure 3.2 and pairwise comparisons yielded significant differences among TE rates at 7, 14, and 28 DAIT. On each date that significant differences were detected, mean clipping weights with TE at 26.3 g a.i. ha^{-1} were not significantly different from those measured with TE at 52.6 or 105.2 g a.i. ha^{-1}. These results suggest that under the conditions of our experiment there was not a benefit to applying TE at rates greater than 26.3 g a.i. ha^{-1} in a single application; however, field validation of this response is warranted.

Peak growth regulation occurred 21 DAIT for TE rates of 3.3, 13.1, 26.3, 52.6, and 105.2 g a.i. ha^{-1}, which corresponded with a 26 to 39% reduction in clipping weights from 7 to 21 DAIT (Figure 3.2). Additionally, mean clipping weights for OTC1 decreased 18% over the same period, whereas Champion, TifEagle, OTC2, MiniVerde, and OTC3 decreased 35, 34, 30, 20, and 20%, respectively. Applications of TE were reported to reduce clipping yield 32 to 69% on Champion, Floradwarf, MiniVerde, MS-Supreme, Tifdwarf, and TifEagle (Baldwin et al. 2009; McCarty et al. 2011; McCullough et al. 2006b; McCullough et al. 2005). The lower amount of regulation measured in this experiment may be due to TE being applied singly in a greenhouse while other researchers have focused on sequential application programs in the field. Kreuser and Soldat (2011) stated that frequent applications of TE without altering application rate could increase the amount of growth suppression on putting greens. TE was applied singly in our experiments to detect differences in sensitivity among commercial cultivars and OT grasses across a wide rate range under controlled conditions.

Increases in clipping production (27 to 75%) were observed 21 to 28 DAIT for the majority of TE rates (Figure 3.2). Clipping production increased 52 to 75% for TE rates < 52.6 g a.i. ha^{-1} compared to only 27 and 28% for 52.6 and 105.2 g a.i. ha^{-1}, respectively. Additionally, mean clipping weights for Champion and OTC3 increased 38 and 47%, respectively, whereas clipping production for the other four grasses increased 18 to 26% from 21 to 28 DAIT. This response, often termed a "rebound effect," has been attributed to increased GA_{20} and total non-structural carbohydrates after TE is metabolized within the plant (Ervin et al. 2008; Kreuser and Soldat 2011). This phase of growth following regulation has been observed in Kentucky bluegrass (*Poa pratensis* L.) and creeping bentgrass (*Agrostis stolonifera* L.) (Beasley and

Branham 2005); however, reports of this on ultradwarf hybrid bermudagrasses and off-type grasses are limited.

Proper timing of PGR application keeps plants in the "suppression phase" and avoids the "rebound phase", thus resulting in consistent, season-long growth regulation (Kreuser and Soldat 2011). Kreuser and Soldat (2011) developed a growing degree-day (GDD) model for timing TE applications on creeping bentgrass putting greens based on estimating TE metabolism. The development and use of a GDD model for ultradwarf bermudagrass cultivars would benefit OT management in putting greens due to increased accuracy of TE applications leading to more consistent growth regulation of OT grasses and desirable cultivars. However, more research is needed to develop this model, because hybrid bermudagrass and creeping bentgrass response to TE likely vary.

The main effect of grass sample was significant in the TE rate response experiment (Table 3.1). Table 3.2 outlines the results of the pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment among grass samples across all TE rates. Mean clipping weight [ln(mg cm^{-2})] for OTC1 was significantly greater than the mean clipping weights for Champion, MiniVerde, OTC2, and OTC3 (Table 3.2). OTC1 (longer internode length phenotype) produced 27, 24, 24, and 21% more clippings than MiniVerde, OTC2, Champion, and OTC3, respectively, in response to TE rates ranging from 0 to 105.2 g a.i. ha^{-1}. In addition, OTC1 produced 16% more clippings than 'TifEagle'; however, this response was not statistically significant (Table 3.2). Figures 2.2 and 2.3 suggest that the three OT grasses and three commercial cultivars are genetically similar despite exhibiting differential responses to applications of TE in this study, similar to McCullough et al. (2005). Off-type grasses present in putting greens similar to OTC1 have the potential to disrupt the functional and aesthetic

characteristics due to differential susceptibility to TE. However, the results of this experiment suggest that this effect may be more muted in TifEagle putting greens.

Conclusions

Morphologically different OT grasses are commonly present in ultradwarf bermudagrass putting greens. Nitrogen and TE applications are typical management practices on ultradwarf bermudagrass putting greens that may impact growth and management of OT grasses. In the current study, N rates $\geq$ 18 kg ha^{-1} wk^{-1} increased clipping production 0 to 8% over a four week time period in the greenhouse; however few differences among desirable cultivars and OTs were observed. Data collected in this study suggest no benefit to applying TE at rates above the maximum-labeled use rate of 26.3 g a.i. ha^{-1}. Moreover, we observed that peak growth regulation occurred 21 DAIT for the majority of TE rates tested. Peak growth regulation varied among grasses due to clipping weights decreasing 18 to 35% 7 to 21 DAIT. Across all TE rates tested, we also observed a period of increased clipping production 18 to 47% from 21 to 28 DAIT for all grasses. Trinexapac-ethyl sensitivity and metabolism differences between cultivars and OT grasses could potentially worsen the issue with OT grasses in ultradwarf putting greens, but field research is needed to explore this hypothesis. It is important to maintain consistent growth among phenotypically different grasses in order to minimize any competitive growth advantage an OT grass may possess over a desirable cultivar; however, the greenhouse culture of this experiment may limit the application of these results to golf course superintendents managing OT infestations in ultradwarf bermudagrass putting greens.

CONCLUSIONS

Off-type (OT) grasses have been identified in hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting greens since the release of 'Tifgreen' and 'Tifdwarf' and they continue to be problematic in ultradwarf bermudagrass (e.g., 'Champion', 'MiniVerde', and 'TifEagle') putting greens. Identifying, rouging, and eradicating OT grasses in nursery production is challenging for many reasons. Phenotypic similarities between OT grasses and commercial cultivars under typical nursery management practices can impede identification. In addition, field maintenance practices to facilitate profitable production are not often aligned with those that facilitate identification of OT grasses. It is likely that OT grasses escape detection during production, expand and spread across a nursery, and then can be widely spread during the establishment of new putting greens.

