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The Effects of DMI Fungicide Applications on Secondary Metabolites in Creeping Bentgrass (Agrostis stolonifera) and Kale (Brassica oleracea var. acephala).

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To the Graduate Council:

I am submitting herewith a thesis written by David P. Shell entitled "The Effects of DMI Fungicide Applications on Secondary Metabolites in Creeping Bentgrass (Agrostis stolonifera) and Kale (Brassica oleracea var. acephala).." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

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Effects of DMI Fungicide Applications on Secondary Metabolites in Creeping Bentgrass (*Agrostis stolonifera*) and Kale (*Brassica oleracea* var. *acephala*)

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DEDICATION

To my parents, Dean and Elizabeth who have loved and supported me in everything I have done. I would not be here today without them.

To my sister, Staci for all the advice she has given me, and the opportunities to laugh, which are usually at her expense.

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ABSTRACT

Propiconazole and tebuconazole are two triazole fungicides that were evaluated for their impact on the carotenoid pathway in creeping bentgrass (*Agrostis stolonifera)* and kale (*Brassica oleracea* var. *acephala)* when applied to plant foliage. Changes in carotenoid pigment concentrations in creeping bentgrass were investigated to determine if applications of propiconazole and tebuconazole could lead to an increase in stress protection. Kale was examined to determine if triazole applications could lead to an increase in carotenoids, which have been well documented for their role in human health. Because of the growing market for immature or baby leafy greens versus mature greens, two different maturation stages were used in this study. Plant pigment concentrations were determined using high performance liquid chromatography (HPLC) following two applications of propiconazole and tebuconazole.

Applications of propiconazole and tebuconazole significantly reduced the concentration of zeaxanthin in creeping bentgrass compared to the untreated control suggesting that treated plants were experiencing less stress than the untreated plants. Minor changes were observed in chlorophyll pigment concentrations, with only chlorophyll *b* being significantly increased in treated plants compared to the untreated control. Baby kale showed significantly increased neoxanthin, lutein, and total carotenoid concentrations, as well as increases in chlorophyll *a* and *b*, and total chlorophyll. Pigment concentrations in mature kale all decreased substantially compared to baby kale and no significant differences were found between treated and untreated plants. This suggests the possibility that triazoles could have a more pronounced effect on pigment concentrations in younger plants.

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INTRODUCTION

The effect of demethylation inhibiting (DMI) fungicide applications on plants has received increasing attention recently. However, most studies have used cereal crops as their basis. Fungicide applications are commonly made on golf courses to control diseases, but little is known regarding how fungicide applications affect the physiology of golf course turfgrass. Based on published studies, it is possible that applications of DMI fungicides may improve stress protection, along with disease control. The use of fungicides is also common in vegetable crops to protect the grower's investment. It is known that DMI fungicides interfere with the production of gibberellins in the plant. What isn't known is what happens when one branch in the isoprenoid pathway is blocked. If it possible to shift the energy in the isoprenoid pathway from gibberellin production to the production of chlorophyll or carotenoid pigments, it may be possible to improve stress tolerance and increase the nutrition level in crops. Therefore, the following studies will attempt to determine what effects DMI fungicides have on both golf course turfgrass and a vegetable crop.

CHAPTER 1

LITERATURE REVIEW

CREEPING BENTGRASS

Creeping bentgrass (*Agrostis stolonifera*) is a perennial, cool season turfgrass species commonly used on golf course greens, tees and fairways. Creeping bentgrass has several qualities that make it suited for use on golf courses such as a fine texture, dark green color and ability to tolerate close mowing heights. However, because of the high quality putting surfaces that creeping bentgrass is able to provide, it is becoming a more common choice in warmer climates (Christians, 1998). During the summer, the quality of creeping bentgrass often declines when golf course greens receive their maximal use in transitional and warm climate regions (Lucas, 1995; Carrow, 1996). The optimum air temperature ranges for creeping bentgrass shoot and root growth are 12 to 20 °C and 10 to18 °C, respectively. When temperatures increase above these ranges, they are referred to as being supraoptimal. Dernoeden (2002) used the term "complex" to describe the decline and death of plants by two or more causal agents, including both biotic (living) and abiotic (nonliving). One common abiotic agent is drought stress.

Antioxidant activity is closely associated with stress tolerance and turfgrass quality (Zhang and Schmidt, 2000). Bowler et al. (1992) reported that environmental stresses damage plant cells due to increased production of reactive oxygen species (ROS) (superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen). Antioxidant metabolites and enzymes involved in stress tolerance, such as superoxide dismutase, help protect plants by scavenging ROS (Zhang and Schmidt, 2000). When conditions of severe plant stress exist, the production of ROS may exceed the scavenging capacity of the antioxidant defense system (van Breusegem et al., 1998). When ROS begin to accumulate, this can lead to cellular damage such as lipid peroxidation or the oxidation of

phospholipids and other unsaturated lipids (DaCosta and Huang, 2007). High antioxidant activity is associated with increased drought stress in grasses (Price and Hendry, 1989).

