ACCASE HERBICIDE RESISTANCE IN BERMUDAGRASS

by

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(Under the Direction of Brian Schwartz)

ABSTRACT

Bermudagrass (*Cynodon dactylon* (L.) Pers.) is a widely used turfgrass in home lawns, golf courses, and athletic fields. The turf industry faces challenges regarding the issues of environmental impact and increasing expense of turf management. Fertilizer and herbicide application has been linked with non-point pollution of fresh water systems. Creating new turf cultivars with potential to reduce environmental impacts is critical for continued success of the industry. Herbicide resistant bermudagrass cultivars are a way to modernize the turf industry, making management more cost efficient for operators. Studies were performed to identify and attempt to create ACCase herbicide resistant bermudagrass cultivars. A possible herbicide tolerant genotype (93-175) exhibited less injury to ACCase herbicide applications. Also, a lysimeter study was completed to analyze fertilizer leaching characteristics of several fertilizers and determined an experimental fertilizer (E 15-0-0) had a greater potential to leach nitrogen than two other commonly used fertilizers.

INDEX WORDS: Bermudagrass, ACCase, Sethoxydim, Clethodim, Fluazifop, Resistance

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Purpose of Study

The turf breeding programs in Georgia have had a rich and productive history of producing cultivars for the turf industry. Recently, economic and social issues have driven the need for plant breeders to develop new cultivars that address a different arrangement of situations. The turf industry will require new solutions to stay viable and environmentally sound. The University of Georgia turf program has a goal to not only aid Georgia growers, but those around the globe as well. New cultivar development and selection has shifted in the past few years from finding the best quality genotypes under high levels of management to also identifying those with traits that reduce inputs, including irrigation, herbicides, or labor. The following studies include research aimed at identifying and solving a subset of the future needs of the turf industry as a whole.

Literature Review

Herbicide Resistance in Bermudagrass

Bermudagrass (*Cynodon spp.* L.) is a warm-season perennial grass that is widely used in the southern United States, India, Australia, Africa and South America. In turf areas of higher intensity management, improved varieties such as the sterile hybrids made from crosses of *Cynodon dactylon* (L.) Pers. and *Cynodon transvaalensis* Burtt Davy are preferred for superior aesthetics, quality, and compared to traditional varieties (Bunnell et al., 2005; Webster et al., 2009). Common bermudagrass *(Cynodon dactylon*) is traditionally a problem weed within these

cultivars. Currently there are no selective herbicides to control common bermudagrass that has contaminated a hybrid cultivar, and due to common's competiveness, and infestations can become a large issue if left untreated. The only method currently available to remove the infested areas is by sod cutting or glyphosate applications followed by replanting of new sod (Bigelow, 2008; Lowe and Foy, 2012).

Herbicides that inhibit acetyl-CoA carboxylase (ACCase) such as the cyclohexanedione (CHD) and aryloxyphenoxypropionates(AOPP) families are used to aid in the control of bermudagrass in various agronomic conditions. This includes sethoxydim (2-[1-(ethoxyyimino)butyl]-5-[2-(ethylthio)propyl]-3-3hydroxy-2-cyclohexen-1-one), clethodim (-2-[(E)-3-chloroallyloxyimino]propyl]-5-(2-ethylthiopropyl)-3-hydroxy-cyclohex-2-enone), and fluazifop (2-[4-(5-trifluoromethyl-2-pyridyloxy)-phenoxy]propionate). These herbicides lead to plant injury by causing rapid necrosis of plant meristematic tissue through fatty-acid synthesis inhibition. The incorporation of herbicide resistance into an improved hybrid bermudagrass would give turf managers the ability to remove common bermudagrass or other grassy weed contamination from their turf without extensive damage or labor inputs.

Callus Induction in DT-1 Bermudagrass

Bermudagrass is an important warm-season turfgrass used throughout the world on home lawns, sports fields, and golf courses. Using the technique of tissue culture to induce somaclonal mutations has been explored as a method to improve bermudagrass germplasm, but it was found that physiological differences in the mutant genotypes were not different enough to warrant the efforts (Goldman et al., 2004). Also, Zhang et al. (2007) determined that a universal protocol for inducing callus in different bermudagrass genotypes has not been successful, and that techniques specific to each genotype likely need to be developed.

Acetyl-CoA carboxylase (ACCase) is a key enzyme that catalyzes the formation of malonyl-CoA from the carboxylation of acetyl-CoA. ACCase is recognized in two forms, one being the heteromic prokaryotic, and the other being the homomeric eukaryotic form. In dicots, the plastidic ACCase is not sensitive to ACCase inhibiting herbicides and results in a lack of activity for this class of herbicides. In grasses, the plastidic homomeric form is the target site of ACCase-inhibiting herbicides which include sethoxydim, clethodim, and flauzifop. ACCase herbicide resistance has been identified in grassy weeds as a gene mutation in the carboxyl transferase (CT) domain of plastidic ACCase. There have been eight mutation sites found, the most common being a Leu-1781-Ile. Many of these mutation sites have shown plant fitness costs. No fitness costs however have been observed for the Leu-1781-Ile or Ile-2041-Asn mutations. In one case, the Leu-1781-Ile showed a fitness advantage. In the species *Poa annua* L., *Festuca rubra* L., and *V. Bromoides* (L.) Gray, the Leu-1781-Ile mutation is the wild type. The different classes of ACCase inhibiting herbicides vary in the mutation sites that confer resistance. In blackgrass (*Alopecurus myosuroides* Huds.), resistance to fluazifop at (Leu-1781-Ile), clethodim at (Asp-2078-Gly), and sethoxydim at (Leu-1781-Ile) has been observed. Wild oat (*Avena fatua* L.), and green foxtail (*Setaria viridis* (L.) P. Beauv.) have also exhibited resistance to sethoxydim due to mutation of the 1781 site (Powles and Yu, 2010).

Resistance to ACCase inhibiting herbicides can develop naturally in weed populations targeted by these herbicides. The frequent use of aryloxyphenoxypropionate and cyclohexaedione herbicides has resulted in 21 weed species globally conferring resistance (Beckie et al., 2000). The genes that encode for both ACCase and metabolism based resistance are nuclear and can be transferred by seed and pollen (Délye, 2005). Resistance has been shown to develop in both self-pollinated and out-crossing species. The genes responsible for resistance

have accumulated at a higher rate in out-crossing populations (Délye, 2005). The increased occurrence of herbicide resistant weeds can eventually limit the usefulness of a cultivar that contains the herbicide resistance. In most integrated pest management (IPM) strategies for turf, the application of one herbicide over a prolonged time frame can cause selection of genes that confer resistance in weed species to that herbicide (Everman et al., 2013).

Development of sethoxydim resistance in corn (*Zea mays* L.) was accomplished by inducing somaclonal variation through tissue culture (Parker et al., 1990). Resistance has also been induced in seashore paspalum (*Paspalum vaginatum* Sw.) through tissue culture and selection of callus from media containing sethoxydim (Heckart, 2010). Fitness issues have been observed in these plants. Some plants with ACCase herbicide resistance and no apparent reduction in fitness were developed but these plants exhibited lower levels of sethoxydim tolerance, and lack known mutations. This resistance was theorized to be a natural metabolism-based mechanism (Tate et al., 2012). Typically, a physiological stress is induced in a susceptible plant which has been exposed to a herbicide (Délye, 2005). This stress can cause the plant to trigger alternative metabolic pathways in response to sethoxydim exposure. In resistant grasses, this response is viewed as a deviation of enzymes involved in normal plant operations to prevent a buildup of herbicide molecules at a target site, or to degrade the herbicide to a non-toxic product (Délye, 2005; Cotterman and Saari, 1992). These types of responses normally occur in grassy weeds with resistance (Délye, 2005). Seashore paspalum resistant varieties without known gene mutations may have the ability to recognize the presence of a herbicide more quickly, and in turn, natural pathways for detoxification of the herbicide respond at a higher rate than in a wild-type plant (Tate, personal communication).

Influence of Soil Type on Nitrogen Leaching of Controlled Release Fertilizers

Environmental stewardship is an important consideration in turf management. Public and political groups have increased their interest and resolve regarding water resource contamination in the United States. In the past few decades, consumers of turf landscapes have demanded higher levels of quality, which led to higher use and application of nitrogen fertilizers (Osmond and Hardy, 2004). This has resulted in turfgrass areas being targeted by regulatory agencies as potential runoff or leaching sources of water contamination (Duncan and Carrow, 2000). The coastal plains of the United States are susceptible to leaching due to the fluid dynamics in sandy soils with low water holding capacity (Wang and Alva, 1996). Thus, practitioners managing bermudagrass in these areas may face regulatory issues regarding management inputs required for successful culture.

Nitrate (NO_3-N) from turfgrass fertilizers is one of the main components associated with groundwater contamination. In recent years this has encouraged fertilizer developers to create new controlled and slow release fertilizers that could reduce the problems associated with over application. Studies have shown improper use of fertilization along with irrigation can lead to the leaching of nitrogen from a turfgrass system (Petrovic, 1990). It is in the interest of fertilizer companies and their stakeholder groups to be viewed as environmentally aware and proactive on the issue.

CHAPTER 2

HERBICIDE RESISTANCE IN BERMUDAGRASS[1]

[1] Grimshaw, A.G., B.M. Schwartz, T.L. Grey, P.L. Raymer, P.E. McCullough, T.M. Webster, and W.A. Parrott. To be submitted to *Weed Science.*

Abstract

Contamination of newly planted bermudagrass (*Cynodon spp.* L.) varieties by undesirable offtype bermudagrass genotypes is an ever increasing concern for turf managers, and selective control options are limited. University of Georgia turf breeding programs are working towards the development of new high quality, herbicide tolerant varieties. In 2009 a possible ACCase herbicide resistant bermudagrass genotype was found in the University of Georgia turf nursery. During 2012, DNA sequencing, along with both greenhouse and field trials were performed in Tifton, GA to identify and confirm the possible ACCase herbicide resistance of the bermudagrass genotype 93-175. A comparison was made to known susceptible genotypes 'Tifway' and common bermudagrass. Sethoxydim, clethodim, and fluazifop were applied to these genotypes at varying rates in both experiments. Results from the greenhouse indicated that 93-175 generally had higher levels of tolerance to sethoxydim than Tifway and common bermudagrass. Field experiments confirmed tolerance at various application rates 42 d after treatment and during green-up in the following spring. At the field application rate of sethoxydim (280 g ai ha^{-1}), 93-175 was 50 to 87 % less injured when compared to the other two genotypes across the evaluation dates using data obtained from colormetric image analysis. Similar results were noted during spring green-up where 93-175 reached 100% recovery during the same rating period as non-treated controls when treated with a field rate of sethoxydim, while common and Tifway had only 48 and 60 % recovery, respectively. DNA sequencing of ACCase regions known to contain herbicide resistance point mutations revealed no differences between 93-175 and the susceptible genotypes. 93-175 will continued to be studied to determine transferability of herbicide tolerance to progeny and the mechanism of the observed tolerance.

Introduction

Bermudagrass (*Cynodon spp.* L.) is a warm-season perennial grass that is used widely in the southern United States, India, Australia, Africa and South America. It is found in over 100 countries within tropical and sub-tropical regions throughout the world. Bermudagrass is generally regarded as having exceptional tolerance to heat and drought, but poor tolerance of shade and freezing temperatures. Improved bermudagrasses provide durable turf on golf courses, sports fields, recreational areas, forages and home lawns (Beard, 1973). Common bermudagrass (*Cynodon dactylon* (L.) Pers.) is the most prevalent around the globe and is mostly regarded as an invasive weed in many crops, including improved varieties of bermudagrass. Non-cultivated common bermudagrasses typically are tetraploid ($2n = 4x = 36$), although hexaploid ($2n = 6x = 54$), pentaploid ($2n = 5x = 45$), and triploid ($2n = 3x = 27$) accessions have been collected (Harlan et al., 1970b; Wu et al., 2006). The genetic variability found within this genus of grasses has allowed for their widespread adaptation and utilization (Harlan et al., 1970a). Tetraploid genotypes tend to be more fertile than the diploid ($2n = 2x = 18$) species such as African Bermudagrass (*Cynodon transvaalensis* Burtt Davy), which generally do not produce many viable seeds (Taliaferro, 2003).

In most intensely managed turf areas, improved varieties such as the sterile hybrids made from crosses between *Cynodon dactylon* and *Cynodon transvaalensis* are preferred because their superior aesthetics and performance which has been confirmed over many years and in different situations (Bunnell et al., 2005; Webster et al., 2009). These sterile hybrids are clonally propagated through rhizomes and stolons and transplanted as sod or sprigs when established in new turf areas. Currently there are no selective herbicides to control common bermudagrass that has contaminated a hybrid cultivar due to a lack of differential resistance. For example, control

of bermudagrass is achieved in centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.) and zoysiagrass (*Zoysia japonica* Steud.) through the application of ACCase herbicides that have selectivity between the species (Cox et al., 1999; Lewis et al., 2010). Due to common bermudagrass's persistence, competiveness and difficulty of eradication by fumigation, unwanted infestation is a normal occurrence and can become a large issue if left untreated. The only method currently available to remove the infested areas is by sod cutting or glyphosate applications followed by replanting of new sod (Bigelow, 2008; Lowe and Foy, 2012).