Off-type grasses sampled from ultradwarf bermudagrass putting greens were genetically evaluated using genotyping-by-sequencing (GBS), which only distinguished five OT grasses from standard hybrid bermudagrass cultivars. The analysis also failed to completely distinguish standard hybrid bermudagrass cultivars such as Champion, MiniVerde, Tifdwarf, TifEagle, and Tifgreen from one another. However, the final bioinformatics analysis did yield 93,188 nucleotide variants that offer the potential to be useful in distinguishing among these standard cultivars. Additional research beyond the scope of this project would be needed to determine which nucleotide variants are diagnostic. Genotyping-by-sequencing was successful in determining triploid hybrid bermudagrass cultivars from diploid and tetraploid progenitor species. Additionally, GBS was also successful in determining triploid hybrid bermudagrass cultivars with lineage to 'Tifgreen' from those not developed from 'Tifgreen' (e.g., 'Tifway'). Despite being classified as genetically similar using GBS, morphological characteristics varied

among OT grasses, which allowed them to be clustered into three distinct phenotypic groups varying in internode length and leaf length.

The response of three OT grasses (one from each morphological cluster) and three desirable cultivars (Champion, MiniVerde, and TifEagle) to six increasing nitrogen (N) and eight increasing trinexapac-ethyl (TE) rates was evaluated in greenhouse studies. The least three N rates (0, 6, and 12 kg N ha^{-1} wk^{-1}) decreased weekly clipping production 18 to 29%, whereas N rates $\geq$ 18 kg N ha^{-1} wk^{-1} increased clipping production 0 to 8% throughout a four week time period; however, few significant differences were detected among desirable cultivars and OT grasses. The TE rate response experiment had similar results as the N rate response experiment, with the addition of a significant grass sample main effect. We observed that peak growth regulation occurred 21 DAIT for the majority of TE rates tested where clipping weights decreased 18 to 35% from 7 to 21 DAIT. We also observed a period of increased clipping production 18 to 47% from 21 to 28 DAIT for all grasses tested. Trinexapac-ethyl sensitivity and metabolism differences between cultivars and OT grasses could potentially worsen the issue in ultradwarf putting greens, but field research is needed to further explore this hypothesis. Moreover, our results suggest minimal benefit to applying TE at rates above the maximum-labeled use rate of 26.3 g a.i ha^{-1}. It is important to maintain consistent growth among phenotypically different grasses in order to minimize any competitive growth advantage an OT grass may possess over a desirable cultivar in a golf course putting green.

FUTURE RESEARCH

There are many topics that future research should address regarding OT grasses in ultradwarf bermudagrass putting greens. Molecular genetics and cytology could shed light on the genetic differences between 'Tifgreen'-derived cultivars and other hybrid bermudagrasses. In addition to DNA, phenotypically different grasses that are genetically similar warrant more research regarding RNA sequence and gene expression. Measuring differential gene expression in OT grasses and cultivars may explain differences in morphology or responses to practices such as daily mowing at heights < 5 mm or treatment with TE. Research on nursery management procedures that aid in OT identification and eradication could reduce the incidence of OT grasses in putting greens; however, research should also focus on managing OT grasses in putting greens. Evaluating OT and cultivar responses to N and TE applications in a field setting for an entire growing season would be more applicable for golf course superintendents. It is also important to explore other PGR active ingredients for OT management. Testing OT responses to other management techniques such as mowing, vertical mowing, sand topdressing, aerification, wetting agents, and lightweight rolling would also benefit golf course superintendents. The continuous and enhanced multidisciplinary research regarding OT grasses in bermudagrass putting greens is critical for the development of a completely integrated OT management program.

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APPENDIX

TABLES

Table 2.1. Plant material used in genetic and phenotypic evaluation of off-type grasses in ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting greens. Selections included 62 desirable (DS) and off-type (OT) bermudagrasses sampled from golf course putting greens in TN, MS, AR, FL, AL, GA, and SC. Six standard (ST) hybrid bermudagrass cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), Tifgreen (TG1-6), and Tifway (TW1-6)] and two progenitor species [(*C. dactylon* (TA1-3 and TB1-3) and *C. transvaalensis* (DA1-3 and DB1-3)] were included in the analysis for comparison. Ploidy level was confirmed using flow cytometry.

Sample	Sample Origin[a]	USA State	Ploidy[b]
S1	Champion (DS)	TN	2n=3x=27
S2	Champion (DS)	MS	2n=3x=27
S3	Champion (DS)	TN	2n=3x=27
S4	Champion (DS)	TN	2n=3x=27
S5	Champion (DS)	TN	2n=3x=27
S6	Champion (DS)	TN	2n=3x=27
S7	Champion (DS)	TN	2n=3x=27
S8	Champion (DS)	MS	2n=3x=27
S9	Champion (DS)	TN	2n=3x=27
S10	Champion (DS)	MS	2n=3x=27
S11	Champion (DS)	AR	2n=3x=27
S12	Champion (DS)	TN	2n=3x=27
S13	MiniVerde (DS)	FL	2n=3x=27
S14	MiniVerde (DS)	FL	2n=3x=27
S15	MiniVerde (DS)	TN	2n=3x=27
S16	MiniVerde (DS)	TN	2n=3x=27
S17	TifEagle (DS)	AL	2n=3x=27
S18	TifEagle (DS)	TN	2n=3x=27
S19	MiniVerde (OT)	FL	2n=3x=27
S20	MiniVerde (OT)	FL	2n=3x=27
S21	Champion (OT)	TN	2n=3x=27
S22	Champion (OT)	MS	2n=3x=27
S23	Champion (OT)	TN	2n=3x=27
S24	Champion (OT)	TN	2n=3x=27
S25	Champion (OT)	TN	2n=3x=27
S26	Champion (OT)	TN	2n=3x=27
S27	Champion (OT)	TN	2n=3x=27
S28	Champion (OT)	TN	2n=3x=27
S29	Champion (OT)	TN	2n=3x=27