The availability of water for irrigation of turfgrasses is becoming increasingly limited, thereby making water conservation a prime concern of turfgrass growers and managers (DaCosta and Huang, 2005). Water is essential to plants serving as a catalyst for all chemical reactions for photosynthesis and as a mechanism for managing heat accumulation in plant tissue (Bell, 2011).

KALE

Kale (*Brassica oleracea* L. var. *acephala* DC) is a member of the Brassicaceae family. Vegetable crops within the *Brassica* genus contain high nutritional and medicinal values. Studies within the *Brassica* genus have shown that consumption of spinach and other green leafy vegetables has been associated with decreased incidence of cataracts (Mares-Perlman, 1997; Lyle et al., 1999). Kale has been specifically cited by Chasan-Taber et al. (1999) and Seddon et al. (1994) as a food source that reduces cataract occurrence. Environmental and growing conditions can affect the growth and nutritional content of kale. Mercandante and Rodriguez (1991) found that the Brazilian grown kale variety "Tronchuda" had significantly higher carotenoid content during the summer than winter months. Conversely, they found that the variety "Manteigna" had significantly higher carotenoid content during the winter than summer.

PLANT PIGMENTS

Sunlight is required for photosynthesis but excessive light can also be one of the most common abiotic stresses affecting plants. Havaux et al. (1998) found that when barley (*Hordeum vulgare* L.) plants were exposed to strong light, a substantial amount of carotenoids bound to the light-harvesting pigment complexes of the photosystems ceased to work as efficient accessory pigments in leaves. Plant pigments are categorized as either primary or accessory based on their role in light harvesting. Primary pigments like chlorophyll *a* and *b* are found within the chloroplasts and play an active role in photosynthesis (Taiz and Zeiger, 2002). Carotenoids act as accessory pigments and are integrated into light-harvesting complexes (LHC) along with chlorophyll (Croce et al., 1999a, 1999b).

Chlorophylls also act as antenna complexes by collecting light energy and transferring it to reaction centers. Duysens et al. (1961) characterized two different reaction centers, classified as Photosystem I (PSI) and Photosystem II (PSII) based on their work with the red algae, *Porphyridium cruentum*.

Carotenoids are C_{40} compounds that are formed in the isoprenoid pathway. The formation of the carotenoid pathway begins with dimerization of the C_{20} compound geranyl-geranyl pyrophosphate to produce the first carotenoid, phytoene, via phytoene synthase (Buchanan et al., 2002). Phytoene is then desaturated to form lycopene. Branching of the pathway occurs when lycopene is cyclized into α-carotene or β-carotene; α-carotene then forms lutein and epoxy-lutein, whereas β-carotene forms the xanthophyll cycle pigments (zeaxanthin, antheraxanthin, and violaxanthin) and

neoxanthin (Buchanan et al., 2000; Demmig-Adams et al., 1996). Carotenoids have two principal roles in the process of photosynthesis: either photoprotection or light collection (Demmig-Adams et al., 1996). Carotenoids function in light collection by channeling photons not absorbed by chlorophyll molecules to the reaction center for photosynthesis (Niyogi, 1999; Goodwin, 1980). Photoprotection is primarily achieved through the xanthophyll cycle. When conditions of excess light occur, reversible de-epoxidation of violaxanthin to zeaxanthin dissipates heat through non-photochemical quenching (NPQ) (Demmig-Adams et al., 1996).

In addition to their roles in photosynthesis and photoprotection, carotenoids are important in human diets. However, since carotenoids cannot by synthesized *de novo* by mammalian systems, consumption of fruits and vegetables rich in carotenoids is important (Perez-Rodriguez, 2009). Consumption of carotenoids in the human diet has been associated with reductions in certain diseases. Le Marchand et al. (1993) reported that dietary intake of lutein and β-carotene can reduce the risk of eye diseases and lung cancer in humans.

Carotenoid accumulation and biosynthesis in plants is affected by a variety of environmental factors. Lefsrud et al. (2005) reported a linear increase in maximum tissue lutein and β-carotene concentrations in kale as temperature increased incrementally from 15 °C to 30 °C. However, they also reported a linear decrease in lutein and β-carotene tissue concentrations in spinach (*Spinacia oleracea* L.) over the same temperature range. McElroy et al. (2006) exposed creeping bentgrass to high and low irradiance and found that as plants were moved from low light to high light, there was an increase in the concentration of zeaxanthin. Zeaxanthin is viewed as the primary carotenoid for NPQ

(Demmig-Adams et al., 1999) because it removes oxygen from violaxanthin and antheraxanthin, and then dissipates heat accumulation (Muller, 2001).