Herbicide resistance has been developed in many agronomic crops to control weeds (Mazur and Falco, 1989). However, many of these plants were developed using transgenic approaches which are deemed impractical when developing turfgrass resistance systems due to the extensive regulations associated with the production and release of a transgenic crop. Costs also could be extensive, limiting the feasibility for public turfgrass breeding programs to use this approach (Bradford et al., 2005). However, plants conferring herbicide resistance developed by other non-transgenic techniques of breeding are not currently regulated.

Sethoxydim (2-[1-(ethoxyyimino)butyl]-5-[2-(ethylthio)propyl]-3-3hydroxy-2-cyclohexen-1-one) is a selective postemergence herbicide used for control of annual and perennial grasses. Manufacturers state sethoxydim can be used to control bahiagrass (*Paspalum notatum* Fluegge), crabgrass (*Digitaria sanguinalis* (L.) Scop.), downy brome (*Bromus tectorum* L.), quackgrass (*Elymus repens* (L.) Gould), annual ryegrass (*Lolium multiflorum* Lam.), wild oats (*Avena spp*. L.), and witchgrasses (*Panicum capillare* L.). Sethoxydim does not control broadleaf weeds or sedges at labeled use rates for turf (Tu et al., 2001). It is part of the cyclohexanedione (CHD) family which inhibit acetyl-CoA carboxylase (ACCase) (Burton et al., 1987), associated with lipid synthesis.

Sethoxydim does not bind or incorporate well with soil particles, but binding increases with organic matter in soil (WSSA, 2002). Due to this low binding rate, sethoxydim is highly mobile within the soil and has the potential for leaching. However, there are no reports of sethoxydim contamination which may be due to its short half-life of 4-5 days in soils (WSSA, 2002). Sethoxydim is also highly degraded by soil microbial metabolism and photodegradation. It has relatively low toxicity to birds and aquatic species, and has little or no effect on soil microbe populations (Tu et al., 2001).

Clethodim (-2-[(E)-3-chloroallyloxyimino]propyl]-5-(2-ethylthiopropyl)-3-hydroxy-cyclohex-2-enone), is also in the CHD family and inhibits ACCase activity in the same manner as sethoxydim. It is registered for use on many agronomic crops and vegetables to control annual and perennial grasses. Appropriate spray volumes are required for effective results, with a minimum carrier volume of 19 to151 L ha^{-1} to maximize foliar penetration. The registered rate varies among weeds, for annual grasses the rate is 79 to 254 g ai ha^{-1}. For perennial species the rate is higher at 124 to 471 g ai ha^{-1}. Symptoms are slow to develop and require 7 to 10 days before initial signs are apparent. Initial phytotoxicity appears on new leaves and full control may take up to 21 days. Clethodim has low persistence in soils due to a 3 day half-life, and breakdown is mainly attributed to aerobic processes. Solubility for clethodim is considered high and it has potential for soil movement with irrigation following application. Clethodim toxicity to birds is very low and it is slightly toxic to freshwater fish (Valent, 2006).

Fluazifop (2-[4-(5-trifluoromethyl-2-pyridyloxy)-phenoxy]propionate) is a grass specific post-emergent herbicide. It is part of the aryloxyphenoxypropionates (AOPP) family, and acts by causing rapid necrosis of plant meristematic tissue. This damage is caused by action on acetyl-CoA carboxylase (ACCase) resulting in fatty-acid synthesis inhibition. Fluazifop is

registered to control barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv. Fernald), bermudagrass, johnsongrass (*Sorghum halepense* (L.) Pers.), and volunteer cereals in resistant crops. It is recommended to be applied as a ground broadcast spray of 47 to 374 L ha^{-1} at a rate of 280 to 426 g ai ha^{-1}. Spraying a second application is recommended after re-growth for complete control. No more than 1268 g ai ha^{-1} of fluazifop should be applied within a 12 month period to any field, and it is limited for use on non-livestock feed fields. Fluazifop is toxic to fish, and slightly toxic to birds. It is immobile in soil buts has a half-life of one day and therefore does not persist in soil or water (Syngenta, 2007).

The incorporation of herbicide resistance into an improved hybrid bermudagrass would give turf managers the ability to selectively control warm-season grassy weeds from their turf without extensive damage to turf and large amounts of labor intensive work. The objectives of these experiments were to 1) evaluate relative susceptibility to ACCase inhibiting herbicides among an experimental variety (93-175), Tifway (Burton 1966), and common bermudagrass; 2) confirm plant responses observed in greenhouse trials also occur in field trials; and 3) identify mutations through DNA sequencing that confer suspected differential tolerance among 93-175, Tifway, and common bermudagrass.

Materials and Methods

Plant Material for Greenhouse and Field Experiments

Tifway, common, and 93-175 bermudagrasses were obtained from the University of Georgia germplasm collection in Tifton, Georgia. Tifway was included in this research because it is a popular variety used on home lawns, sports fields, and golf courses. Common bermudagrass was also evaluated because it and Tifway are presently the primary contaminants of new bermudagrass cultivars after planting. 93-175 is an experimental tetraploid ($2n = 4x =$

36) bermudagrass genotype chosen from a Tiflawn progeny population in 1993. The population was evaluated for turf quality and seed production. 93-175 did not meet requirements for seed production and was kept for use as a potential parent due to its turf quality. For this study 93-175 was chosen for its potential herbicide resistance.

Preliminary field trials indicated that 93-175 was resistant to a labeled rate (280 g ai ha^{-1}) of sethoxydim in Tifton, GA during 2009 (Schwartz, Unpublished). Tolerance was confirmed during 2011 in a greenhouse dose response experiment in conetainers at varying rates of sethoxydim application from 25 g ai ha^{-1} to 800 g ai ha^{-1}. The evaluation identified Tifway as sustaining 96% injury at the 400 g ha^{-1} rate, whereas 93-175 exhibited no more than 16% injury in the 400 to 800 g ha^{-1} treatment 28 days post application.

Spray Chamber Dose Response Experiment

A spray chamber dose response experiment was performed at the University of Georgia Tifton Campus from June to September 2012. The greenhouse was maintained at 35±4^{o}C, with natural sunlight as the light source. In 2011 December, plant materials were vegetatively established into 7.65 cm round pots containing commercial potting media, and were grown to 90% coverage at a 3.8 cm canopy height prior to herbicide application. Plants were fertilized at a monthly rate of 2.4 g N m^{-2} with Miracle-Gro 20-20-20 Water Soluble All Purpose Plant Food (The Scotts Company, LLC, New York, NY) and irrigated as needed to maintain quality growth.

Plants were treated using three herbicide chemistries (sethoxydim x=280 g ai ha^{-1}, clethodim x=300 g ai ha^{-1}, fluazifop x=350 g ai ha^{-1}) applied using eight rates (0.25x, 0.5x, 1x, 2x, 4x, 6x, 8x,and non-treated) of each herbicide. Herbicides were applied with a mechanical spray chamber to give consistent application rates. The experimental design was a randomized complete block design with treatments replicated five times, and the experiment was repeated

once. The first trial was conducted 26 June 2012, and the second began 19 July 2012. At 21, 28, and 60 d after treatment (DAT) turf quality and health were evaluated by visual ratings of damage (1= 99% injury, 9= 0% injury) as well as with digital photographs that were analyzed for percent green cover with SigmaScan® as described by Richardson et al. (2001). Immediately after the visual and digital image ratings at 21 d, 28 d, and 60 d, leaves and stems growing above the 3.8 cm canopy height were trimmed and collected from all plants and fresh weights were measured. Each sample was dried 60^{o}C for two weeks, and then dry weight was measured. Results were used to identify the appropriate rates of herbicide to be used in subsequent field trials.

Field Experiment

Field experiments were conducted at two locations (+31º28'33.65", -83º31'43.55"; +31º28'59.10", -83º31'12.99") in Tifton, GA during 2012. Soil at both locations was Tifton loamy sand (fine-loamy, kaolinitic, thermic Plinthic Kandiudults). Tifway, common, and 93-175 bermudagrasses were established with vegetative plant material in a strip plot design with four replications in August 2011 and May 2012. The trials were planted so that a maximum of 15 herbicide/rate sub-plots could be evaluated per genotype (whole-plot) in each replication along with a control. Each genotype strip was 14.6 m x 1.8 m allowing for sub-plots of 0.9 m x 1.8 m. Turf was established to 95% coverage at each location before herbicide treatments began, and was managed with sprinkler irrigation and fertilized monthly at 4.9 g N m^{-2} with Rainbow 16-4-8 Plant Food (Agrium US Inc., Denver, CO) to maintain plant health. The experiments were mown weekly at 3.8 cm with a rotary mower.

Herbicide treatments consisted of (sethoxydim x=280 g ai ha^{-1}, clethodim x=300 g ai ha^{-1}, fluazifop x=350 g ai ha^{-1}) with a non-treated control applied at 1x, 2x, 4x, 6x, 8x with a CO_2-

pressurized backpack sprayer equipped with flat-fan nozzles delivering 140 L ha^{-1} at 165 kPa and 4.8 km h^{-1} on 11 September 2012. Turf quality and percent injury was evaluated by visual ratings (TQ, 1= dead, 5=acceptable, 9= excellent; % injury, 0%-99%) of damage as well as with photographs which were analyzed for percent green cover through SigmaScan® as described by Richardson et al. (2001) at 14, 21, 28, 35, and 42 days post treatment. Percent injury was determined for photographic data by dividing percent green cover of the treatment plot by the non-treated control within its replication. A second application of all treatments was applied on 8 November 2012 to both trials in the same manner as described above. Turf recovery during spring green-up of the turf was measured visually and with digital image analysis between March and April 2013.

DNA Extraction and Sequencing

Vegetative samples of approximately 0.5 g total of fresh leaf tissue were taken from 10-15 leaves of each accession. Fresh leaf tissue was place in a screw cap microcentrifuge tube with three ceramic grinding beads and 700 µL CTAB buffer and pulverized using a Retsch MM300 mixer mill (RETSCH Inc., USA). DNA was extracted according to the CTAB method (Reichardt and Rogers, 1997). DNA quality was verified by running an aliquot of each sample in a 1% agarose gel (DNA grade agarose [FischerBiotech]).

1. Primer Development and PCR Conditions

Two mutation containing regions were amplified. The first region contained the 1781 Ile to Leu mutation site and the second contained the 1999 Trp to Cys through 2096 Gly to Ala mutation sites. The region containing the 1781 Ile to Leu mutation was amplified using the sv384f (CGGGGTTTCAGTACATTTAT) forward primer and the sv384r (GATCTTAGGACCACCCAACTG) reverse primer (Heckart et al., 2010). The region

containing the 1999 Trp to Cys through 2096 Gly to Ala mutation sites was amplified using the POA3F (CATGTGATCCTCGTGCAG) forward primer and the WS1R (AGAACCTCCAGGAGACTAG) reverse primer (Tate, 2012). All primers were obtained from Integrated DNA Technologies (San Diego, CA, USA, http://www.idtdna.com/). PCR was performed using 20 nanograms of genomic DNA in a 20 μL total volume with a final concentration of 1X PCR buffer, 0.8 pmol μL^{-1} each primer, 0.2 mM dNTPs, 1.5 mM MgCl2 and 0.05 U μL^{-1} *Taq* DNA polymerase (Promega). The PCR conditions for the sv384f/sv384r primer pair were 4' at 95°C, 35x(30" at 95°C, 30" at 52.7°C, 30" at 72°C), 7' at 72°C (Heckart et al., 2010). The conditions for the POA3F/WS1R primer pair were 5' at 95°C, 35x(30" at 95°C, 30" at 54°C, 30" at 72°C), 7' at 72°C (Tate, 2012).

2. PCR Product Verification, Purification, and Sequencing

An aliquot of each PCR product was run in a 1% agarose gel (DNA grade agarose [FischerBiotech]) to verify a single product of the expected size for the primer pair used. The remaining PCR product was then purified using the DNA Clean & Concentrator 5 kit (Zymo Research Inc., Orange, Calif.). Each purified DNA sample was sequenced both forward and reverse. Briefly, a 15 μL volume containing 10 nanograms of purified DNA mixed with 25 pmol of corresponding primer were loaded in plates and shipped per instruction of GENEWIZ Inc. (South Plainfield, NJ). Sequences were analyzed using Geneious v5.4 software (Drummond et al., 2011). Sequences were aligned and compared to *Alopecurus myosuroides* Huds. nucleotide sequence AJ310767 as a reference with all known mutation sites annotated. Sequence data from all accessions were then translated and compared with the translation of *Alopecurus myosuroides* AJ310767 as a reference.