Table 2.1. Continued

Sample	Sample Origin[a]	USA State	Ploidy[b]
S30	Champion (OT)	TN	2n=3x=27
S31	Champion (OT)	MS	2n=3x=27
S32	Champion (OT)	MS	2n=3x=27
S33	Champion (OT)	TN	2n=3x=27
S34	Champion (OT)	MS	2n=3x=27
S35	Champion (OT)	AR	2n=3x=27
S36	Champion (OT)	TN	2n=3x=27
S37	Champion (OT)	TN	2n=3x=27
S38	MiniVerde (OT)	TN	2n=3x=27
S39	TifEagle (OT)	AL	2n=3x=27
S40	TifEagle (OT)	AL	2n=3x=27
S41	MiniVerde (OT)	TN	2n=3x=27
S42	MiniVerde (DS)	TN	2n=3x=27
S43	MiniVerde (OT)	FL	2n=3x=27
S44	MiniVerde (OT)	MS	2n=3x=27
S45	MiniVerde (OT)	MS	2n=3x=27
S46	MiniVerde (OT)	TN	2n=3x=27
S47	TifEagle (DS)	AL	2n=3x=27
S48	TifEagle (DS)	AL	NA
S49	Champion (OT)	TN	NA
S50	Champion (OT)	TN	NA
S51	Champion (OT)	GA	NA
S52	Champion (OT)	GA	NA
S53	Champion (OT)	TN	NA
S54	Champion (OT)	TN	NA
S55	MiniVerde (DS)	TN	NA
S56	Champion (DS)	SC	NA
S57	Champion (DS)	GA	NA
S58	TifEagle (OT)	MS	NA
S59	Champion (OT)	GA	NA
S60	TifEagle (DS)	TN	NA
S61	TifEagle (DS)	MS	NA
S62	MiniVerde (DS)	TN	NA
CH1-6	Champion (ST)	GA	2n=3x=27
MV1-6	MiniVerde (ST)	GA	2n=3x=27
TD1-6	Tifdwarf (ST)	GA	2n=3x=27
TE1-6	TifEagle (ST)	GA	2n=3x=27
TG1-6	Tifgreen (ST)	GA	2n=3x=27
TW1-6	Tifway (ST)	GA	2n=3x=27
TA1-3	*C. dactylon* (ST)	GA	2n=4x=36
TB1-3	*C. dactylon* (ST)	GA	2n=4x=36
DA1-3	*C. transvaalensis* (ST)	GA	2n=2x=18
DB1-3	*C. transvaalensis* (ST)	GA	2n=2x=18

[a] Desirable and off-type samples were harvested from golf course putting greens. Standard samples were provided by the University of Georgia Coastal Plain Experiment Station in Tifton, GA

[b] Ploidy was confirmed using flow cytometry. Ploidy was not confirmed for samples with NA

Table 2.2. Number of nucleotide variants with different genotypes (homozygous for the reference allele, homozygous for the alternate allele, or heterozygous) between each pair of triploid hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] cultivars Champion, MiniVerde, Tifdwarf, TifEagle, Tifgreen, and Tifway. Variants with differing heterozygosity between or within the two subgenomes (i.e. 0/0/1 or 0/1/1) are not included.

Cultivar	Number of nucleotide variants[a]				
	Champion	MiniVerde	Tifdwarf	TifEagle	Tifgreen
Champion	-	-	-	-	-
MiniVerde	146975	-	-	-	-
Tifdwarf	146280	140388	-	-	-
TifEagle	137869	127295	129690	-	-
Tifgreen	150109	140547	141041	129072	-
Tifway	455860	438521	457970	442106	454237
	Number of nucleotide variants[b]				
Champion	-	-	-	-	-
MiniVerde	4003	-	-	-	-
Tifdwarf	4086	3404	-	-	-
TifEagle	4281	3489	4299	-	-
Tifgreen	3969	3028	4476	4088	-
Tifway	35104	29614	37802	36796	36838

[a] The total number of nucleotide variants shared between each pair of cultivars

[b] Nucleotide variants that were filtered for a read depth of at least 40, but did not exceed 100 per cultivar

Table 3.1. Analysis of variance (ANOVA) of the linear-mixed model fit on clipping weights (mg cm^{-2}) that were natural logarithm transformed due to non-constant variance for both nitrogen (N) and trinexapac-ethyl (TE) rate response experiments. The linear-mixed model and ANOVA were performed R (v3.3.2; R Core Team; Vienna, Austria).

	N Rate Response		TE Rate Response	
Source of Error	Significance Level	F-Value	Significance Level	F-Value
Blocking factor	***	103.01	***	172.77
Selection	ns[a]	1.68	***	4.98
Rate	***	8.06	***	6.73
Time after treatment (TAT)	***	38.13	***	45.09
Selection × Rate	ns	1.07	ns	0.73
Selection × TAT	ns	1.58	ns	0.95
Rate × TAT	***	7.61	***	5.06
Selection × Rate × TAT	ns	0.62	ns	0.62

*** Significant at the 0.001 probability level.
[a] ns, non-significant at the 0.05 probability level.

Table 3.2. Pairwise comparison t-tests with pooled standard deviation and Bonferroni p-value adjustment on natural logarithm transformed clipping weights [ln(mg cm^{-2})] on the significant main effect of grass sample in the trinexapac-ethyl rate response experiment in Knoxville, TN. Clippings were harvested 7, 14, 21, and 28 days after treatment and then reported as the natural log of mg cm^{-2}. Commercial cultivars Champion, MiniVerde, and TifEagle were included in the experiment with three off-type selections from golf course putting greens.

	OTC1	Champion	MiniVerde	TifEagle	OTC2	OTC3
Champion	**	-	-	-	-	-
MiniVerde	***	ns	-	-	-	-
TifEagle	ns[a]	ns	ns	-	-	-
OTC2	***	ns	ns	ns	-	-
OTC3	**	ns	ns	ns	ns	-

** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.
[a] ns, non-significant at the 0.05 probability level.

FIGURES

Figure A. Off-type grasses (lighter in color and noted by red circle) present in an ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting green. The difference in turfgrass color between desirable and off-type grasses disrupts aesthetic uniformity of the putting green. Photo is courtesy of Mr. Rodney Lingle. Figure was generated using Keynote (v6.6.2).

Figure B. Close-up of an off-type grass patch (noted by red circle) present in an ultradwarf hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] putting green. The difference in growth rate between the desirable and off-type grasses has the potential to disrupt the functional uniformity of putting greens with off-type infestations. Figure was generated using Keynote (v6.6.2).