TRIAZOLES

Sterol biosynthesis inhibitors (SBI) represent the largest group of fungicides in use today (Latin, 2011). Fungi contain sterols, specifically ergosterol, that are different from sterols found in plants and animals which allows for the development of broad spectrum fungicides targeting sterol biosynthesis for disease control (Kuck et al., 2012). Ergosterol was first described in 1889 by Tanret as a result of studies with the ergot pathogen, *Claviceps purpurea* (Windaus, 1928). Ergosterol is an important constituent of cell walls that regulates the selective absorption and expulsion of certain substances. SBIs cause a disruption in membrane function, which leads to electrolyte leakage, suppression of fungal growth, and the death of fungal cells (Latin, 2011).

SBI's are organized into four groups: Group G1, which contains demethylation inhibitors (DMI), Group G2, which contains the amines, Group G3, which contains the hydroxyl-anilides and Group G4 which contains the squalene epoxidase inhibitors (FRAC, 2012). Only the first three groups have an importance in crop protection, with DMIs playing the greatest role. The DMIs are broken down further into five chemical classes: the piperazines, the pyridines, the pyrimindines, the imidazoles, and the triazoles (Kuck et al., 2012). The triazoles constitute the largest class within the DMIs not only in numbers, with 26 compounds, but also market importance (FRAC, 2012; Kuck et al., 2012).

In addition to disrupting ergosterol synthesis in fungi, triazoles also inhibit the hormone gibberellin (GA) in plants. There are several physiological roles that GAs play in the development of a plant including seed germination, starch metabolism, and cell elongation (Graebe, 1987). The effect on GA biosynthesis has been characterized as an inhibition of ent-kaurene to ent-kaurenoic acid (Koller, 1987; Rademacher et al., 1987). The oxidation of ent-kaurene to ent-kaurenoic acid is cytochrome P-450 dependent (Rademacher et al., 1987). Studies have examined the effect of triazoles on abiotic stress tolerance in plants. Gilley and Fletcher (1997) applied paclobutrazol, propiconazole and tetraconazole to wheat (*Triticum aestivum* L. cv. Katepwa) seedlings and found that treated plants had significantly more shoot regrowth after being subjected to heat stress than untreated plants. Berova et al. (2002) found that wheat plants treated with paclobutrazol had longer shoot length, and increased fresh and dry weight than untreated plants after being exposed to a chilling stress. In plants, GAs are synthesized in the isoprenoid pathway and it is hypothesized that the protective properties provided by the triazoles are a result of a shift in products in the isoprenoid pathway (Gilley and Fletcher, 1997).

In the event of publishing, this paper is based on contributions by David Shell, Brandon Horvath, Dean Kopsell and Jim Brosnan:

My primary contributions to this paper include (i) conducting the experiments, (ii) processing, analyzing and interpreting data, (iii) reading literature, (iv) writing the manuscript.

CHAPTER 2

EFFECTS OF DMI FUNGICIDE APPLICATIONS ON SECONDARY METABOLITES IN CREEPING BENTGRASS (*AGROSTIS STOLONIFERA*).

Abstract

Creeping bentgrass **(***Agrostis stolonifera*) is a common turfgrass species widely used on golf courses. Demethylation inhibiting (DMI) fungicides are routinely applied to creeping bentgrass to control diseases and maintain overall turfgrass quality. It has been suggested that one class of DMI fungicides, the trialzoles, play an important role in plant protection. This study was conducted to determine the effects triazole fungicides have on creeping bentgrass carotenoid content. Two applications of propiconazole and tebuconazole were made to creeping bentgrass var. 'Penn A-1' grown in a greenhouse on a 7-day interval and clippings were collected to determine chlorophyll and carotenoid concentrations through the use of high performance liquid chromatography (HPLC). Zeaxanthin was significantly reduced in treated plants compared to the untreated control. Zeaxanthin is viewed as the primary carotenoid in stress protection; therefore, reductions in zeaxanthin concentrations following triazole treatment indicate that treated plants were experiencing less stress. The only significant difference found among the chlorophyll pigments was an increase in chlorophyll *b*.

Introduction

Creeping bentgrass is an important turfgrass species throughout the world and specifically in the golf course industry of the United States (McElroy et al., 2006). It is one of the most researched and intensively managed turfgrass species in the world (McElroy and Kopsell 2009). Due to several important characteristics such as tolerance to low mowing heights and a desirable color, it is often grown in areas it is not well adapted to, thereby subjecting it to numerous abiotic stresses. It is the primary cool-season grass used on golf course greens (Christians, 1998). Lyman et al. (2007) reported that creeping bentgrass ranked highest in total area planted with over 11,000 hectares, with annual bluegrass second at 4,000 hectares.

Carotenoids are C_{40} isoprenoid polyene plant compounds divided into two groups; the oxygenated xanthophylls (i.e., zeaxanthin, antheraxanthin, and violaxanthin) and the hydrocarbon carotenes (i.e., β-carotene and α-carotene) (Zaripheh and Erdman, 2002). Carotenoids are incorporated into light-harvesting complexes (LHC) along with chlorophyll and perform the physiological roles of photoprotection and light harvesting (Croce et al., 1999a, 1999b). The six primary carotenoids found in plants are zeaxanthin, antheraxanthin, violaxanthin, lutein, β-carotene and α-carotene (Sandmann 2001).