Statistical Analysis

Greenhouse data were subjected to a mixed model ANOVA, with trial as random factors, while bermudagrass types and herbicide rates were fixed factors. The relationship between amount of green cover and herbicide rates fit to a log-logistic regression model,

$$y = c + \left[\frac{d-c}{1+e^{\left(\frac{x}{I_{50}}\right)^{b}}}\right] \quad [1]$$

where y is the amount of green cover, d is the upper limit of the regression, c is the lower limit of the regression, x is the herbicide rate, I_{50} is the herbicide rate that provides median weed control, and b is the slope of the regression at I_{50} (Ritz et al., 2013; Seefeldt et al., 1995). An approximate $R^2_{nonlinear}$ (Askew and Wilcut, 2001; Jasieniuk et al., 1999) was determined using:

$$R^2_{nonlinear} = 1 - \left(\frac{Residual\ sum\ of\ squares}{Corrected\ total\ sum\ of\ squares}\right) \quad [2]$$

Data from field experiments were assessed with a histogram and normal probability plot. Pearson correlation coefficients were computed using the CORR procedure in SAS (SAS Institute Inc., Cary, NC) to test whether any of the traits were predictors of other characteristics. An analysis of variance was performed on each of the measured variables from the field trials with PROC GLM (SAS Institute Inc., Cary, NC) using a mixed model where trials and replications were considered random effects and genotype and herbicide treatment were fixed effects (Table 2.1). Genotypic and herbicide treatment means were separated using a Fisher's least significant difference (LSD) at the 0.05 level of probability where appropriate.

Results and Discussion

Spray Chamber Dose Response Experiment

Due to the correlation of computer analyzed green turf cover with both fresh clipping weight (0.63, P<0.001) and visual ratings (0.87, P<0.0001), only percent green turf cover as

determined by digital image analysis is reported here to describe herbicide injury observed on the bermudagrasses. There was a log-logistic relationship between the amount of green cover and herbicide rate for each of the tested herbicides and bermudagrass genotypes (Tables 2.2, 2.3, 2.4). Based on the green cover data obtained from photographic images, 93-175 was observed to have a slightly higher tolerance to sethoxydim (Figure 2.1), clethodim (Figure 2.2), and fluazifop (Figure 2.3) as compared to Tifway and common bermudagrass at 21 days after herbicide application. 93-175 had more green cover than either Tifway or common at all rates (1x-8x) of the sethoxydim (Figure 2.1) and fluazifop (Figure 2.3) herbicide treatments. This is confirmed by the log-logistic parameters for the genotypes within these herbicides. For sethoxydim, 93-175 exhibits an I_{50} of 1220 g ai ha^{-1} followed by Tifway (I_{50} = 833 g ai ha^{-1}) and common (I_{50} = 422 g ai ha^{-1}) (Table 2.2). Although the I_{50} values were similar for each genotype when treated with fluazifop, the minimum value *c* was 26 for 93-175, 20 for common and 0 for Tifway (Table 2.3) which demonstrates the potential for survival of 93-175 at higher rates of fluazifop. When increasing rates of clethodim were applied to the three bermudagrass genotypes, 93-175 exhibited higher tolerance up to the 6x (1800 g ai ha^{-1}) rate (Figure 2.2).

At the 60 day evaluation date (Figure 2.4), the 4x, 6x, and 8x sethoxydim herbicide treatments triggered an increase of vegetative growth in 93-175 when compared to that of the non-treated control and the other two bermudagrasses. The overall morphological response of 93-175 to initial sethoxydim injury can be further characterized by an increase in leaf size, elongation of internodes, and etiolation of the plant structure. With this information it was concluded to adopt the treatments used in the greenhouse experiment to confirm herbicide tolerance in larger plots under field conditions similar to those that turfgrass managers could encounter.

Field Experiment

1. Practical Application

The overall ANOVA (Table 2.1) shows that all "Trial" interactions were significant, however this was largely explained by the slightly higher herbicide injury levels observed in the second trial and not by changes in the rankings of the genotypes. It is theorized the later planting date of trial two caused this effect. Therefore, data from both field experiment trials were combined. Due to high correlation (0.97, $P<0.0001$) between visual and computer generated ratings, results of herbicide injury as measured by computer analyzed green turf cover were only reported.

For a herbicide resistant or tolerant turfgrass cultivar to have practical application, injury levels and length of recovery must be acceptable for end users (Radko, 1957). In these field experiments, 93-175 had significantly ($P<0.05$) less injury than both Tifway and common bermudagrass at the 1x field rate (280 g ai ha^{-1}) of sethoxydim on all evaluation days (Figure 2.5). At 14 d post application, 93-175 showed 27.5% injury compared to the non-treated control, whereas Tifway and common showed 60.5% and 67.2% injury, respectively. At 21, 28, 35, and 42 d post application 93-175 exhibited only 8.7%, 1.7%, 8.5%, and 1.2% injury, respectively. The three week period that 93-175 required to recover to pre-sethoxydim application turf cover is likely an acceptable timeframe for most turf managers who face problematic offtype bermudagrass contamination. At these same rating dates, Tifway (66.4%, 76.4%, 63%, 47.4%) and common (68.9%, 68.2%, 58.2%, and 50.9%) show significantly higher levels of injury and only began to slowly recover at 35 and 42 DAT. 93-175 exhibited the ability to recover faster and be actively growing well before the other two genotypes. These responses indicate that

turfgrass managers would be able to treat a stand of 93-175 with sethoxydim, reducing the ability of contaminating bermudagrasses to spread and thrive.

At higher sethoxydim rates, 93-175 also showed less injury than the susceptible genotypes at all rating dates. At a rate of 1120 g ai ha^{-1} (4x), 93-175 exhibited the ability to recover to a 9.7 % injury level at 42 days post treatment with a peak injury level at 14 days of 59.7% (Figure 2.6). At that same rating date, Tifway and common bermudagrass showed 89.9% and 81.8% injury with peaks at 21 days of 96.1% and 91.5% injury, respectively. 93-175 appears tolerant to excessive sethoxydim rates and would allow turf managers greater application flexibility if overlaps or miscalibration occurs.

2. Reduced Absorption/Translocation Tolerance

Even though 93-175 did not show a practical level of tolerance to field rate applications of both clethodim (Figure 2.7) and fluazifop (Figure 2.8), it did show a significant reduction ($P<0.05$) in injury as compared to common bermudagrass and Tifway at all but the 35d rating date in the clethodim herbicide treatment (Figure 2.7). No known mutations associated with resistance to ACCase herbicides in other weed and crop species were found when sequencing targeted gene regions of 93-175. No differences were seen in the 1781 Leu to Ile sequences of 93-175 and Tifway (Figure 2.9) or the 1999 Trp to Cys through 2096 Gly to Ala region (Data not shown). In other crops there have been reports of mutations conferring resistance to several herbicides in the ACCase family, an example being blackgrass (*Alopecurus myosuroides* Huds.) where resistance to fluazifop at (Leu-1781-Ile), clethodim at (Asp-2078-Gly), and sethoxydim at (Leu-1781-Ile) has been observed (Powles and Yu, 2010). 93-175 only had reduced injury to the three different herbicide chemistries and not complete resistance, suggesting a metabolic response or reduction in uptake of ACCase herbicide applications as the mechanism of tolerance

rather than a point mutation. Further study of 93-175 regarding the fate of radio-labeled ACCase herbicides after application or the inheritance of herbicide tolerance in offspring may clarify the mechanism of tolerance and provide a better understanding of the value of this genotype to turfgrass managers and the University of Georgia's turfgrass breeding program.

3. Spring Greenup

Genotypes were evaluated for spring recovery from dormancy in 2013 March and 2013 April following a second application of all herbicide treatments on 8 November 2012. On 24 April 2013 all non-treated control plots had reached full green up from winter. At this date, 93-175 displayed higher recovery rates than either Tifway or common bermudagrass at all rates of sethoxydim application. Recovery of 93-175 in the 280 g ai ha^{-1} treatment interestingly showed faster spring green-up than in the non-treated control, indicating that this herbicide treatment actually stimulated growth in this genotype (Table 2.5). It was observed during spring green up that 93-175 did not suffer increased injury to a second application of herbicide in late fall. The November 2012 application was applied before the onset of winter dormancy, and the resulting herbicide injury was similar to bermudagrass plant response to cooler temperatures, in which the turf turned off-color to a golden brown. This application timing could be a strategy used by turf managers to hide the injury caused by ACCase herbicide applications and subsequent discoloration from turfgrass users by sending the turf into pseudo dormancy prior to the first frost.

Spring green-up in 93-175 was only faster than Tifway and common bermudagrass in two of the fluazifop herbicide treatments (Table 2.6), which corresponded to the higher injury levels generally observed from clethodim and fluazifop as compared to sethoxydim. At 350 g ai ha^{-1} and 700 g ai ha^{-1} treatments of fluazifop, 93-175 exhibited greater levels of green-up than

both common and Tifway bermudagrasses. At the 300 g ai ha^{-1} treatment of clethodim, green-up in 93-175 was statistically greater than Tifway, but not common bermudagrass.

Conclusion

Renovating and replacing turf on a golf course may be an expensive and time consuming task. Loss of revenue plus labor and material costs of such a project can reach into the millions of dollars. Creating a turf variety with the ability to prolong the timeframe between renovations would benefit turf users. A herbicide resistant or tolerant bermudagrass that could give turf managers the ability to control contamination from both common bermudagrass and previously planted cultivars would increase potential for successful culture. Additionally, many golf and sporting complexes demand uniformity and the best playing conditions of their turf (Radko, 1957), and offtypes may reduce of quality and performance of their turf surfaces to unacceptable levels.

This research demonstrated 93-175 has the ability to tolerate herbicide applications that significantly injured non-tolerant varieties. While 93-175 does have some high quality turf characteristics, much of its value lies in that it is a fertile tetraploid and may have the ability to transfer herbicide resistance to offspring. Currently 93-175 is being crossed and its progeny will be evaluated for inheritance of herbicide tolerance. If the tolerance observed in 93-175 is highly heritable the incorporation into a sterile triploid would be a desired form of release of this technology by preventing the possibility of transfer of herbicide tolerance to weedy grasses. Further understanding of whether the mechanism of its tolerance is based on mutations that conferred a change of the active binding sites of ACCase inhibiting herbicides, or a genetic makeup that allows 93-175 to metabolize ACCase herbicides with less injury than other

bermudagrasses. These results would help in transferring tolerance traits to progeny and should be researched in the future.

Table 2.1: Split Plot model for percent green plot cover measured in field trials at Tifton Agricultural Experiment Station.

Source	df	Mean Square
Trial (T)	1	5217***
Error a, Rep(T)	6	566
Genotype (G)	2	44640***
G × T	2	1104***
Error b, G × Rep(T)	12	769
Herbicide Treatment (H)	15	368***
H × T	15	336***
H × G	30	1195***
H × T × G	30	165***
Error c, H × Rep(T × G)	270	137

***Significant at the 0.001 probability level.

Table 2.2: Parameter estimates for the log-logistic model that describes the amount of green area from digital analysis at 21 DAT regressed on rate of sethoxydim

Bermudagrass	Time of rating	I_{50}	s.e.	*b*	s.e.	*c*	s.e.	*d*	s.e.	R^2
93-175	21 DAT	1200	25	6.4	0.3	52	0.8	83	0.2	0.98
Common	21 DAT	422	48	6.9	2.0	43	2.2	83	2.0	0.77
Tifway	21 DAT	833	177	5.0	1.7	23	8.0	72	2.2	0.75

Table 2.3: Parameter estimates for the log-logistic model that describes the amount of green area from digital analysis at 21 DAT regressed on rate of clethodim.

Bermudagrass	Time of rating	I_{50}	s.e.	*b*	s.e.	*c*	s.e.	*d*	s.e.	R^2
93-175	21 DAT	645	192	3.7	1.1	10.0	10.0	72	3.6	0.73
Common	21 DAT	289	113	1.7	0.35	2.0	1.0	84	1.7	0.90
Tifway	21 DAT	420	66	3.3	0.6	0.0	5.0	69	2.6	0.89

Table 2.4: Parameter estimates for the log-logistic model that describes the amount of green area from digital analysis at 21 DAT regressed on rate of fluazifop.

Bermudagrass	Time of rating	I_{50}	s.e.	*b*	s.e.	*c*	s.e.	*d*	s.e.	R^2
93-175	21 DAT	370	400	1.5	0.7	26	18	85	3.2	0.68
Common	21 DAT	251	170	1.5	.06	20	11	78	2.8	0.76
Tifway	21 DAT	374	70	2.6	0.5	0	5	67	2.4	0.89

Table 2.5: Percent green turf cover of three bermudagrass genotypes treated with sethoxydim during spring dormancy recovery on April 23, 2013 in two herbicide field trials when control plots reached 100% recovery in Tifton, GA.

Genotype	Application Rates sethoxydim (x=280gai ha^{-1}).				
	1x	4x	6x	8x	10x
93-175	109.4a[1,2]	86.3a	86.7a	60.5a	81.7a
Common	48.5b	35.0b	22.1b	23.3b	15.9b
Tifway	60.8b	9.5c	7.6b	3.9c	4.1b

[1]% of non-treated control
[2]same letters in column denote non significance, Fisher's LSD,(P>0.05)

Table 2.6: Percent green turf cover of three bermudagrass genotypes treated with fluazifop and clethodim during spring dormancy recovery on April 23, 2013 in two herbicide field trials when control plots reached 100% recovery in Tifton, GA.