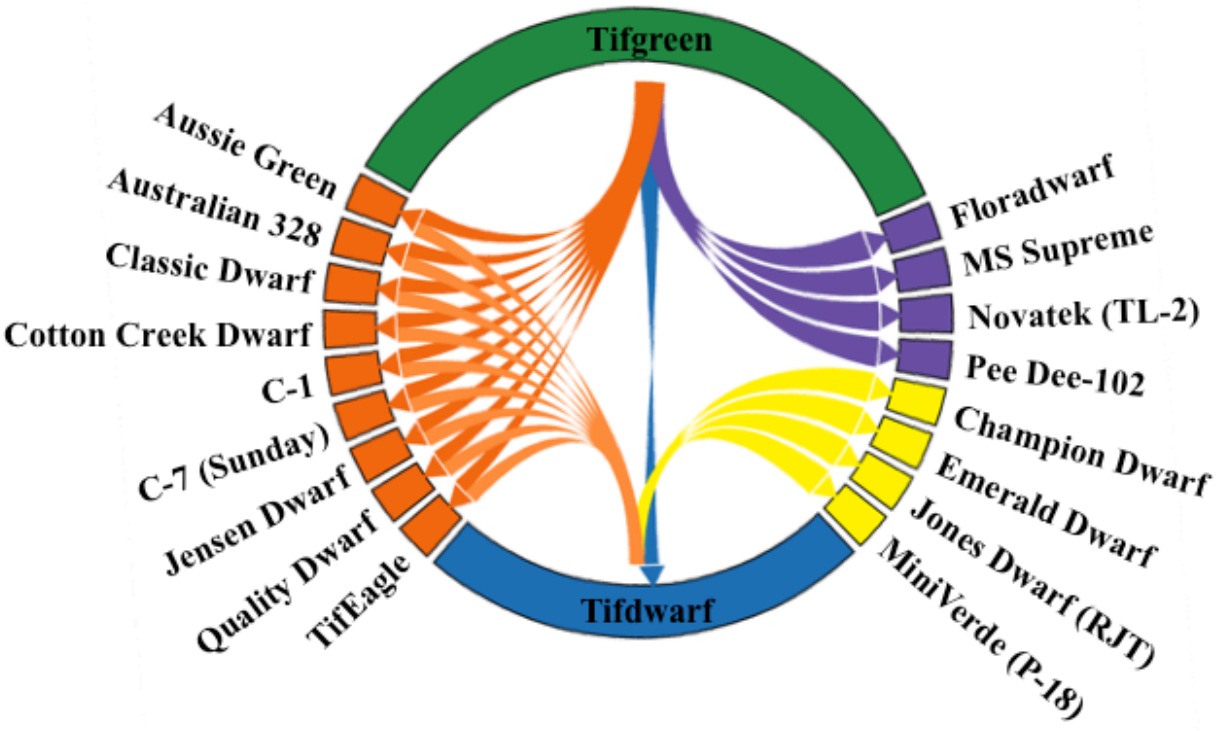

Figure 1.1. Current understanding of the lineage among accessions of interspecific hybrid bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] used on golf course putting greens. The cultivars represented by blue, yellow, and purple colors are those with lineage explicitly reported either in scientific or patent literature. The cultivars represented by orange are those that the true lineage is unknown or are not explicitly reported by scientific or patent literature.