Triazoles represent the largest and most important group of systemic compounds developed originally for the control of fungal diseases in plants and animals (Berova et al. 2002). Triazoles inhibit cytochrome P450 mediated oxidative demethylation reactions necessary for the synthesis of ergosterol and the conversion of kaurene to kaurenoic acid in the gibberellin biosynthesis pathway (Rademacher 1992). Triazoles can protect plants from various environmental stresses (Berova et al., 2002). One possible reason for their

protective properties is that applications shift the balance of important plant hormones in the isoprenoid pathway (Fletcher and Hofstra 1985).

Propiconazole and tebuconazole represent two triazole compounds that are commonly applied to golf courses to control diseases such as dollar spot (*Sclerotinia homoeocarpa*) and brown patch (*Rhizoctonia solani*). However, little is known about the impact these fungicides may have on the carotenoid pathway in creeping bentgrass. Improved knowledge about the effects of triazole compounds have on the carotenoid pathway could lead to more efficient use of this important class of chemicals.

Materials and Methods

Plant cultures

This study was conducted in a greenhouse at the University of Tennessee-Knoxville (Lat. 35° 57'-N). Creeping bentgrass ('Penn A-1'; Tee-2-Green, Hubbard, OR) was seeded into 10-cm diameter pots filled with a peat moss (79%), perlite (15%), and vermiculite (6%) growing medium (Fafard 2 Mix; Conrad Fafard Inc., Agawam, MA) at 48 kg ha^{-1}. Seeding dates were Feb. 1, 2010 and Feb. 3, 2012. After seeding, pots were fertilized weekly for 3 weeks (Howard Johnson's Triple Twenty Plus Minors, Milwaukee, WI) at 40 kg nitrogen ha^{-1} and watered as needed to maintain adequate soil moisture. Hand shears were used to cut plants six times per week to a height of 4 mm with clippings collected. Plants were allowed to grow for approximately 2 months to obtain full coverage and plant maturity before study initiation.

Fungicide treatments

Plants were treated with propiconazole (Banner Maxx®; Syngenta Professional Products, Greensboro, NC) and tebuconazole (Lynx® 2; Bayer CropScience, Research Triangle Park, NC) at low, medium and high application rates. Low, medium and high rates for propiconazole were 0, 976, and 1952 g ha^{-1}, respectively. For tebuconazole, the low, medium and high rates were 0, 1527, and 3054 g ha^{-1}, respectively. Two applications at each rate were made on a 7-day interval using a CO_2-powered boom sprayer containing two flat-fan nozzles (TeeJet 8004 flat fan spray nozzle; Spraying Systems Co., Roswell, GA) calibrated to deliver a spray volume of 815 L ha^{-1}.

Data collection

Assessments of chlorophyll fluorescence (F_v/F_m) were made prior to each application and at the conclusion of the study using a hand held fluorometer (OS1-FL fluorometer; Opti-Sciences, Hudson, NH). Seven days after the second application, leaf tissue was harvested in the greenhouse and analyzed for carotenoid and chlorophyll pigment concentrations using methods similar to (Elmore et al., 2011). Upon harvesting, clippings were immediately frozen in liquid nitrogen (N) and placed on ice for transfer to storage at -80 °C. Tissue pigments were extracted and quantified according to previously published methods (Emenhiser et al., 1996; Kopsell et al., 2007). All clippings were first homogenized in liquid N using a mortar and pestle. A 0.25-g subsample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, NJ) with 0.8 mL of ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, MO), as an internal standard, and 2.5 mL of tetrahydrofuran (THF). Each sample was vortexed and homogenized in the tube using approximately 25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a

drill press (Craftsman 15-inch drill press; Sears, Roebuck, and Co., Hoffman Estates, IL) set at 540 rpm while the tube was immersed in ice to dissipate heat. The tube was then placed into a centrifuge for 5 min at 500 g_n. Precautions were taken to keep the tissue samples on ice during extraction to decrease pigment degradation and increase percent recovery (Kimura and Rodriguez-Amaya, 1999). The supernatant was removed with a Pasteur pipette, placed into a conical 15-mL test tube, capped and held on ice during the remainder of the extraction. The sample pellet was re-suspended in 2 mL THF and the extraction procedure was repeated. By the third or fourth extraction, the supernatant was colorless and one additional extraction was conducted. The remaining sample pellet was discarded and the combined supernatants were reduced to 0.5 mL using nitrogen (N-EVAP 111; Organomation Inc., Berlin, MA). Acetone was added to bring each sample to a 5 mL volume. Samples were vortexed and filtered through a 0.2-µm polytetrafluoroethylene filter (Econofilter PTFE 25/20; Agilent Technologies, Wilmington, DE) before high performance liquid chromatography (HPLC) analysis.