Genotype	Application Rates fluazifop (x=350gai ha^{-1})				
	1x	2x	4x	6x	8x
93-175	74.8a[1,2]	35.7a	7.9a	1.6a	1.7a
Common	14.6b	1.9b	1.3a	1.1a	2.4a
Tifway	5.7b	0.5b	0.3a	0.5a	0.2a
	clethodim (x=300gai ha^{-1})				
	1x	2x	4x	6x	8x
93-175	45.5a	23.5a	9.9a	21.6a	10.6b
Common	32.9a	25.5a	11.8a	22.6a	18.4a
Tifway	6.5b	4.8b	5.0b	3.0b	1.6c

[1]% of non-treated control
[2]same letters in column denote non significance, Fisher's LSD,(P>0.05)

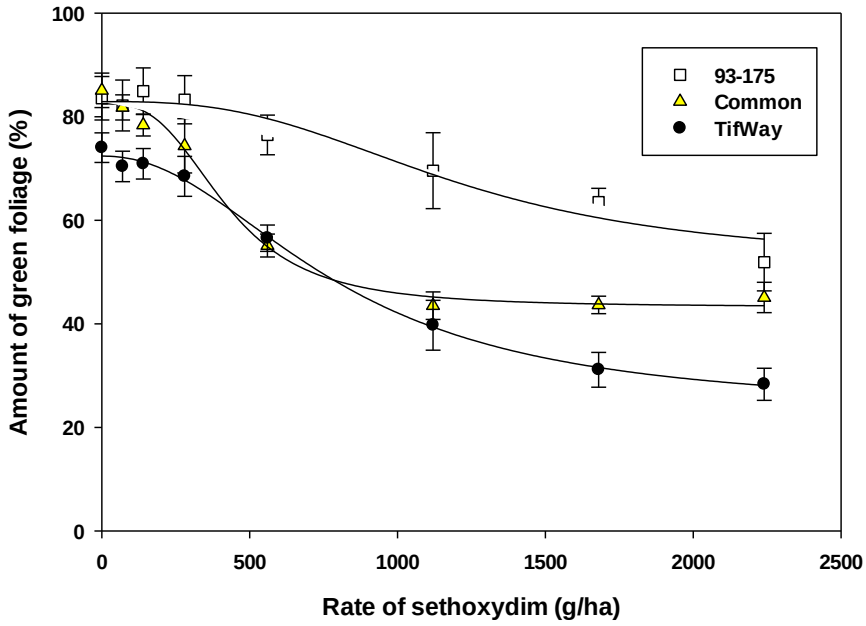

Figure 2.1: Percent green cover of three bermudagrass genotypes at 21 DAT with sethoxydim (x= 280 g ai ha^{-1}) rates in two greenhouse trials conducted during 2012 in Tifton, GA. (Vertical bars represent standard error.)

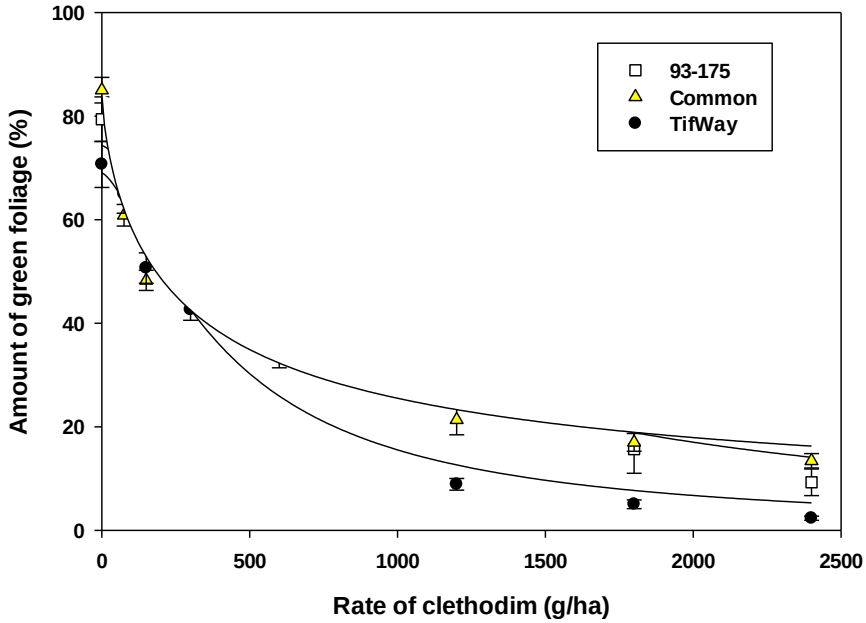

Figure 2.2: Percent green cover of three bermudagrass genotypes at 21 DAT with clethodim (x= 300 g ai ha^{-1}) rates in two greenhouse trials conducted during 2012 in Tifton, GA. (Vertical bars represent standard error.)

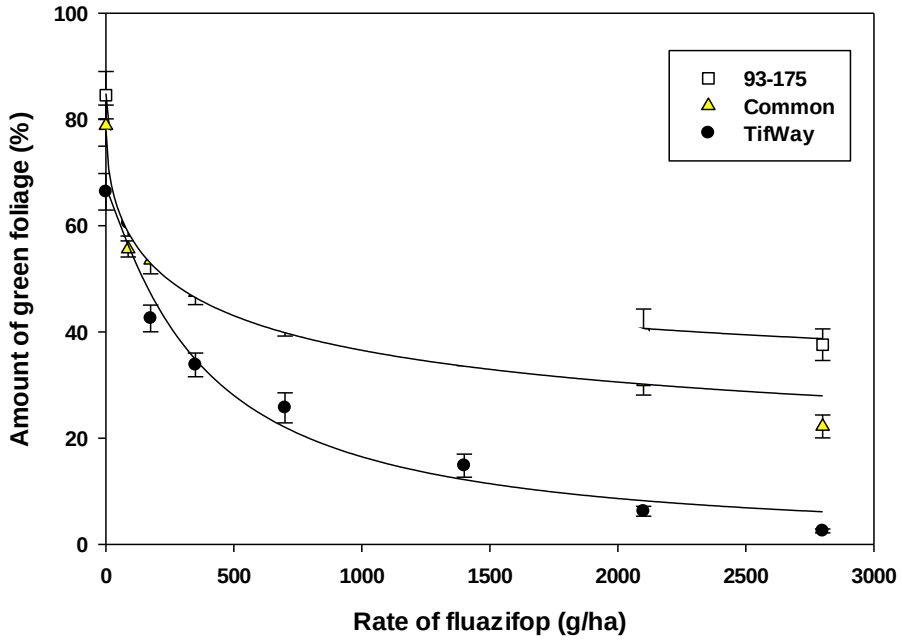

Figure 2.3: Percent green cover of three bermudagrass genotypes at 21 DAT with fluazifop (x= 350 g ai ha^{-1}) rates in two greenhouse trials conducted during 2012 in Tifton, GA. (Vertical bars represent standard error.)

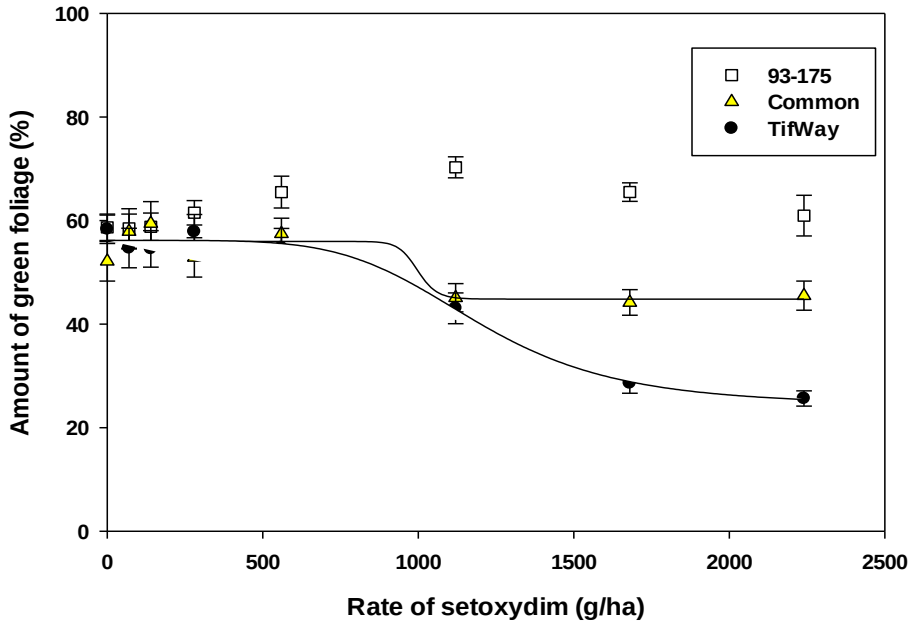

Figure 2.4: Percent green cover of three bermudagrass genotypes at 60 DAT with sethoxydim (x= 280 g ai ha^{-1}) rates in two greenhouse trials conducted during 2012 in Tifton, GA. (Vertical bars represent standard error.)

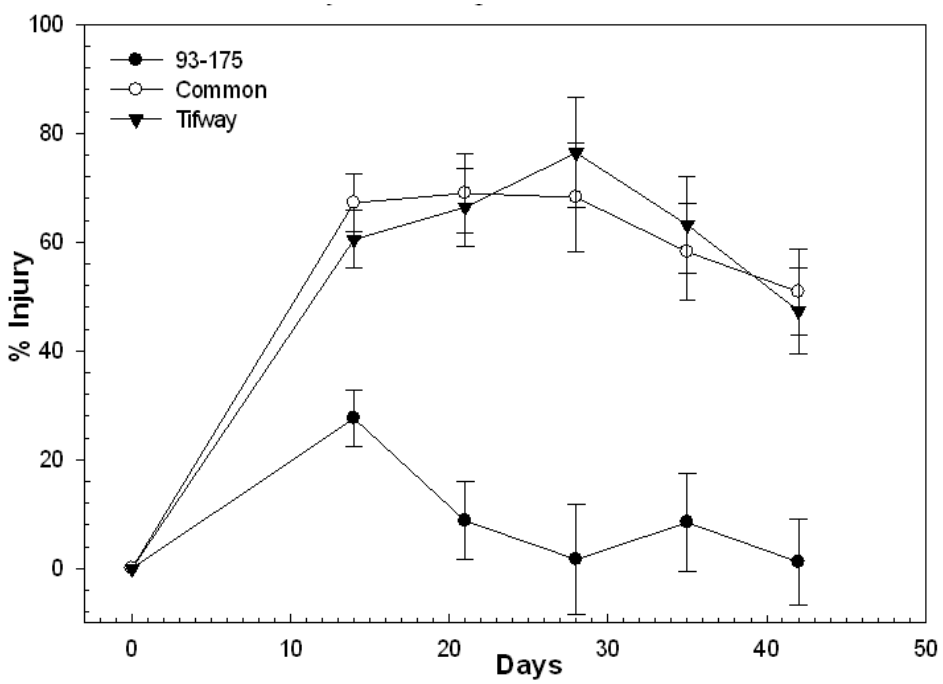

Figure 2.5: Percent injury (compared to the non-treated control) of three bermudagrass genotypes over 42 days after sethoxydim application at a rate of 280 g ai ha^{-1} in two field trials conducted during 2012 in Tifton, GA. Vertical bars represent Fisher's LSDs (P<0.05) on each respective rating date.

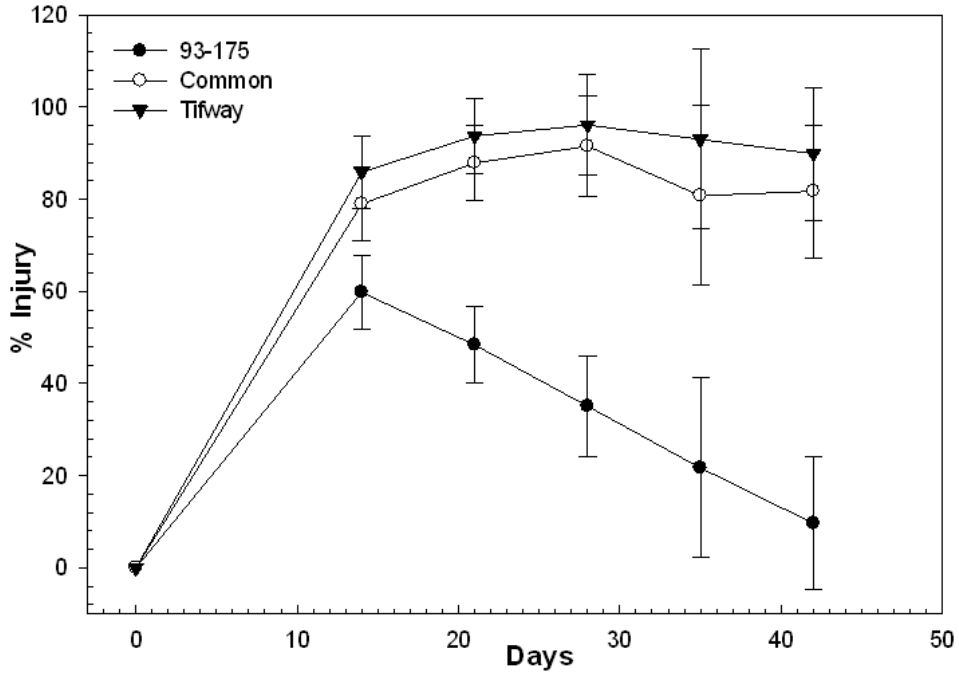

Figure 2.6: Percent injury (compared to the non-treated control) of three bermudagrass genotypes over 42 days after sethoxydim application at a rate of 1120 g ai ha^{-1} in two field trials conducted during 2012 in Tifton, GA. Vertical bars represent Fisher's LSDs (P<0.05) on each respective rating date.