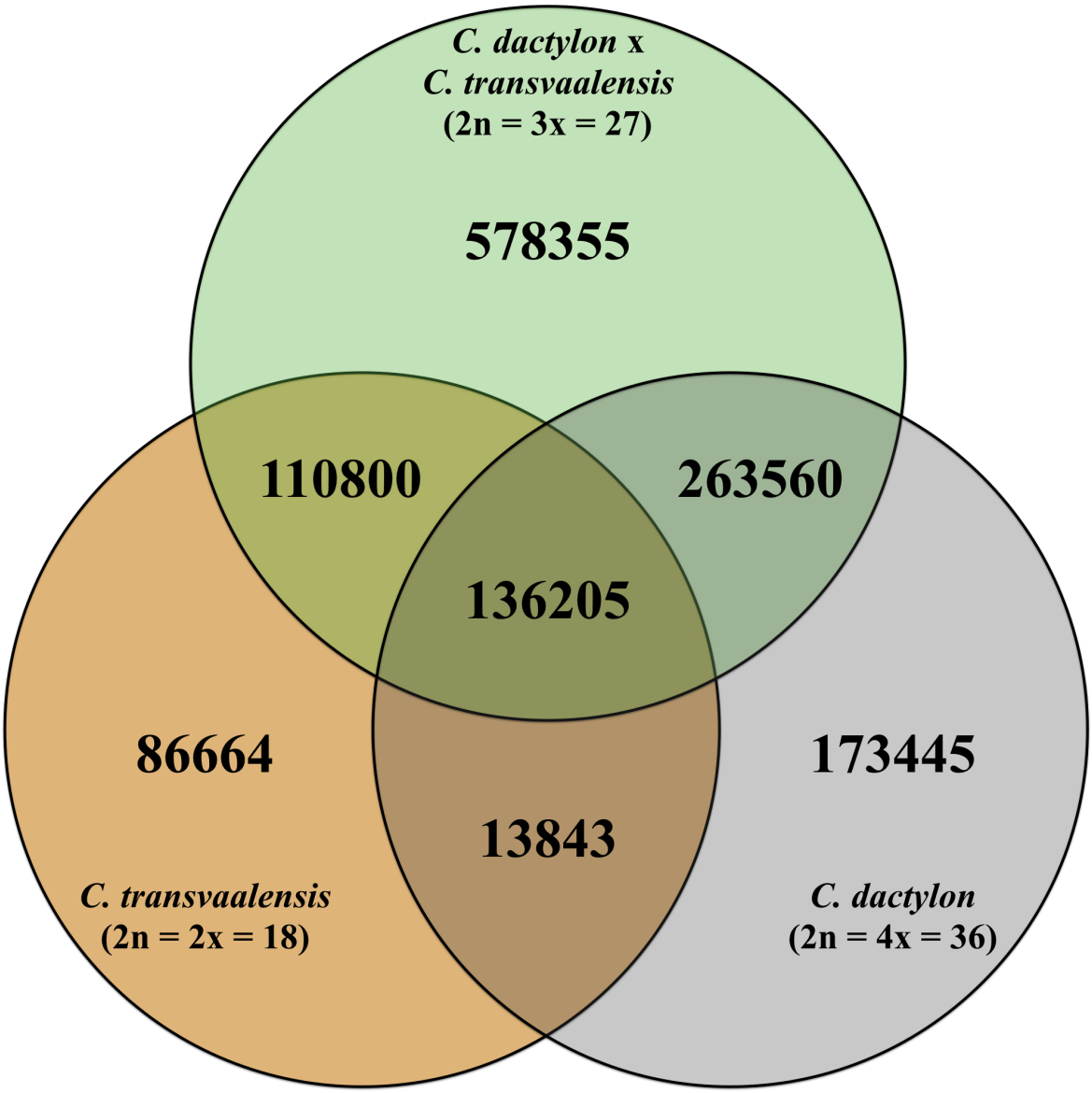

Figure 2.1. Venn diagram showing the number of total and shared nucleotide variants from genotyping-by-sequencing for *Cynodon transvaalensis*, *C. dactylon*, and *C. dactylon* x *C. transvaalensis*. Ploidy levels were confirmed using flow cytometry and are noted in parentheses. Figure was generated using Keynote (v.6.6.2).

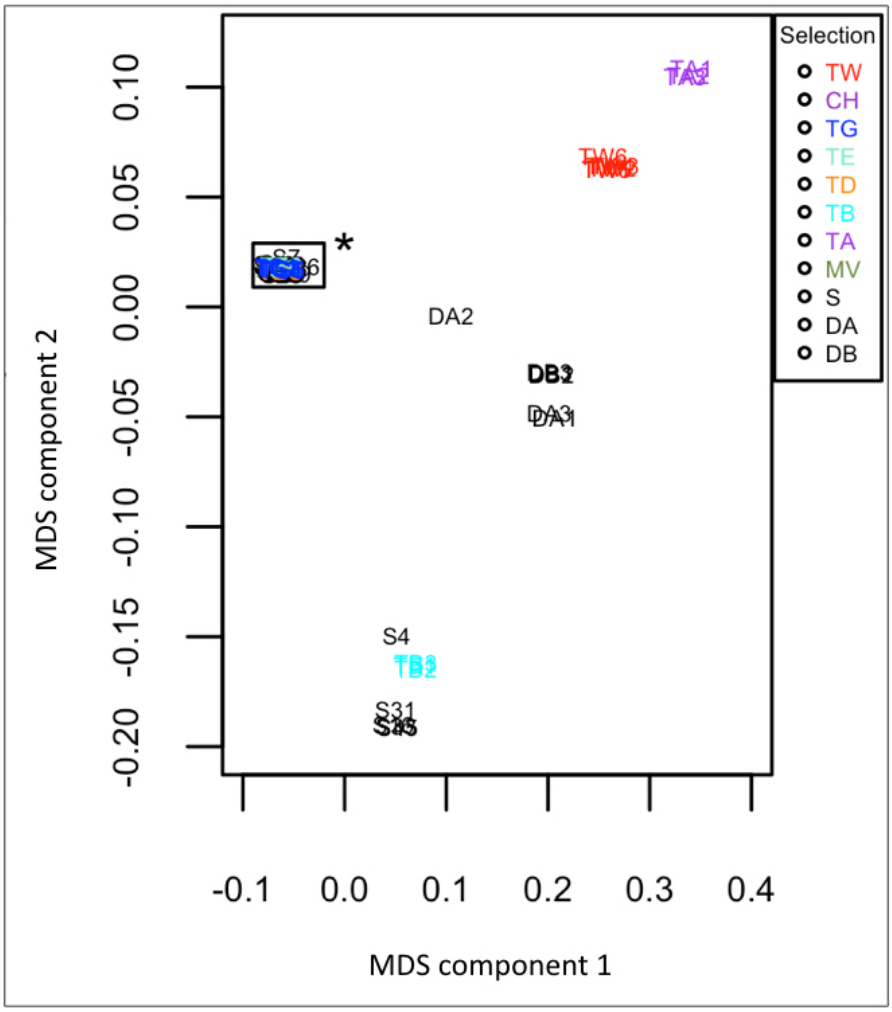

Figure 2.2. Multidimensional scaling plot (MDS) of nucleotide variants from 47 desirable and off-type bermudagrasses sampled from golf course putting greens (S1-47), six hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), Tifgreen (TG1-6), and Tifway (TW1-6)], and two progenitor species [*C. dactylon* (TA1-3, TB1-3) and *C. transvaalensis* (DA1-3, DB1-3)]. Samples S19, S28, S30, S32, and S44 were not included due to lack of read depth. Nucleotide variants were generated using Freebayes, the MDS plot was calculated in Plink, and plotted in R. The asterisk on the box indicates the zoomed region in Figure 2.3.