HPLC pigment separation and quantification were carried out using an Agilent 1200 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA). The column used was a 250 X 4.6 mm i.d., 5-µm analytical scale polymeric RP-C_{30} with a 10 X 4.0-mm i.d. guard cartridge and holder (ProntoSIL; MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar carotenoid compounds. The column was maintained at 30 °C using a thermostatted column compartment. All separations were achieved isocrattically using a binary mobile phase of 11% methyl *tert*-butyl ether, 88.99% MeOH, and 0.01% triethylamine (v/v). The flow rate was 1.0 mL min^{-1} with a run time of 53 min followed

by a 2 min equilibration before the next injection. Eluted compounds from a 10-µL injection loop were detected at 453 nm (carotenoids, chlorophyll *b*, internal standard) and 652 nm (chlorophyll *a*) with data collected, recorded, and integrated using ChemStation Software (Agilent Technologies) (Demmig-Adams et al., 1996; Frank and Cogdell, 1996). Carotenoids evaluated included: β-carotene, antheraxanthin, lutein, neoxanthin, violaxanthin, and zeaxanthin. These carotenoids were selected based on their active roles in photoprotection and light harvesting. Peak assignments were performed by comparing retention times to internal standards and line spectra (250-700 nm) obtained from photodiode array detection with authentic standards (ChromaDex Inc., Irvine, Ca). Concentrations of the authentic standards were determined spectrophotometrically using quantitative spectroscopic data (Davies and Köst, 1998). HPLC recovery rates of ethyl-β-apo-8'-carotenoate (recovery rates averaged 61%) were used to estimate losses during extraction. All carotenoids were calculated on a 100 g fresh weight (FW) of creeping bentgrass leaf blade tissue.

Statistical analysis

Treatments in the two experimental runs were arranged as a 2 x 3 factorial, randomized complete block design, with six replications. Factors included fungicide (propiconazole and tebuconazole) and rate (low, medium and high). All data were subjected to ANOVA (P = 0.05; Statistical Analysis Software, Inc., Cary, N.C.). Lack of significant treatment-by-year interactions allowed for data to be pooled over experimental runs. Measured variables did not vary due to fungicide rate and no fungicide-by-rate interactions were detected; results for each fungicide are pooled over application rate.

Results

When evaluating the xanthophyll cycle pigments, zeaxanthin responded significantly ($P \leq .001$) to fungicide treatment (Table 1). Total zeaxanthin concentrations were 1.25, 1.34 and 1.77 mg 100 g^{-1} FW for propiconazole, tebuconazole and the untreated control, respectively. No significant differences in violaxanthin, antheraxanthin, neoxanthin, lutein, β-carotene, and total pigment concentrations were detected due to fungicide treatment.

DMI fungicides have been associated with a greening effect in plants. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll pigments (Table 2) were all increased within fungicide treated plants, however, chlorophyll *b* was the only pigment that was increased significantly ($P = 0.03$).

As a result of lower zeaxanthin concentrations in treated plants, the total xanthophyll pigment concentration (ZAV) (Table 3) was not significantly different than the untreated control. However, the zeaxanthin + antheraxanthin to zeaxanthin + antheraxanthin + violoxanthin (ZA/ZAV) ratio was significantly lower ($P = 0.003$). Due to the increased chlorophyll *b* in treated plants the carotenoid to chlorophyll (Car/Chl) ratio was also significantly lower ($P = 0.0003$) in treated plants.

Discussion

Zeaxanthin is regarded as the primary carotenoid responsible for photoinhibition prevention through NPQ, with antheraxanthin functioning as a transition state molecule within the xanthophyll cycle (Demming-Adams et al. 1999). Goss et al. (1998) reported that in the absence of zeaxanthin, antheraxanthin functions in photoprotection through

NPQ, which suggests that antheraxanthin plays a greater role than just a transition carotenoid to zeaxanthin. While zeaxanthin is known to accumulate under high irradiance conditions, violaxanthin is known to accumulate under low irradiance (McElroy et al., 2006; Demming-Adams et al., 1996; Thayer and Björkman, 1990). Changes to the xanthophyll cycle that decrease zeaxanthin and increase violaxanthin could potentially lead to plants that are better adapted to stress. Brosnan et al. (2011) hypothesized that stress induced following three HPPD inhibiting herbicides to common bermudagrass (*Cynodon dactylon* L. Pers.) may have influenced violaxanthin de-epoxidase activity, thus increasing the conversion of violaxanthin to the intermediate antheraxanthin and finally zeaxanthin.