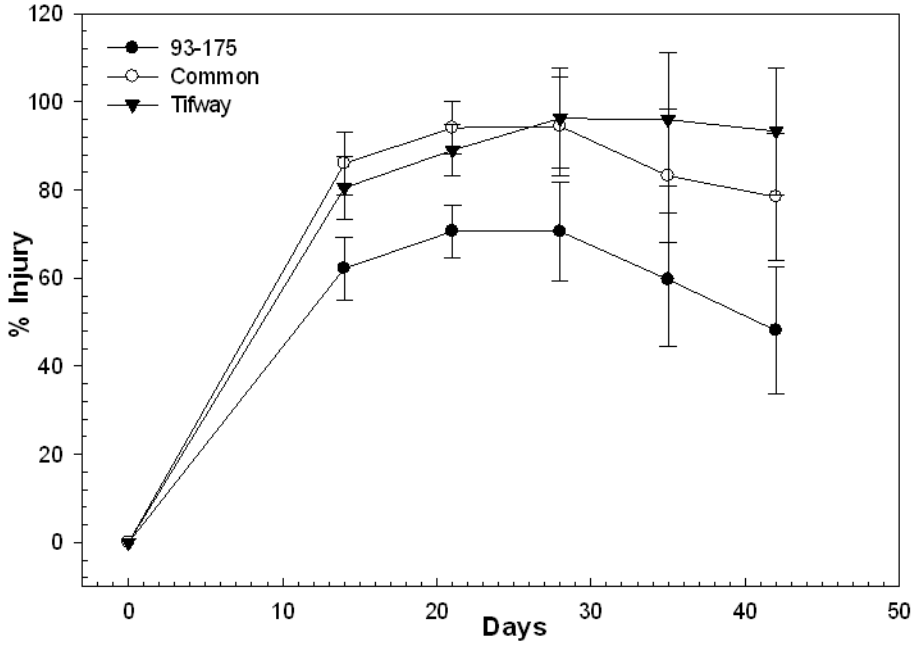

Figure 2.7: Percent injury (compared to the non-treated control) of three bermudagrass genotypes over 42 days after clethodim application at a rate of 300 g ai ha^{-1} in two field trials conducted during 2012 in Tifton, GA. Vertical bars represent Fisher's LSDs (P<0.05) on each respective rating date.

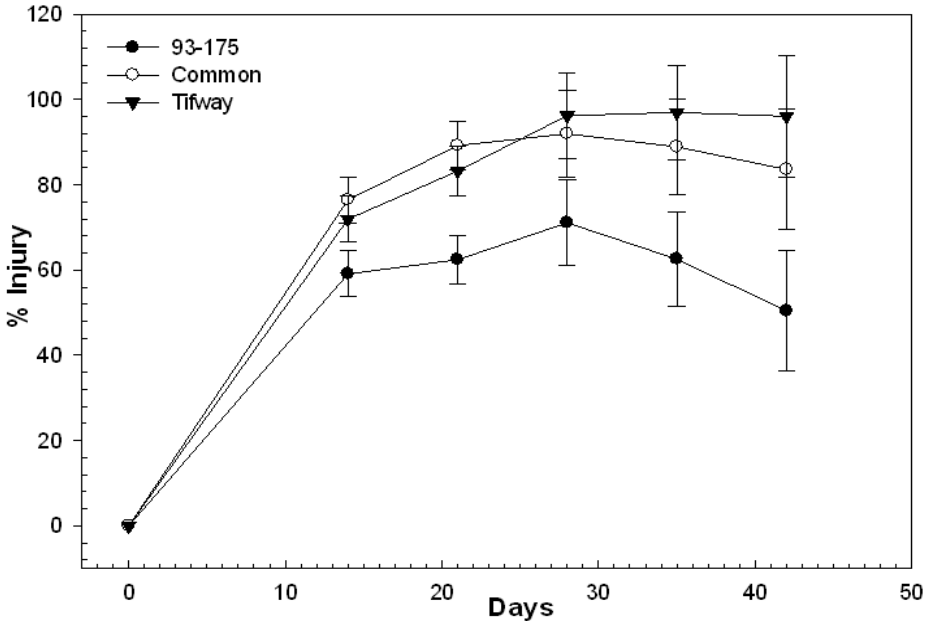

Figure 2.8: Percent injury (compared to the non-treated control) of three bermudagrass genotypes over 42 days after fluazifop application at a rate of 350 g ai ha^{-1} in two field trials conducted during 2012 in Tifton, GA. Vertical bars represent Fisher's LSDs (P<0.05) on each respective rating date.

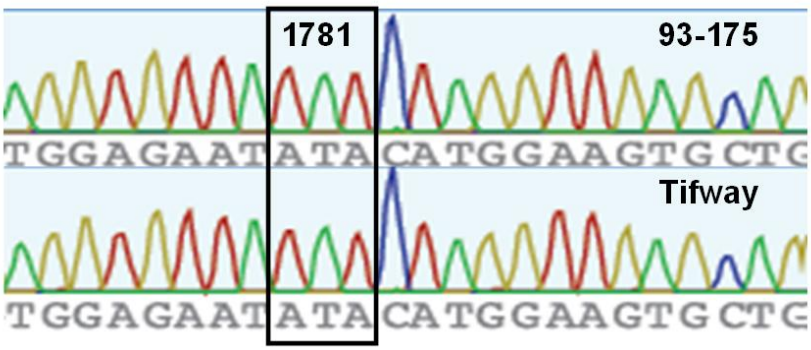

Figure 2.9: Comparison of the sequence read showing no difference of the 1781 Ile to Leu mutation sight of ACCase gene in two bermudagrass genotypes used in greenhouse and field trials in Tifton, GA 2012.

CHAPTER 3

CALLUS INDUCTION IN DT-1 BERMUDAGRASS

Introduction

Improvement of germplasm by traditional breeding methods and selection is often limited by the genes that are represented in a collection that are available for recombination. The technique of using tissue culture and somaclonal variation to create mutations was previously evaluated at the University of Georgia and was found ineffective compared to traditional breeding from a physiological standpoint (Goldman et al., 2004). Tissue culture is time consuming and expensive. However, for a novel trait such as Acetyl-CoA carboxylase (ACCase) resistance the methodology appears useful (Rowe, 1986).

ACCase resistance has been achieved in seashore paspalum (*Paspalum vaginatum* Sw.) through tissue culture and selection of callus from media containing sethoxydim (Heckart, 2010). In bermudagrass (*Cynodon dactylon* (L.) Pers.) however, there have been challenges in the past to develop an efficient protocol to produce plants from tissue culture. Zhang et al. (2007) determined that a universal protocol for inducing callus in different bermudagrass genotypes has not been successful, and that techniques specific to each genotype likely need to be developed. Objectives of this experiment were to develop an in vitro tissue culture and selection protocol to obtain callus from DT-1 bermudagrass genotype which will aid in the future identification of plants that confer resistance to ACCase inhibiting herbicides.

Materials and Methods

Calli production

Immature inflorescences were obtained from the University of Georgia triploid ($2n = 3x = 27$) bermudagrass germplasm line DT-1 in the spring and summer months of 2012 to initiate calli production. Immature inflorescences were identified by the bulging shape of the leaf sheath as early as possible to encourage a higher frequency of calli production (Fig. 3.1A). Inflorescence leaves were removed, but remained in the sheath along with the flag leaf and first node below inflorescence. Previous studies had no report of keeping this node (Goldman et el., 2004; Zhang et al., 2007). The inflorescences were placed in a deionized water wash for one hour prior to 70% ethanol for one minute, followed by 20% bleach (containing 6.15% sodium hypochlorite) for 20 minutes. Five 10- minute sterilized water rinses followed the 20 minute bleach treatment. Once sterilized, the inflorescences were chopped into 3-5 mm sections. These sections were plated on small (100 mm×15 mm) round petri dishes that contained the callus inducing media (Zhang et al., 2007). This media consisted of MS basal medium (Murashige and Skoog, 1962) amended with MS vitamins, 2,4-dichlorophenoxy acetic acid (2,4-D), 0.01 mg l^{-1} 6-Benzyladenine (BAP), plant preservative mixture (PPM), sucrose, and adjusted to a pH of 7.8 (Appendix A). The segments were cultured at 25^{o}C in a growth chamber for 21-28 days in the dark, and then placed on fresh medium.

Regeneration

After 50 days on the induction media, a pretreatment with low light (30 µmol m^{-2} s^{-1}) was imposed on the calli for 10 additional days with a 16/8 h day/night photoperiod. Then calli were transferred to MS medium containing 0.1 mg/0 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP for shoot regeneration. Regeneration was conducted at 25^{o}C under light (100 µmol m^{-2} s^{-1} from cool white

fluorescent bulbs with 16/8 h day/night photoperiod) in a growth chamber. Healthy calli during the regeneration period were transferred to new medium every 21 days.

Results and Discussion

There is not a documented method to effectively induce bermudagrass flowering in the greenhouse or growth chamber. Taliaferro (2003) surmised that bermudagrass flowering in the spring is a result of longer daylengths, and possibly increasing temperatures. The greatest seedhead density in these species is usually found during an initial flush in the spring, although flowering in most bermudagrass genotypes is indeterminate in nature and can last throughout the summer and fall. Flowering in DT-1 bermudagrass in Tifton, GA was more prolific than observed in 'Tifway' (Burton, 1966) in adjacent plots and the greenhouse during 2012.

Initially, inflorescences plated on induction medium were developing 100 percent bacterial contamination after one week when placed in the growth chamber. This level of contamination was not reported before (Zhang et al., 2007). Media containing plant preservative mixture (PPM) delayed contamination 2-3 days, but severity was similar. A one hour deionized water bath prior to sterilization delayed onset of bacteria also. The Tifton, GA area was experiencing a very wet and humid summer and it was theorized the levels of bacterial contamination in the field could be at higher concentrations. Numerous methodology including removing the inflorescence from its sheath prior to and after sterilization, and chopping the inflorescence into 3-5mm sections prior to sterilization were tried until the discovery that leaving the flag leaf and first node of the inflorescence intact along with the sheath during sterilization completely inhibited the onset of any contamination. Keeping the first node proved vital for calli production, and the removal of the inflorescence from the sheath or damaging the sheath prior to sterilization should be prevented.

After contamination issues were resolved, it was observed that all (100%) calli produced using DT-1 as explant material was formed from the single node below the inflorescence that was included during induction. The explant material that only contained the upper inflorescence sections produced no viable calli. In total 80 calli were maintained and underwent light treatment prior to regeneration techniques. A pretreatment with low light has been shown to improve calli performance in regeneration 6-8 fold (Zhang et al., 2007). Of those 80 calli that were placed into regeneration, 27 (33.8%) were white soft callus (Type I), 30 (37.5%) were globular sticky callus embedded in a watery membrane (Type II), and 23 (28.7%) were globular sticky that developed into transparent compact callus (Type III). Of the compact callus, three (1.3%) were white compact callus (Type IV). The type I and II callus without compact callus developed green spots but quickly turned necrotic. Compact callus developed green spots and were healthy with Type IV callus also developing fine white strands. After three weeks, healthy callus was transferred to media containing 0 mg l^{-1} 2,4-D to induce shoot production. However, after four weeks in the regeneration stage the compact calli also turned necrotic without shoot development, likely due to improper levels of either 2,4-D or BAP within the media. If shoots had been observed on the calli, rooting would have been initiated on MS or ½ MS medium without growth regulators (Appendix A).

Conclusion

Prior to an experiment being performed to develop ACCase resistant DT-1 bermudagrass, a stable and consistent tissue culture program needs to be developed. The finding of a consistent callus induction procedure was promising, but regeneration was unsuccessful. Methods to induce successful regeneration in DT-1 bermudagrass need to be optimized prior to conducting experiments to create somaclonal variation to discover mutations that confer herbicide resistance.

If a highly unusual mutation in a single callus is found, it would be wasted without the regeneration step. Researchers would need to take in account the large amount of time required to develop a tissue culture program capable of successfully inducing resistant calli that also contain no other detrimental mutations.

If the development of an ACCase herbicide resistant plant were released to turf mangers, the effectiveness could be on a limited basis for some weed species. An education program that is incorporated into the release of a herbicide resistant cultivar would help prolong the effectiveness of the resistant characteristic by limiting the situations in which weed resistances have been observed to develop. It would be most advantageous if the cultivar had adequate resistance to more than one herbicide within the family, but in most cases it is suggested to rotate herbicides from multiple families. The strategy to remove unwanted common bermudagrass would be suggested for use within areas where other control methods have failed and herbicide application is the only alternative to renovate the area. Heckart (2010) described plant material that expressed minimal initial injury to herbicide applications, but turf managers should also be made aware that there is a possibility they may see some level of damage occur to material produced through these methods. However, decline in turf quality may be acceptable over the effects of glyphosate and replacement. Because ACCase herbicides may take up to 21 days before complete control is achieved, studies which compared the time frame of varying methods needed to remove weedy species would be beneficial. Although preliminary experimentation to induce callus from seed and other explant material has been unsuccessful to date, work will continue to discover the most efficient techniques possible to induce mutations which confer ACCase herbicide resistance in bermudagrass.