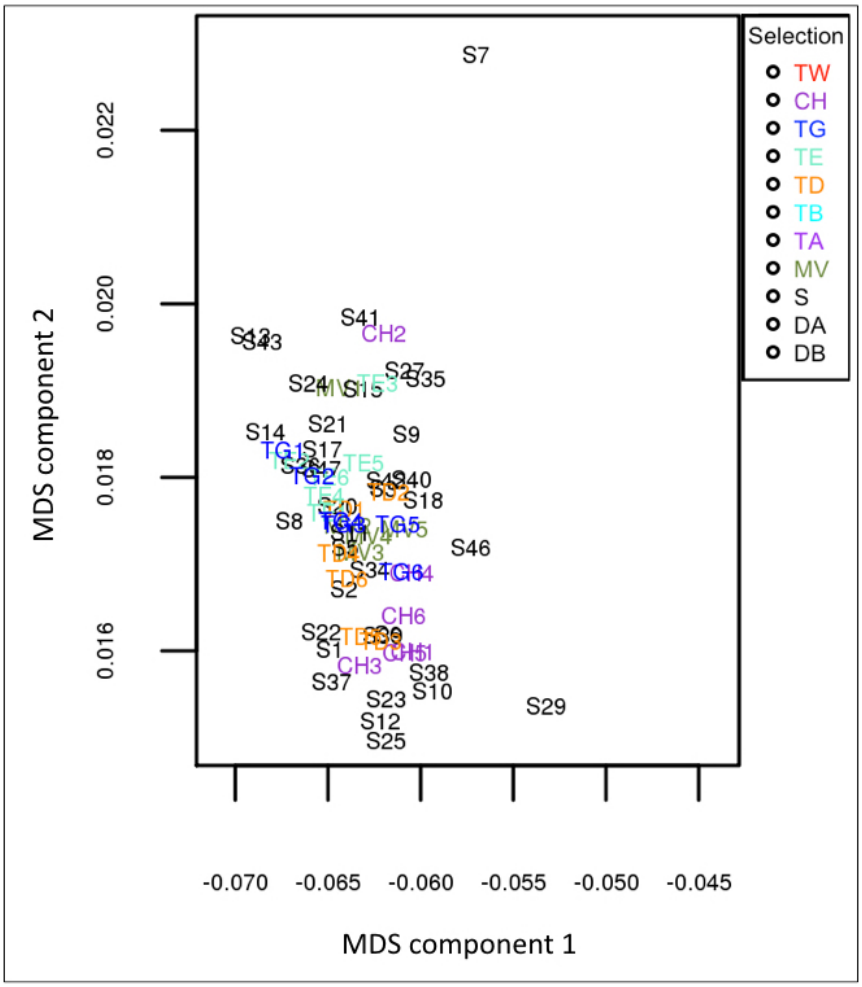

Figure 2.3. Multidimensional scaling plot (MDS) of nucleotide variants from desirable and off-type bermudagrasses [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] sampled from golf course putting greens (S1-47) and cultivars [Champion (CH1-6), MiniVerde (MV1-6), Tifdwarf (TD1-6), TifEagle (TE1-6), and Tifgreen (TG1-6)]. Samples in this MDS plot have been determined to be genetically similar due to the clustering in the noted boxed with an asterisk in Figure 2.2. Desirable and off-type bermudagrasses that were analyzed by genotyping-by-sequencing, but did not cluster in this region included the following samples: S4, S16, S31, S33, and S45. Samples S19, S28, S30, S32, and S44 were not included due to lack of read depth. Nucleotide variants were generated using Freebayes, the MDS plot was calculated in Plink, and plotted in R.

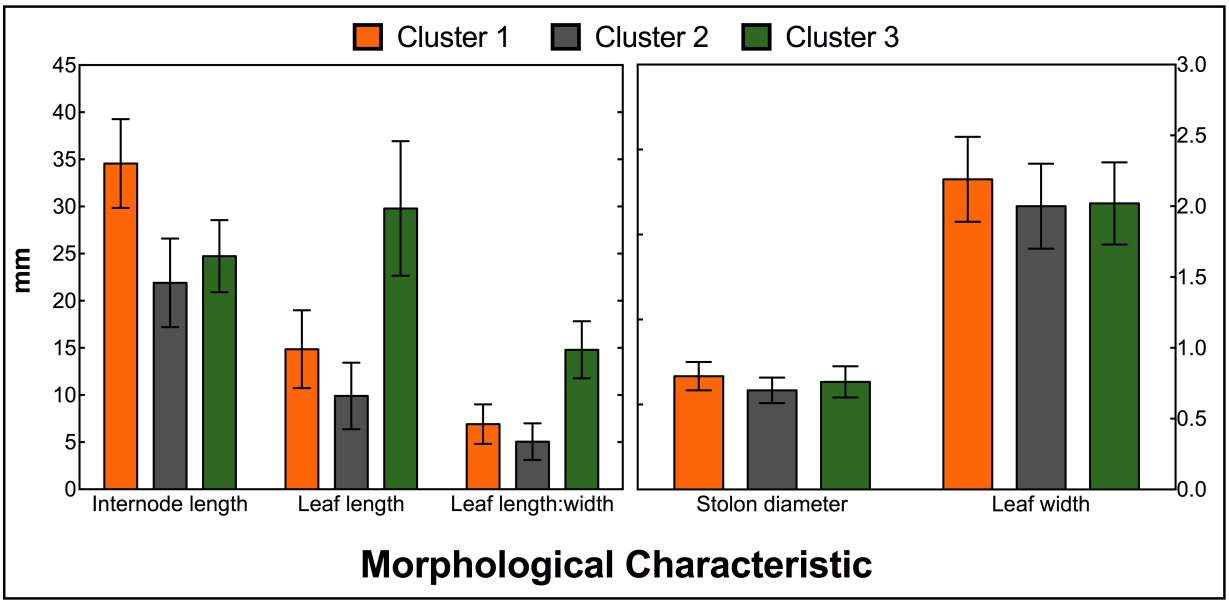

Figure 2.4. Cluster means and standard deviations for internode length, leaf length, leaf length:width ratio, stolon diameter, and leaf width measurements. Morphological parameters were assessed using methods similar to Roche and Loch (2005). Measurements were made on 52 off-type and desirable hybrid bermudagrass [*Cynodon dactylon* (L.) Pers. x *C. transvaalensis* Burtt-Davy] samples harvested from golf course putting greens in the southeastern United States. Cluster means and standard deviations were generated from the K-means algorithm in SAS Enterprise 6.1 and graphed using Prism 6.0 for Mac. Statistical differences were determined using standard deviations.