In this study, lutein was the carotenoid found in most abundance, comprising 43% of the total carotenoids for each of the two treatments. One reason for this could be due to the uneven distribution of carotenoids between the two photosystems. β-carotene is the predominant carotenoid in photosystem I while photosystem II is enriched in lutein (Thayer and Bjokman, 1990; Demmig-Adams et al., 1996). Photosystem II develops earlier than photosystem I in leaf tissue, which allows for the concentrations of lutein to be maximized in leaf tissues before the other pigments (Taiz and Zeiger, 1998). In kale, lutein and B-carotene are the two carotenoids found in the highest concentrations (USDA, 2002). However, in this study neoxanthin was found in higher concentrations in the treated plants than β-carotene. Neoxanthin is a pre-cursor for the apo-carotenoid abscisic acid (ABA). Zeevaart and Creelman (1988) referred to ABA as a "stress hormone" and noted that increases in ABA concentrations were observed during acclimation to abiotic stresses. Therefore, it is possible that higher concentrations of

neoxanthin could result in lower concentrations of ABA. However, testing of both carotenoids and ABA would be required to determine if this was the case.

Chlorophylls are complex molecules well suited for the absorption of light, energy transfer, and electron transfer functions that they perform during photosynthesis (Taiz and Zeiger, 2002). Plants treated with triazoles are shorter and more compact and contain higher chlorophyll content (Fletcher and Hofstra, 1985). Chlorophyll *b* was the only pigment affected by triazole treatment in this study. A greater difference in chlorophyll pigment concentrations was expected prior to the initiation of this study. Because of the goal to maintain the cutting height of the plants close to that which would be found in the field, the yield from the clippings was limited.

Although no difference was found in the ZAV concentration among treatments, there was a difference among the ZA/ZAV ratio among treatments. Plants treated with propiconazole and tebuconazole had a significantly lower ratio than the untreated. Even though the total xanthophyll cycle pigment concentrations from the treated and untreated plants were the same, the ZA/ZAV ratio shows that fungicide treated plants had higher concentrations of violaxanthin than the untreated plants.

In the event of publishing, this paper is based on contributions by David Shell, Brandon Horvath, Dean Kopsell and Jim Brosnan:

My primary contributions to this paper include (i) conducting the experiments, (ii) processing, analyzing and interpreting data, (iii) reading literature, (iv) writing the manuscript.

CHAPTER 3

EFFECTS OF DMI FUNGICIDE APPLICATIONS ON SECONDARY METABOLITES IN KALE (*BRASSICA OLERACEA VAR. ACEPHALA*) AT TWO DIFFERENT MATURATION STAGES.

ABSTRACT

Kale (*Brassica oleracea* var. acephala) is a vegetable crop that contains high concentrations of lutein and β-carotene. Vegetable growers make applications of fungicides to protect against diseases and ensure their products make it to market. One class of chemicals within the demethylation inhibitors (DMI) fungicide group is the triazoles. Application of triazoles has been associated with increased carotenoid concentrations in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L. cv Katepwa). Research was initiated in a greenhouse (Knoxville, TN) during 2012 to determine if applications of triazole fungicides could increase the carotenoid levels in kale at two different maturation stages. Two applications of propiconazole and tebuconazole were made to baby and mature kale plants on a seven-day interval. Plants were harvested and carotenoid and chlorophyll pigments were analyzed using high performance liquid chromatography (HPLC). Fungicide applications increased neoxanthin, lutein, total carotenoids, chlorophyll *a* and *b*, and total chlorophyll in baby kale compared to the untreated control. No significant differences in carotenoid or chlorophyll pigment concentrations due to fungicide treatment were detected in mature kale suggesting that triazole fungicides have a more pronounced effect on younger leaves.

INTRODUCTION

Triazole fungicides are commonly used to control diseases that would aversely affect crop yields. Two common triazoles applied to crops are propiconazole and tebuconazole. Aside from disease control, it has been suggested that triazole fungicides could shift the balance of plant hormones in the isoprenoid pathway of tolerant plants (Fletcher and Hofstra 1985). Triazoles inhibit cytochrome P450 mediated oxidative demethylation reactions including those which are necessary for the synthesis of ergosterol and the conversion of kaurene to kaurenoic acid in the gibberellin biosynthesis pathway (Rademacher 1992).

Zeaxanthin, antheraxanthin, violaxanthin, lutein, β-carotene and neoxanthin, constitute the six primary carotenoids found in higher plants (Sandmann, 2001). Consumption of carotenoids is associated with a reduced risk of lung cancer and chronic eye diseases such as cataract and age-related macular degeneration (Le Marchand et al., 1993). Among, green leafy vegetables, kale (*Brassica oleracea* L. var. *acephala*) ranks highest among all vegetable crops for reported lutein and β-carotene concentrations (USDA, 2002). Despite these high concentrations, consumption of kale remains low with the per capita fresh intake at less than 0.33 kg/year (Lucier and Plummer, 2003). The synthesis of carotenoids is unique only to plants; therefore, consumption of fruits and vegetables that contain a mixture of carotenoids is important.