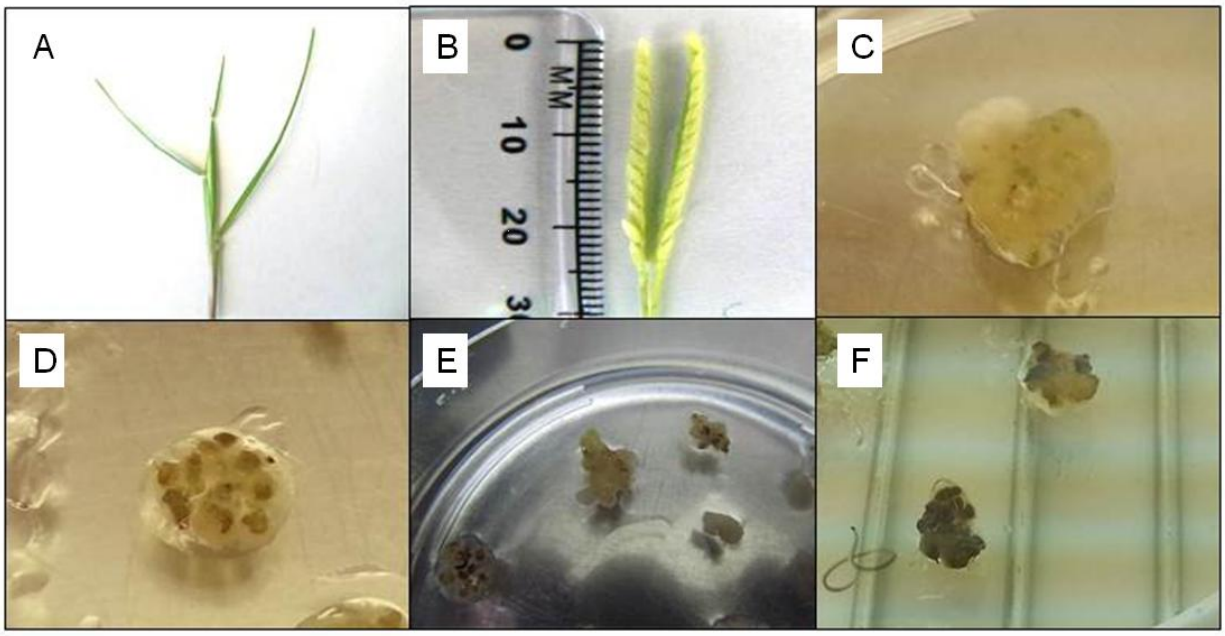

Fig. 3.1: Immature inflorescence (A); Inflorescence removed from sheath (B); Globular sticky callus (Type II) with compact white callus (Type IV) exhibiting green spots (C); Globular sticky callus in watery membrane(Type II) (D); Calli in regeneration media (E); Necrotic calli (F).

CHAPTER 4

INFLUENCE OF SOIL TYPE ON NITROGEN LEACHING OF CONTROLLED RELEASE FERTILIZERS[1]

[1]Grimshaw A.L., B.M. Schwartz, P.L Raymer, A.R. Kowalewski, and T.L. Grey. To be submitted to *Applied Turf Science.*

Abstract

Misuse of nitrogen fertilizers has become an ever increasing problem in management of turfgrass lawns and fields. The environmental impacts of these practices are especially evident in sandy soils that are low in organic matter which can bind nitrogen, or in new turfgrass stands that have not developed mature root systems which are capable of nutrient uptake before leaching occurs. Controlled and slow release fertilizers are designed to release nitrogen over a longer period of time which should improve the efficiency of nitrogen use and reduce leaching. A lysimeter study was performed at the University of Georgia to determine if an experimental fertilizer (E 15-0-0) would reduce leaching compared to UMAXX (47-0-0) and an analog fertilizer (16-4-8). Leached and soil retained nitrate-nitrogen (NO_3-N) and ammonium-nitrogen (NH_4-N) samples were collected from a USGA green's mix, Florida soil, and Maryland soil. The experimental fertilizer leached more NO_3-N than the other fertilizer types in all soils. The analog fertilizer leached more NH_4-N during both trial years in the Florida and USGA soils. Soil retention of NO_3-N and NH_4-N nitrogen was more variable than leachate data with less uniformity of treatment results, and was more affected by plant health and environmental factors. However, the E 15-0-0 fertilizer did show higher retention of nitrate in the Florida and USGA soils in 2012. Developing an improved controlled release fertilizer would decrease the likelihood of leached nutrients. Further study should be performed in the field to more accurately simulate real world conditions that affect fertilizer application and fate to effectively evaluate nitrogen loss characteristics of these fertilizers.

Introduction

Interest from both the public sector and various political groups has grown in recent years regarding the contamination of water resources in the United States. Prior to this concern,

consumer expectations for turfgrass quality and performance in home lawns and landscapes began to rise, leading to greater fertilizer use and water consumption (Osmond and Hardy, 2004). Turfgrass landscapes have been targeted by environmental agencies as a point source of water contamination due to the use and possible runoff or leaching of nitrogen fertilizers (Duncan and Carrow, 2000). This issue is prevalent in areas such as the Coastal Plains of the United States that have sandy soils, which are prone to leaching due to basic soil dynamics and their lower water holding capacity (Wang and Alva, 1996). The issue of groundwater contamination is further amplified as microbes in the soil break down ammonium (NH_4-N) into nitrate (NO_3-N), a negatively charged particle that is more easily flushed out of a soil system during water infiltration due to reduced affinity with the soil (Wolkowski, 1995).

Nitrate (NO_3-N) from turfgrass fertilization is one of the proposed sources of groundwater contamination, but researchers have hypothesized that turfgrass can reduce groundwater contamination by reducing runoff and erosion, while promoting metabolism of chemicals before they infiltrate the soil (Erickson et al. 2001). Research has demonstrated that improper irrigation and fertilization, specifically the over-use of quick release fertilizers on turfgrass systems, can lead to excess leaching and groundwater contamination (Petrovic, 1990). To combat this occurrence, researchers have focused on developing best management practices that mitigate the improper use of inputs (Dietz et al., 2004).

Fertilizer developers have improved slow and controlled release fertilizers to combat the issues of water contamination as awareness has increased. These fertilizers use less labor resources and were first developed to reduce the dependence on quick release fertilizers that required a shorter interval between applications (Robbins, 2005). As an added benefit, slow and controlled release fertilizers also were shown to reduce nitrogen leaching (Killian, 1966). Brown

et al. (1982) found that NO_3-N levels leached from golf course greens dropped from 8.6% - 21.9% to 0.2% -1.6% when slow-release sources such as isobutylidene diurea and ureaformaldehyde were used instead of ammonium nitrate.

Both field and greenhouse methodologies have been developed to collect and quantify nitrogen which has been leached from a soil/root system. Lysimeters of varying sizes have primarily been used in these methodologies. Some studies have used pots with holes drilled in the sides (Saha et al., 2007), while others have used polyvinyl chloride (PVC) pipes (Grey et al., 2009). Additionally, water samples from underground basins have been collected in the field (Erickson et al., 2008), and from drains under golf course greens (Lisi et al., 2004).

Fertilizer companies have a growing interest in developing environmentally friendly products that meet the expectation of public and private stakeholder groups. The objective of this research was to conduct a greenhouse lysimeter nitrogen leaching study to evaluate the nitrogen leaching potential of an experimental (E 15-0-0) and proprietary (UMAXX) 47-0-0 controlled release fertilizers versus a generic (analog) 16-4-8 granular fertilizer in three growing mediums: native Florida soil, Chesapeake Bay Maryland soil, and a United States Golf Association (USGA) specified root zone mix.

Materials and Methods

Experimental Design

Nitrogen leaching characteristics of three fertilizers, an experimental controlled release fertilizer (E 15-0-0), a proprietary controlled release fertilizer (UMAXX) 47-0-0 (AGROTAIN International, L.L.C., St. Louis, MO), and a generic fertilizer (analog) 16-4-8 (10.3% ammonical nitrogen, 5.7% urea nitrogen) were studied in comparison to an unfertilized control during two greenhouse trials at the University of Georgia, Griffin and Athens campuses. Trial one was

performed between 22 July 2011 and 26 August 2011, and trial two between 6 March 2012 and 17 April 2012. Lysimeters were used to study leachate contents collected from three soils, a (Florida) Immokalee fine sand (sandy, siliceous, hypothermic Aremic Alaquods) , a (Maryland) Annapolis sandy loam (fine-loamy, glauconitic, mesic Typic Hapludults), and a (USGA) green's mix which has maximum 10% coarse sand, a minimum of 60% medium, maximum 20% fine sand particles, and no more than 5% silt and 3% clay (Tables 4.1 and 4.2). The experimental design was a 4 by 3 factorial, with the three fertilizer treatments and soil types organized in a randomized complete block with two replications (Table 4.3).

Leachate Collection and Analysis

The lysimeter columns were 10.2 cm by 30.5 cm design, with a funneling cap on the bottom made from polyvinyl chloride (PVC). Leachate was funneled using rubber tubing into 750 ml bottles placed below the racks holding the lysimeters. Each of the three soils was added to the lysimeters to obtain a bulk density of 1.4 to 1.5 g cm^{-3} for consistency by compacting as they were filled. The field capacity of each lysimeter was determined prior to the experiments by calculating the difference in weight between each when completely dry, and after they had been allowed to drain for 24 hours following an irrigation event that brought soil water to field capacity. Kentucky Bluegrass (*Poa pratensis* L.) cv. 'Everest' was seeded (14.5 g m^{-2}) and allowed to reach 70% coverage at the three leaf stage before initiation of fertilizer treatments, and was maintained at a height of 7.6 cm by clipping every three days. The four fertilizer treatments were applied at 4.9g N m^{-2} every week for six consecutive weeks. All treatments were applied using 100 ml of water as a carrier to uniformly distribute over the entire lysimeter surface. Irrigation was applied daily to the lysimeters to maintain plant health at field capacity in the columns by applying water until a small amount (3-5 ml) of water leached from the bottom

of the column, any leachate volume that occurred during the week was recorded before disposal. 250 ml to 350 ml of irrigation was applied to each column every 7 days until 250 ml of leachate was recovered. Two 125ml high-density polyethylene (HDPE) amber bottles (Thermo Fisher Scientific, Mass.) were filled with the leachate from each column and frozen at 4°C for no longer than 21 days. One leachate sample was tested for nitrogen content, including (NO_3–N) and (NH_4-N) while the second was held in reserve in cold storage at -20°C. Analysis was performed at the University of Georgia Soil, Plant, and Water Analysis Laboratory, Athens, Ga. Milligrams of nitrogen in the leachate were determined by converting the parts per million (ppm) level of each sample based on the amount of water recovered per column during that week for the leachate data. Soil columns were allowed to dry for three weeks at the conclusion of each six week leaching experiment, the entire soil column was removed from the lysimeter and split into thirds, and soil samples were taken from the upper 10.2 cm, middle 10.2 cm, and lower 10.2 cm of each column. Samples were then analyzed for (NO_3–N) and (NH_4-N) content, and reported as averages from the three sections in mg kg^{-1} of soil.

Statistical Analysis

The distribution of data for cumulative (NO_3-N) and (NH_4-N) recovered in the leachate over the course of the six week experiment, as well as that which was retained in the soil, was assessed with a histogram and normal probability plot for normality. An analysis of variance was performed on each of the measured traits to test whether fertilizer treatments and soil types varied (Table 3). Fertilizer treatments and soil types were separated using a Fisher's least significant difference (LSD) at the 0.05 level of probability.

Results and Discussion

Cumulative Leachate Nitrate-Nitrogen (NO_3-N) and Ammonium-Nitrogen (NH_4-N)

Leachate collected weekly throughout the study was analyzed for differences at each sampling date for effects due to fertilizer source in each of the three soils to access the amount of nitrogen lost from each lysimeter (data not shown). Results are reported as a cumulative six week total for the experiment. In the overall analysis of variance for NO_3-N (Table 3), soil and treatments were significant at ($P \leq 0.01$). Trial interactions were not significant, thus results are combined for the two years.