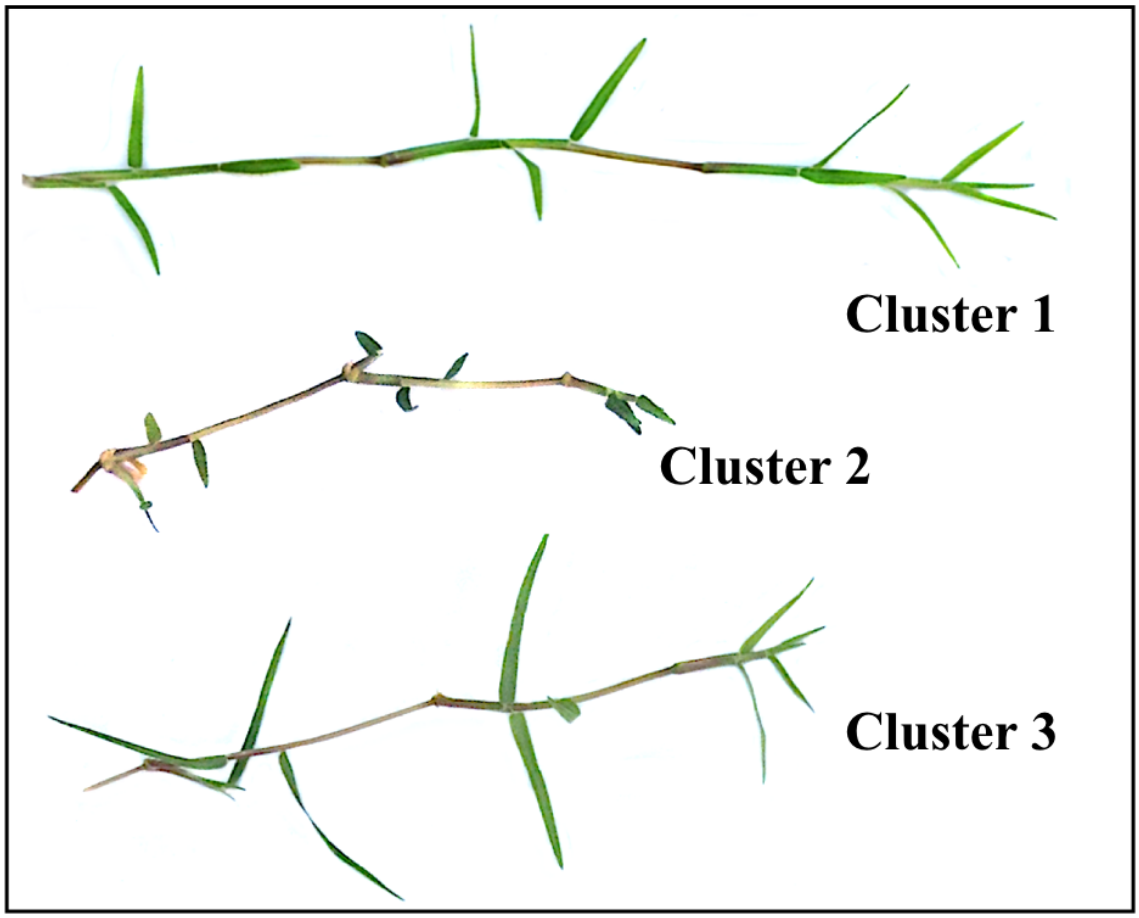

Figure 2.5. Photographs of bermudagrass samples representative of each morphological cluster in Figure 2.4. Cluster analysis was performed using a K-means algorithm in SAS Enterprise Guide (Version 6.1, SAS Institute, Cary, NC, USA) with cluster means and standard deviations graphed in Prism (Prism 6 for Mac OS X; GraphPad Software, Inc.) to determine statistical differences. Grasses in cluster one had significantly longer internode lengths than those within clusters two and three. Grasses in cluster three had significantly longer leaves than those in clusters one and two. Figure was generated using Keynote (v6.6.2).

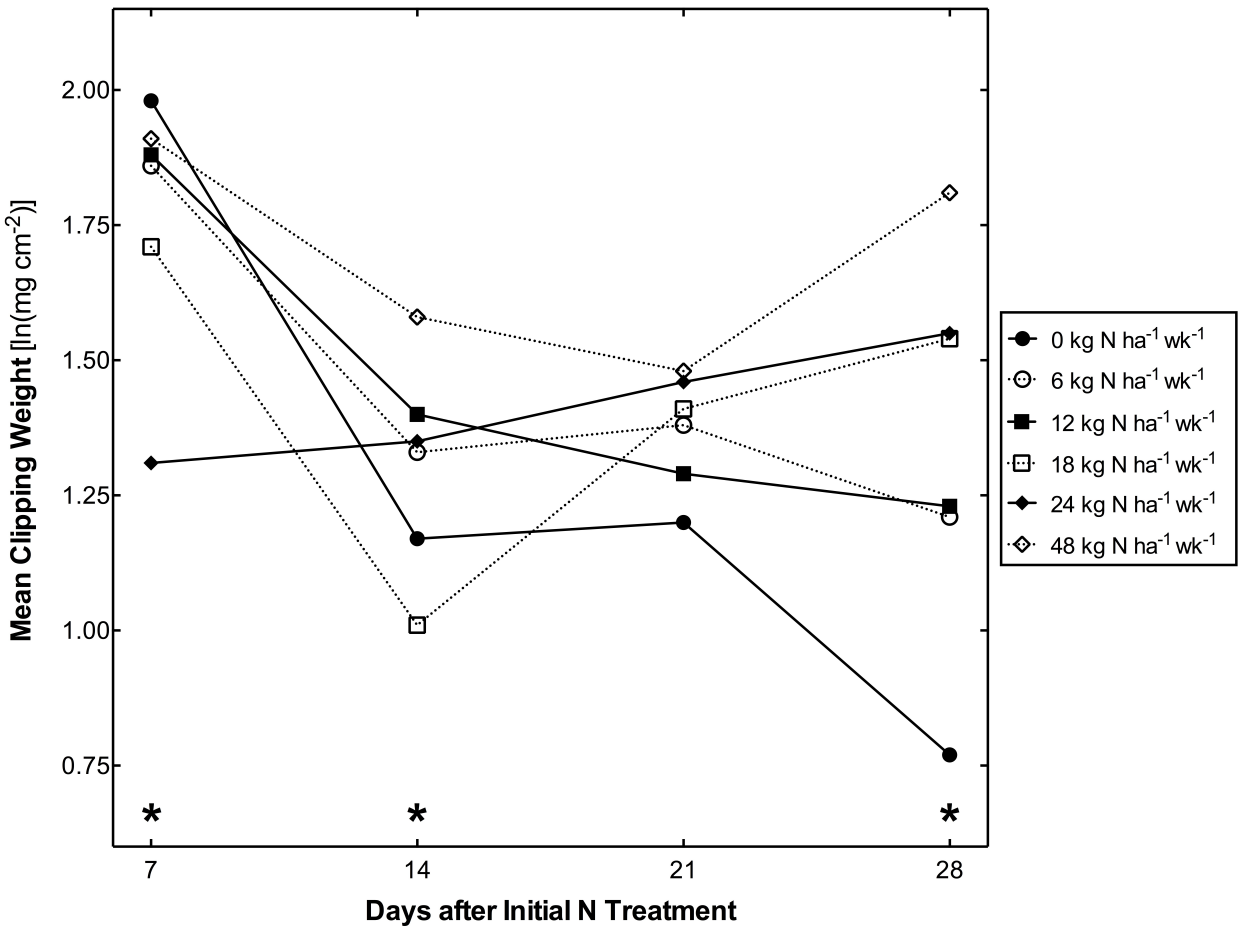

Figure 3.1. Mean clipping yield of nitrogen rates 0, 6, 12, 18, 24, and 48 kg N ha^{-1} wk^{-1} at 7, 14, 21, and 28 days after initial N treatment. Mean clipping yields were pooled across three commercial bermudagrass cultivars Champion, MiniVerde, and TifEagle and three off-type grasses. The clipping weights (mg cm^{-2}) were natural logarithm transformed due to non-constant variance and then plotted as ln(mg cm^{-2}) in Prism software (Prism 6.0 for Mac OSX. GraphPad Software. La Jolla, CA). Days after initial treatment marked with an asterisk (*) has N rates that are significantly different from pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment in R (v3.3.2; R Core Team; Vienna, Austria).