Pena (2004) reported that the consumption of leafy green vegetables has doubled in the last two years. The majority of the salad market now includes baby, or immature, leafy greens. The USDA (2002) reported that kale has higher concentrations of lutein and β-carotene than any other vegetable. However, immature kale leaves often contain

less lutein and β-carotene than those found on mature plants (de Azevedo and Rodriguez-Amaya, 2005). Therefore, ways to maximize carotenoid concentrations in the growing baby kale marked warrants investigation.

MATERIALS AND METHODS

Plant cultures

This study was conducted in a greenhouse at University of Tennessee-Knoxville (Lat. 35° 57'-N).. Kale variety 'Red Russian' (Johnny's Selected Seed, Winslow, ME) was seeded into a 96 cell flat (Hummertt International, Earth City, MO) filled with a peat-moss (79%), perlite (15%), and vermiculite (6%) growing medium (Fafard 2 Mix; Conrad Fafard Inc., Agawam, MA). Seeding dates were Feb. 1 2010 and Feb. 3 2012. Once the plants formed their first true leaf, they were then transferred to a 10-cm diameter pot, filled with the same media. After the plants were transferred, pots were fertilized on a weekly basis (Howard Johnson's Triple Twenty Plus Minors, Milwaukee, WI) at 200 mg L^{-1} and watered as needed to maintain adequate soil moisture. Two separate studies were conducted to determine the effects on immature and mature kale. Baby kale was harvested after 25 days and the mature kale was harvested after 50 days.

Fungicide treatments

Plants were treated with propiconazole (Banner Maxx®; Syngenta Professional Products, Greensboro, NC) and tebuconazole (Lynx® 2; Bayer CropScience, Research Triangle Park, NC) at low, medium and high application rates. Low, medium and high rates for propiconazole were 0, 24, and 48 g ha^{-1}, respectively. For tebuconazole, the low, medium and high rates were 0, 35, and 70 g ha^{-1}, respectively. Two applications

were made 7 days apart using a CO_2-powered boom sprayer containing two flat-fan nozzles (TeeJet 8004 flat fan spray nozzle; Spraying Systems Co., Roswell, GA) calibrated to deliver a spray volume of 815 L ha^{-1}. A non-ionic surfactant (Capsil; Auquatrols, Paulsboro, NJ) was added to each fungicide at a rate of 0.05% v/v.

Data collection

Assessments of chlorophyll fluorescence (F_v/F_m) were made prior to each application and at the conclusion of the study using a hand held fluorometer (OS1-FL fluorometer; Opti-Sciences, Hudson, NH). Seven days after the second application, leaves were harvested in the greenhouse for carotenoid and chlorophyll analysis in the lab. Upon harvesting, leaves were immediately frozen in liquid nitrogen (N) and placed on ice for transfer to storage at -80 °C. Frozen kale samples were lyophilized for a minimum of 72 hours (Model 6L FreeZone, LabConCo, Kansas City, MO). Tissue pigments were extracted and quantified according to previously published methods (Emenhiser et al., 1996; Kopsell et al., 2007). All clippings were first homogenized in liquid N using a mortar and pestle. A 0.1 g subsample was placed into a Potter-Elvehjem tissue grinder tube (Kontes, Vineland, NJ) with 0.8 mL of ethyl-β-apo-8'-carotenoate (Sigma Chemical Co., St. Louis, MO), as an internal standard, and 2.5 mL of tetrahydrofuran (THF). Each sample was vortexed and homogenized in the tube using approximately 25 insertions with a Potter-Elvehjem tissue grinder pestle attached to a drill press (Craftsman 15-inch drill press; Sears, Roebuck, and Co., Hoffman Estates, IL) set at 540 rpm while the tube was immersed in ice to dissipate heat. The tube was then placed into a centrifuge for 5 min at 500 g_n. Precautions were taken to keep the tissue

samples on ice during extraction to decrease degradation and increase percent recovery (Kimura and Rodriguez-Amaya, 1999). The supernatant was removed with Pasteur pipette, placed into a conical 15-mL test tube, capped and held on ice during the remainder of the extraction. The sample pellet was resuspended in 2 mL THF and the extraction procedure was repeated. By the third or fourth extraction, the supernatant was colorless and one additional extraction was conducted. The remaining sample pellet was discarded and the combined supernatants were reduced to 0.5 mL using nitrogen (N-EVAP 111; Organomation Inc., Berlin, MA). Each sample was brought up to a 5 mL volume with acetone. Samples were vortexed and filtered through a 0.2-µm polytetrafluoroethylene filter (Econofilter PTFE 25/20; Agilent Technologies, Wilmington, DE) before high performance liquid chromatography (HPLC) analysis. HPLC pigment separation and quantification were carried out using an Agilent 1200 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA). The column used was a 250 X 4.6 mm i.d., 5-µm analytical scale polymeric RP-C_{30} with a 10 X 4.0-mm i.d. guard cartridge and holder (ProntoSIL; MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar carotenoid compounds. The column was maintained at 30 °C using a thermostatted column compartment. All separations were achieved isocrattically using a binary mobile phase of 11% methyl *tert*-butyl ether, 88.99% MeOH, and 0.01% triethylamine (v/v/v). The flow rate was 1.0 mL min^{-1} with a run time of 53 min followed by a 2 min equilibration before the next injection. Eluted compounds from a 10-µL injection loop were detected at 453 nm (carotenoids, chlorophyll b, internal standard) and 652 nm (chlorophyll a) and data were collected, recorded, and integrated using ChemStation