Soils responded differently ($P \leq 0.01$) in the amount of leachate collected based on the treatments. In all cases the mean NO_3-N leachate from the E 15-0-0 treatment was the greatest but was not significant in the Maryland sandy loam soil (Table 4). In the Florida soil, fertilizing with the E 15-0-0 resulted in leachate containing a total of 34.4 mg NO_3–N, approximately seven times that found in the leachate collected from the other treatments. Leachate from lysimeters fertilized with the analog (4.8mg NO_3-N) and UMAXX (4.6mg NO_3-N) products did not contain a statistically different amount of NO_3-N than found in the unfertilized control (1.4mg). The overall leachate collections of NO_3-N in the USGA sand were higher than found in the Florida sand and may be explained by the higher percentages of smaller particles (8.2% silt, 2% clay) in the Florida sand soil. A higher concentration of silt and clay has been shown to be correlated with reduced NO_3-N concentrations in leachate from different sources of nitrogen (Guertal and Howe, 2012). The highest losses of NO_3-N were found in leachate collected from the E 15-0-0 treatment (120.8mg NO_3-N), followed by UMAXX (44.1mg NO_3-N), analog (25.2mg NO_3-N), and the unfertilized control (2.8mg NO_3-N). Leachate from the Maryland sandy loam soil contained the highest mean concentrations of NO_3-N of the three soils, although variability

prevented statistical separation from the other fertilizer treatments. The Maryland sandy loam soil was composed of 18% silt, 12.1% clay and more than double the organic matter of either the Florida sand or USGA sand (Table 4.2). Strictly based on soil classification and organic matter content, the Maryland sandy loam soil should have a greater ability to prevent leaching. However, the initial soil analysis of the Maryland sandy loam soil indicated it contained 28.08mg kg^{-1} NO_3–N as opposed to 0.93mg kg^{-1} and 1.29mg kg^{-1} NO_3–N in the USGA sand and Florida sand soils, respectively (Table 4.1). The initial NO_3-N level may have been high enough to saturate the CEC of the Maryland sandy loam soil, thereby minimizing the possibility for soil binding to occur when fertilizer was applied, which would allow the flushing of NO_3-N particles in a leaching event.

For leached NH_4-N (Table 4.5), all interactions were significant at $P \leq 0.01$ (Table 4.3), thus results are reported separately for soil types and trials conducted in 2011 and 2012. Generally, the results for NH_4-N leachate in the Florida and USGA soils were very similar, with levels in 2012 being more pronounced than in 2011. In both of these soils, all fertilizer treatments leached statistically equal amounts of NH_4-N during 2011, ranging from 20.1mg to 30.0mg in the Florida sand soil and 20.5mg to 31.8mg in the USGA sand. During 2012, the greatest amount of NH_4-N was leached from the analog fertilizer treatment, followed by the E 15-0-0 fertilizer, and finally the UMAXX in both the Florida sand and USGA sand soils. In comparison, the Maryland sandy loam showed lower average amounts of NH_4-N leaching than observed in the other two soils. Differences in the magnitude of NH_4-N leaching in loamy sands and clay soils have been observed when compared to sandy soils (Guertal and Howe, 2012). The analog treatment showed the largest variability from 2011 to 2012 in the Maryland sandy loam soil, and as a result, significant differences between fertilizer treatments were only observed

during 2011, although the level and range of these differences was very small compared to the NH_4-N leaching seen during 2012 in the Florida sand and USGA sand soils. Because the analog treatment is a granular formula, there is a greater chance for unequal distribution of nitrogen in a small area. Another possibility is that analog applications made during 2011 may have contained less nitrogen by weight than during 2012 due to unforeseen sampling error when measuring out only a few grams of a granular fertilizer to be applied.

Soil Nitrate-Nitrogen (NO_3-N) and Ammonium-Nitrogen (NH_4-N)

Results for residual soil NO_3-N and NH_4-N content were not consistent between soils and are also reported separately for 2011 and 2012 due to variation between the years (Table 4.3). No differences were seen during 2011 in the Florida sand and USGA sand soils for soil NO_3–N retention (Table 4.6). In 2012 there were differences in which the E 15-0-0 fertilizer exhibited retention of NO_3–N in the Florida sand soil (11.6 mg kg^{-1} NO_3–N) that was significantly different from the other three treatments: UMAXX (1.7mg kg^{-1} NO_3–N), analog (0.3mg kg^{-1} NO_3–N), and control (0.2mg kg^{-1} NO_3–N). The same trend was observed in the USGA sand soil with the E 15-0-0 in 2012 having significantly higher NO_3–N content (15.0 mg kg^{-1} NO_3–N) compared to UMAXX (6.6 mg kg^{-1} NO_3–N), although in this soil the analog (0.3mg kg^{-1} NO_3–N) and control (0.2mg kg^{-1} NO_3–N) were significantly lower than both. In the Maryland sandy loam soil, differences were seen both years, however greater amounts of nitrate again were retained in the 2012 trial. During 2011, the UMAXX fertilizer treatment resulted in the highest mean NO_3–N soil concentration (3.0mg kg^{-1} NO_3–N) and the E 15-0-0 was the lowest (1.4mg kg^{-1} NO_3–N). Conversely, in 2012 the E 15-0-0 treatment had the highest mean concentration (11.1mg kg^{-1} NO_3–N) and the control was the lowest (0.2mg kg^{-1} NO_3–N).

For residual soil NH_4-N, differences among fertilizer treatments were found in both years with a trend of higher concentrations in 2012 over 2011 being observed (Table 4.6). In the Florida sand soil, UMAXX showed the highest retention in both years. The Florida sand soil showed the largest changes between years also. Differences in NH_4-N retention in the other two soils were significant for some of the treatments, but were not as great in magnitude.

Overall, the trend for soil retention of nitrogen was greater in 2012 than 2011. Anecdotally, there was poorer plant health in terms of lower biomass and necrosis during the 2012 experiment when compared to 2011, primarily attributed to increased stress from insect pressure. If plants were not as healthy in 2012, this could lead to a situation where they would not be using all the available nutrients in the soil during the dry down period at the conclusion of the study. Non-stressed turf is essential to prevent the loss of nitrogen after a fertilizer application. Bowman et al. (1989) reported that after 48 hours, 75% of applied ammonium was absorbed by a turfgrass stand in good condition. Because fertilizer applications during a time of high stress could lead to an inefficiency of the plant to utilize nitrogen, it would be beneficial to educate home owners and turfgrass managers about proper fertilization practices that could promote turf health with minimal environmental risks.

Conclusion

The E 15-0-0 fertilizer leached the largest amounts of NO_3-N on average from the two trials performed in 2011 and 2012. Designed as a possible new controlled fertilizer, it is evident the proprietary experimental mixture underperformed from its predicted results of reducing NO_3-N leaching. Do to the unknown composition of this fertilizer it is impossible to speculate the reasons for negative results. For NH_4-N, the analog fertilizer showed the highest amounts of leaching in the Florida sand and USGA sand soils, indicating an inefficiency for conversion to

the more plant-available form of NO_3-N. Residual NO_3-N and NH_4-N remaining in the soil at the conclusion of the tests may have been more dependent on plant health than soil type. The treatments showed similar levels of retention in all soil types. However, the E 15-0-0 fertilizer did retain more NO_3-N in 2012 within both the Florida sand and USGA sand soils which would show an ability to retain plant available nitrogen over time, while UMAXX retained the highest NH_4-N in the Florida sand soil for both trials. Many environmental factors can affect the plant utilization, or possible leaching of a fertilizer. Input from turf managers would be important to determine the timing and environmental conditions which are commonly considered when applying fertilizer so that additional experiments can be designed to further study the impact of these fertilizers in conditions outside of the scope of this research.

Table 4.1: Soil type composition of three soils prior to nitrogen leaching studies conducted at the University of Georgia during 2011 and 2012.

Soil Name	Soil Type	pH	Ca	K	Mg	Mn	P	Zn	NO_3-N
			----------	----------	----------	mg kg^{-1}	----------	----------	----------
USGA	Sand	6.43	64.1	7.01	24.78	1.78	2.02	0.95	0.93
Maryland	Sandy Loam	4.30	257.0	44.5	71.87	5.20	20.96	1.93	28.08
Florida	Sand	4.27	24.6	5.22	6.50	<0.05	6.57	0.79	1.29

Table 4.2: Soil mineral concentrations of three soils prior to nitrogen leaching studies conducted at the University of Georgia during 2011 and 2012.

Soil Name	Soil Type	Sand	Silt	Clay	N	OM [3]
		----------	----------	----------	%	----------
USGA	Sand	95.90	2.1	2.0	0.009	0.80
Maryland	Sandy Loam	69.90	18.0	12.1	0.062	1.80
Florida	Sand	89.80	8.2	2.0	0.013	0.80

Table 4.3. Mean squares for nitrate nitrogen (NO_3-N) measured in greenhouse leaching trials performed at the University of Georgia in Griffin, GA and Athens, GA during 2011 and 2012.

		Mean squares			
Source	df	NO_3-N Leachate	NH_4-N Leachate	NO_3-N Soil	NH_4-N Soil
Trial	1	1449	19270**	194**	652**
Error a, Rep(Trial)	2	194	68	0.2	0.8
Soil	2	28925**	14127**	26**	692**
Treatment	3	23714**	12986**	85**	260**
Soil × Trial	2	437	5478**	3	444**
Treatment × Trial	3	606	7101**	86**	86**
Treatment × Soil	6	4186**	3265**	10*	198**
Treatment × Trial × Soil	6	144	1933**	6	77**
Error b	22	1007	89	3	5

* Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

Table 4.4: Nitrate-Nitrogen (NO_3-N) leached from the soil over six weeks in studies conducted at the University of Georgia during 2011 and 2012

	Florida Sand	USGA Sand	Maryland Sandy Loam
Treatment	2011-2012	2011-2012	2011-2012
	----------mg----------		
E 15-0-0	34.4a†	120.8a	170.6a
UMAXX	4.6b	44.1b	132.0a
Analog	4.8b	25.2c	76.4ab
Control	1.4b	2.8d	5.1b

† Means within a column followed by the same letter are not significantly different at P< 0.05 according to Fisher's LSD.

Table 4.5: Ammonium-Nitrogen (NH_4-N) leached from the soil over six weeks in studies conducted at the University of Georgia during 2011 and 2012.

	Florida Sand		USGA Sand		Maryland Sandy Loam	
Treatment	2011	2012	2011	2012	2011	2012
	----------mg----------					
E 15-0-0	20.1ab†	107.0b	20.5a	64.11b	2.6b	1.3a
UMAXX	30.0a	60.6c	27.7a	23.9bc	3.9a	6.1a
Analog	43.3a	210.6a	31.8a	187.4a	3.5a	0.8a
Control	1.2b	1.4d	1.8a	0.2c	1.0c	0.2a

† Means within a column followed by the same letter are not significantly different at P< 0.05 according to Fisher's LSD.

Table 4.6: Nitrate-Nitrogen (NO_3-N) retained in the soil over six weeks in studies conducted at the University of Georgia during 2011 and 2012

	Florida Sand		USGA Sand		Maryland Sandy Loam	
Treatment	2011	2012	2011	2012	2011	2012
	----------mg kg^{-1}----------					
E 15-0-0	0.5a†	11.6a	1.2a	15.0a	1.4b	11.1a
UMAXX	0.5a	1.7b	2.2a	6.6b	3.0a	8.4ab
Analog	0.5a	0.3b	0.7a	0.3c	2.0ab	7.8ab
Control	0.4a	0.2b	0.6a	0.2c	2.2ab	0.2b

† Means within a column followed by the same letter are not significantly different at P< 0.05 according to Fisher's LSD.

Table 4.7: Ammonium-Nitrogen (NH_4-N) retained in the soil over six weeks in studies conducted at the University of Georgia during 2011 and 2012.

	Florida Sand		USGA Sand		Maryland Sandy Loam	
Treatment	2011	2012	2011	2012	2011	2012
	----------mg kg^{-1}----------					
E 15-0-0	1.9b†	23.7b	4.8a	4.7a	1.6a	3.8a
UMAXX	12.3a	50.4a	2.8ab	4.6a	1.6a	3.3ab
Analog	1.6b	19.1b	0.8b	3.5a	1.3ab	3.0ab
Control	0.7b	1.5c	0.7b	0.4b	1.2b	1.9b

† Means within a column followed by the same letter are not significantly different at P< 0.05 according to Fisher's LSD.

CHAPTER 5

CONCLUSIONS

Turf managers are required to be cost effective and efficient while dealing with end user expectations, usage, and environmental policies. For the turf industry to be sustainable, new turfgrass varieties will have to meet the challenges of reduced inputs and limited economic support, while being able to be maintained with more environmentally conscious practices. Herbicide resistant and tolerant cultivars and reduced nitrogen leaching fertilizers have promising implications for long-term sustainable turf culture.

ACCase herbicide resistance or tolerance in bermudagrass partially solves the issue of renovating and replacing turf on golf courses, home lawns, and sports fields and help control problem weeds. In a golf course situation, loss of revenue and labor costs of a major refurbishment can be into the millions of dollars. An ACCase herbicide resistant or tolerant variety would allow turf managers to increase lifespan and improve efficiency in managing their turfgrass systems.

ACCase inhibiting herbicide applications applied to 93-175 demonstrated this genotype's higher levels of herbicide tolerance compared to sensitive varieties. Because 93-175 does not have complete resistance to sethoxydim, clethodim, or fluazifop, further study will be needed to determine if tolerance is suitable to turf managers. Meanwhile, the inheritance of ACCase herbicide tolerance from 93-175 to progeny will be tested. Further understanding and research of this tolerance, whether it is metabolic or a mutation conferring change in the ACCase binding sites, will help identify the potential of 93-175.