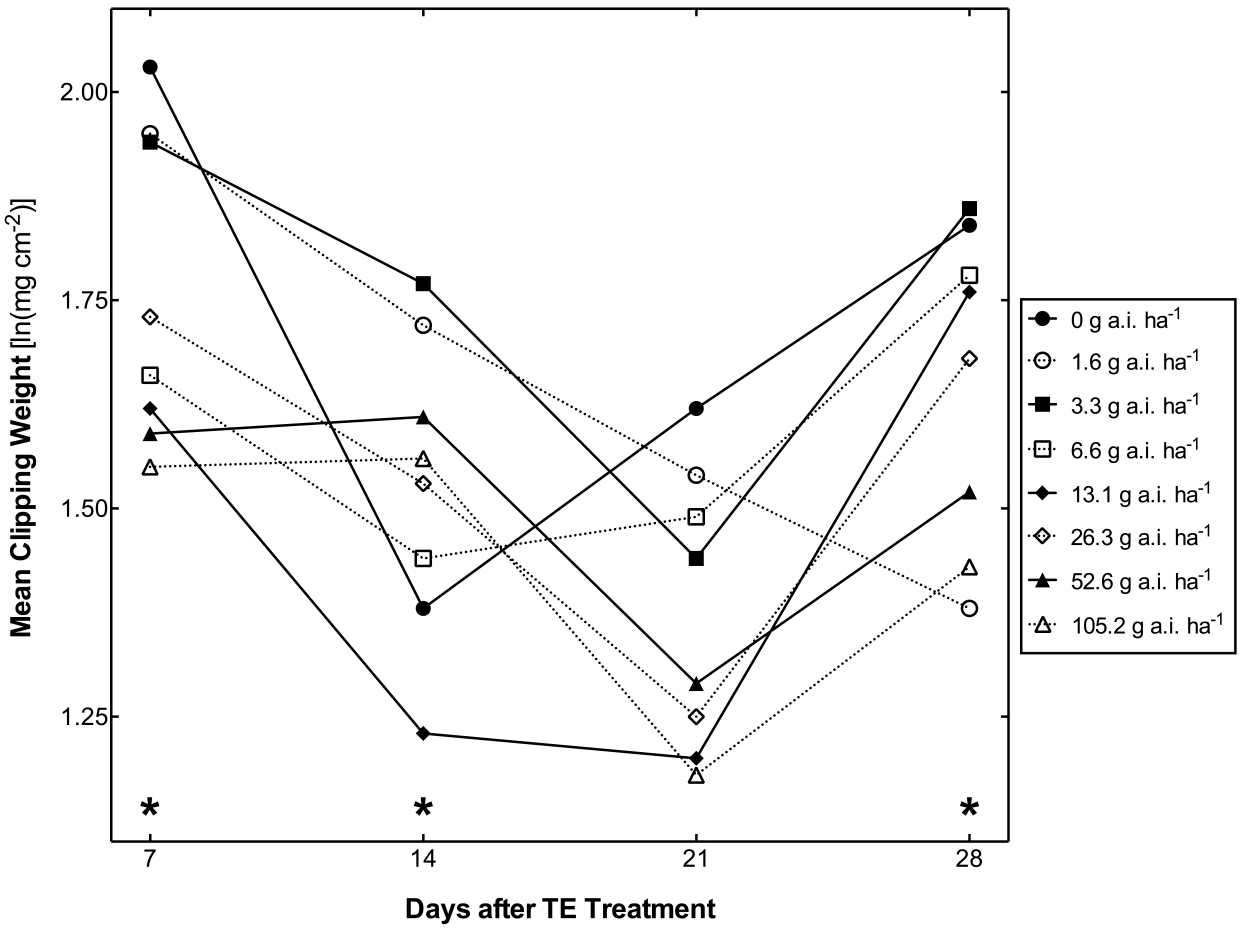

Figure 3.2. Mean clipping yield of trinexapac-ethyl (TE) rates 0, 1.6, 3.3, 6.6, 13.1, 26.3, 52.6, or 105.2 g a.i. ha^{-1} at 7, 14, 21, and 28 days after initial N treatment. Mean clipping yields were pooled across three commercial bermudagrass cultivars Champion, MiniVerde, and TifEagle and three off-type grasses. The clipping weights (mg cm^{-2}) were natural logarithm transformed due to non-constant variance and then plotted as ln(mg cm^{-2}) in Prism software (Prism 6.0 for Mac OSX. GraphPad Software. La Jolla, CA). Days after initial treatment marked with an asterisk (*) has TE rates that are significantly different from pairwise comparisons using t-tests with pooled standard deviation and Bonferroni p-value adjustment in R (v3.3.2; R Core Team; Vienna, Austria).

VITA

Eric Hall Reasor was born on April 19, 1991 in Wytheville, VA to George and Jennifer Reasor. Raised in Rural Retreat, VA, he attended Rural Retreat High School and graduated in 2009. He went on to pursue a degree in Crop and Soil Environmental Sciences at Virginia Tech with an emphasis in Turfgrass Management, in which he graduated in 2012 with a Bachelor of Science degree. He began the pursuit of his Masters of Science degree under the direction of Dr. John Sorochan in 2012 at the University of Tennessee and graduated in May 2014. Also in May 2014, Eric enrolled in a Ph.D. program in turfgrass weed science under the direction of Dr. Jim Brosnan. Eric will begin July 2017 as an assistant professor of turfgrass science in the Department of Plant and Soil Sciences at Mississippi State University.