Studies using somaclonal variation to discover mutations that confer herbicide resistance have been used to developed plant material that exhibits minimal to complete ACCase herbicide resistance (Heckart, 2010). However, a stable and consistent tissue culture methodology needs to be developed to confer resistance. This research identified a method for consistent callus induction, but efforts to regenerate plants were unsuccessful. Optimized methods for regeneration need to be determined before somaclonal variation can be used to induce resistance to ACCase herbicides. Researchers would need to develop a tissue culture program capable of successfully inducing resistant calli that also contain no other detrimental mutations. Although preliminary experimentation to induce callus from seed and other explant material has been unsuccessful to date, work will continue to discover the most efficient techniques possible to induce mutations which confer ACCase herbicide resistance in bermudagrass.

If an ACCase herbicide resistant cultivar were released to turf mangers, the effectiveness could be on a limited basis for some weed species due to resistance development. An education program that is incorporated into the release of that cultivar would help prolong the effectiveness of the resistant characteristic by limiting the situations in which weed resistances have been observed to develop. It would be most advantageous if the cultivar had adequate resistance to more than one herbicide family to allow for proper rotation of active ingredients. Ultimately, the incorporation of ACCase herbicide resistance into a new bermudagrass cultivar could allow for the removal of unwanted common bermudagrass and other grassy weeds within areas where other control methods have failed. This process would be more environmentally and economically sustainable than a complete renovation, which is currently the only long-term alternative for maintaining a pure stand of bermudagrass.

Another sustainability issue turf managers are facing is nitrogen losses, and fertilizer companies are working to develop new products that meet the demand of managers. An experimental (E 15-0-0) fertilizer studied in this research leached the largest amounts of nitrate (NO_3-N) on average from the two trials performed in 2011 and 2012. Concerning the fate of ammonium (NH_4-N), the analog (16-4-8) fertilizer exhibited higher losses indicating inefficiency of nitrification. Residual NO_3-N and NH_4-N remaining in the soil at the conclusion of the tests may have been more dependent on plant health than soil type. Many environmental factors can affect the plant utilization, or possible leaching of a fertilizer. A consistent evaluation protocol to determine the nutrient efficiency of new fertilizers as they are being developed warrants further research. Information and knowledge from turf managers would be important to determine the timing, biotic, and abiotic factors which are commonly considered when applying fertilizer so these factors are evaluated in a variety of environmental conditions.

REFERENCES

Anonymous. 1993. USGA recommendation for a method of putting green construction: The 1993 revision. USGA Green Section Record 31(2):1-3.

Askew S.D., and J.W. Wilcut. 2001. Tropic croton interference in cotton. Weed Science. 49:184-189.

Beard J.B. 1973. Turfgrass: Science and Culture, 1st ed. Prentice-Hall, Engelwood Cliffs, NJ.

Beckie H.J., I.M. Heap, R.J. Smeda, and L.M Hall. 2000. Screening for herbicide resistance in weeds. Weed Technology 14:428-445.

Bigelow C.A. 2008. Problem Solver: Common bermudagrass. The Lawn Problem Solver, Available http://ksuturf.org/.

Bowman, D.C., J.L. Paul, W.B. Davis, and S.H. Nelson. 1989. Rapid depletion of nitrogen applied to Kentucky bluegrass turf. Horticulture Science 114:229-233.

Bradford, K. J., A. Van Deynze, N. Gutterson, W. Parrott, and S. H. Strauss. 2005. Regulating transgenic crops sensibly: Lessons from plant breeding, biotechnology and genomics. Nature Biotechnology. 23(4): 439-444.

Brown, K., J. Thomas, and R. Duble. 1982. Nitrogen source effect on nitrate and ammonium leaching and runoff losses from greens. Agronomy Journal 74:947-950.

Bunnell, B. T., L. B. McCarty, and Bridges Jr. 2005. Evaluation of three bermudagrass cultivars and Meyer japanese zoysiagrass grown in shade. International Turfgrass Society Research Journal. 10(2): 826-833.

Burton, G.W. 1966. Tifway (Tifton 419) bermudagrass (Reg. No. 7). Crop Science. 6: 93-94.

Burton J., J. Gronwald, D. Somers, J. Connelly, B. Gengenbach, and D. Wyse. 1987. Inhibition of plant acetyl-coenzyme A carboxylase by the herbicides sethoxydim and haloxyfop. Biochemical and Biophysical Research Communications. 148:1039-1044.

Cotterman, J. C., and L. L. Saari. 1992. Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in diclofop-methyl-resistant rigid ryegrass (*Lolium rigidum*) biotype SR484. Pesticide Biochemistry and Physiology. 43(3):182-192.

Cox C. J., L. B. McCarty, and J. K. Higingbottom. 1999. Envoy (clethodim) for bermudagrass control and centipedegrass tolerance. Proceedings Southern Weed Science. 52:68.

Délye C. 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. Weed Science 53:728-746.

Dietz, M.E., J.C. Clausen , and K. Filchak. 2004. Education and changes in residential nonpoint source pollution. Environmental Management 34:684-690.

Drummond A.J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and A. Wilson. 2011. Geneious v5.4, Available from http://www.geneious.com/

Duncan, R. R., and R.N. Carrow. 2000. Seashore paspalum: The environmental turfgrass. John Wiley & Sons, Hoboken, NJ.

Erickson, J.E., J.L. Cisar, J.C. Volin, and G.H. Snyder. 2001. Comparing nitrogen runoff and leaching and between newly established St. Augustine grass turf and an alternative residential landscape. Crop Science 41:1889-1895.

Erickson, J., J. Cisar, G. Snyder, D. Park, and K. Williams. 2008. Does a mixed-species landscape reduce inorganic-nitrogen leaching compared to a conventional St. Augustinegrass lawn. Crop Science 48:1586.

Everman W.J., D.L. Jordan, and F.H. Yelverton. 2013. Herbicide resistance managemnet. North Carolina Agricultural Chemicals Manual p.279.

Goldman J.J., W.W. Hanna, and P. Ozias-Aikins. 2004. Plant regenration and analysis of somaclonal variation from TifEagle and TifSport bermudagrass cultivars. Horticulture Science 39(6):1381-1384.

Grey, T.L., M. Czarnota, T. Potter, and B.T. Bunnell. 2009. Timed release of flurprimidol from a granular formulation in mulches and sand. Horticulture Science 44:512-515.

Guertal, E.A. and J. A. Howe. 2012. Nitrate, ammonium, and urea leaching in hybrid bermudagrass as affected by nitrogen source. Agronomy Journal 104:344-352.

Harlan J.R., J.M.J. de Wet, W.W. Huffine,and J.R. Deakin. 1970a. A guide to the species of *Cynodon* (Gramineae) Bulletin B-673, OSU Experimental Station.

Harlan J.R., J.M.J. de Wet, K.M. Rawal, M.R. Felder,and W.L. Richardson. 1970b. Cytogenetic studies in *Cynodon* L. C. Rich. (Gramineae). Crop Science. 10:288-291.

Heckart D.L., W.A. Parrott, and P.L. Raymer. 2010. Obtaining sethoxydim resistance in seashore paspalum. Crop Science. 50:2632-2640

Jasieniuk, M., I. N. Morrison, and A. L. Brûlé-Babel. 1995. Inheritance of dicamba resistance in wild mustard (*Brassica kaber*). Weed Science. 192-195.

Killian, K.C., O.J. Attoe, and J.C. Engelbert. 1966. Urea formaldehyde as a slowly available form of nitrogen for Kentucky bluegrass. Agronomy Journal:204-206.

Lewis, D. F., J. S. McElroy, J. C. Sorochan, T. C. Mueller, T. J. Samples, and G. K. Breeden. 2010. Efficacy and safening of aryloxyphenoxypropionate herbicides when tank-mixed with triclopyr for bermudagrass control in zoysiagrass turf. Weed Technology. 24(4):489-494.

Lisi, R., J. Park, and J. Stier. 2004. Mitigating nutrient leaching with a sub-surface drainage layer of granulated tires. Waste Management 24:831-839.

Lowe T. and J. Foy. 2012. Off-Types in Ultradwarf Putting Greens. USGA Green Section Record. 50(2).

Osmond, D.L., and D.H. Hardy. 2004. Characterization of turf practices in five North Carolina communities. Journal of Environmental Quality 33(2):565-575.

Mazur, B. J., and S.C. Falco 1989. The development of herbicide resistant crops. Annual Review of Plant Biology. 40(1): 441-470.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Parker W.B., L.C.Marshall, J.D. Burton, D.A. Somers, D.L. Wyse, J.W. Gronwald, and B.G. Gengenbach. 1990. Dominant mutations causing alterations in acetyl-coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize. Proceedings of the National Academy of Sciences 87:7175.

Petrovic, A.M. 1990. The fate of nitrogenous fertilizers applied to turfgrass. Journal of Environmental Quality 19:1-14.

Powles S.B., and Q. Yu. 2010. Evolution in action: plants resistant to herbicides. Annual Review of Plant Biology 61:317-347.

Radko A.M. 1957. Relationship between green committee and superintendent. USGA Journal and Turf Management. p. 30-32.

Richardson, M. D., D. E. Karcher, and L.C. Purcell. 2001. Quantifying turfgrass cover using digital image analysis. Crop Science. 41(6): 1884-1888.

Ritz C., C.B. Pipper, and J.C. Streibig. 2013. Analysis of germination data from agricultural experiments. European Journal of Agronomy. 45:1-6

Robbins, J. 2005. Slow-release fertilizers as tools. IFA International Workshop on Enhanced-Effiecency Fertilizers.

Rowe, W. J. 1986. In Tissue culture as a plant production system for horticultural crops, New technologies in plant tissue culture. Springer, Netherlands. pp 35-51.

Saha, S.K., L.E. Trenholm, and J.B. Unruh. 2007. Effect of fertilizer source on nitrate leaching and St. Augustinegrass turfgrass quality. Horticulture Science 42:1478-1481.

Seedfelt S.S., J.E. Jensen, and E.P. Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Technology. 9:218-227.

Syngenta. 2007. Fusilade II Turf and Ornamental [Material Safety Data Sheet]. Available. http://www.syngenta.com/

Taliaferro, C.M. 2003. Bermudagrass (*Cynodon* (L.) Rich). In: M.D. Casler and R.R. Duncan, editors, Turfgrass biology, genetics, and breeding. John Wiley & Sons, Hoboken, NJ. p. 235-256.

Tate, T.M., P. Raymer, M. Harrison-Dunn, and P. McCullough. 2012. Characterization of acetyl coenzyme a inhibitor resistance in turfgrass and grassy weeds. Available http://www.libs.uga.edu/

Tu M., Hurd C., and Randall J. 2001. Weed Control Methods Handbook, The Nature.

Valent. 2006. ENVOY Plus herbicide [Material Safety Data Sheet]. Available http://www.valent.com/

Wang, F.L., and A.K. Alva. 1996. Leaching of nitrogen from slow-release urea sources in sandy soils. Soil Science Society of America Journal, 60(5):1454-1458.

Webster, T.M., C.W. Bednarz, and W.W. Hanna. 2009. Sensitivity of triploid hybrid bermudagrass cultivars and common bermudagrass to postemergence herbicides. Weed Technology. 17(3):509-515.

Wolkowski, R.P., K.A. Kelling, and L.G. Bundy. 1995. Nitrogen management on sandy soils. Available http://corn.agronomy.wisc.edu/.

WSSA Herbicide Handbook Committee. 2002. Herbicide handbook of the weed science society of America. Weed Science Society of America, Champaign, IL.

Wu Y.Q., C.M. Taliaferro, G.H. Bai, D.L. Martin, J.A. Anderson, M.P. Anderson, and R.M. Edwards. 2006. Genetic analyses of Chinese *Cynodon* accessions by flow cytometry and AFLP markers. Crop Science. 46:917-926.

Zhang S., W. Hanna,and P. Ozias-Akins. 2007. Comparison of callus induction and plant regeneration from different explants in triploid and tetraploid turf-type bermudagrasses. Plant Cell, Tissue and Organ Culture 90:71-78.

APPENDIX A

TISSUE CULTURE MEDIA

Table 1: Callus induction media used for DT-1 calli formation at the University of Georgia, 2012 (all recipes refer to 1L of medium).	
MS Macro**	100ml
MS Micro**	1ml
Ca**	10ml
Fe**	10ml
MS vitamin**	1ml
2,4-D*	1.5mg
Sucrose*	30g
Proline*	1.16g
Gelzan*	2.0g
Agar*	5.0g
PPM*	1ml
BAP*	0.01mg
**Murshiage and Skoog. 1962 *(Zhang et al. 2007)	

Table 2: Regeneration Medium used for DT-1 shoot formation at the University of Georgia, 2012 (all recipes refer to 1L of medium).	
MS Macro**	100ml
MS Micro**	1ml
Ca**	10ml
Fe**	10ml
MS vitamin**	1ml
2,4-D*	0.1mg/0mg
Sucrose*	30g
Proline*	1.16g
Gelzan *	2.0g
BAP*	2mg
**Murshiage and Skoog. 1962 *(Zhang et al. 2007)	

Table 3: Regeneration Medium used for DT-1 root formation at the University of Georgia, 2012 (all recipes refer to 1L of medium).	
MS Macro**	100ml
MS Micro**	1ml
Ca**	10ml
Fe**	10ml
MS vitamin**	1ml
Gelzan*	2.0g
Sucrose*	30g
**Murshiage and Skoog. 1962 *(Zhang et al. 2007)	