Software (Agilent Technologies) (Demmig-Adams et al., 1996; Frank and Cogdell, 1996). Carotenoids evaluated included: β-carotene, antheraxanthin, lutein, neoxanthin, violaxanthin, and zeaxanthin. These carotenoids were selected based on their active roles in photoprotection and light harvesting. Peak assignments were performed by comparing retention times to internal standards and line spectra (250-700 nm) obtained from photodiode array detection with authentic standards (ChromaDex Inc., Irvine, Ca). Concentrations of the authentic standards were determined spectrophotometrically using quantitative spectroscopic data (Davies and Köst, 1998). HPLC recovery rates of ethyl-β-apo-8'-carotenoate (average recovery rates were 72% and 74% for baby and mature kale, respectively) were used to estimate losses during extraction. All carotenoids were calculated on a 100 g dry weight (DW) of kale leaf blade tissue.

Statistical analysis

Treatments were arranged in a 2 x 3 factorial, randomized complete block design, with six replications. Two experimental runs were conducted. Factors included fungicide (propiconazole and tebuconazole) and rate (low, medium and high). All data were subjected to ANOVA (P = 0.05; Statistical Analysis Software, Inc., Cary, N.C.). Lack of significant treatment-by-year interactions allowed for data to be pooled over experimental runs. Measured variables did not vary due to fungicide rate and no fungicide-by-rate interactions were detected; results for each fungicide are pooled over application rate.

RESULTS

Study one, baby kale pigments

The carotenoid and chlorophyll content of vegetable crops is usually reported on a fresh weight (FW) basis to equate to typical consumption patterns (Holden et al. 1999); however, due to the increasing popularity of dried materials in dietary supplements as sources of antioxidants, the accumulations of the six primary carotenoids were calculated on a dry weight (DW) basis. When comparing fungicide treated plants to the untreated control, chlorophyll *a* responded significantly (Table 6). Chlorophyll *a* increased 18 and 12%, respectively, following propiconazole and tebuconazole treatment.. Total chlorophyll and chlorophyll *b* concentrations responded similarly with 11 to 15% increases compared to the untreated control.

With regards to carotenoid pigments, applying a fungicide increased total carotenoids, lutein and neoxanthin concentrations compared to the untreated control (P = 0.04, 0.04, and 0.003, respectively; Table 4). The total xanthophyll pigment (ZAV) concentration in treated plants was also increased (P = 0.052) compared to the untreated control; however, no significant differences in the zeaxanthin + antheraxanthin to zeaxanthin + antheraxanthin + violaxanthin (ZA/ZAV) ratio were detected due to fungicide treatment. Increases in chlorophyll *a*, chlorophyll *b*, and total chlorophyll concentrations significantly (P = 0.02) reduced the carotenoid to chlorophyll (CAR/CHL) ratio compared to the untreated control.

Study two, mature kale pigments

No significant differences in any measured parameter were detected due to fungicide treatment in mature kale (Table 7).

DISCUSSION

In this study, total carotenoid content was higher in baby kale compared to the mature kale. These finding are similar to those of Lefsrud et al. (2007) who found that maximum concentrations of β-carotene, lutein, chlorophyll *a* and chlorophyll *b* in 'Winterboro' kale at 1 to 3 weeks of age; after this point pigment concentrations began to decline. Lutein was the major carotenoid found comprising 41% and 43% of the total carotenoids for baby and mature kale, respectively. β-carotene was the next most prominent carotenoid found at (~ 23%) for both baby and mature kale. One reason for this is due to the uneven distribution of carotenoids between the two photosystems. Photosystem I is enriched in β-carotene while lutein is the dominant carotenoid in photosystem II (Thayer and Bjokman, 1990; Demming-Adams et al., 1996). Since photosystem II develops earlier than photosystem I in leaf tissue, this allows for the concentrations of lutein to be maximized before the other pigments (Taiz and Zeiger, 1998). Although no direct comparison was made in this study between the two different maturation stages in kale, the baby kale had higher pigment concentrations than the mature kale. These findings are different than those reported by Azevedo and Rodriguez-Amaya (2005) in which mature kale leaves had significantly more β-carotene and lutein compared to the young leaves. Because of a longer maturation period, the mature kale had more plant biomass that could result in a dilution of pigment concentrations (Mills and Jones, 1996).

Chlorophyll *a*, chlorophyll *b*, and total chlorophyll concentrations in this study were also greater in the baby compared to the mature. These findings are similar to Lefsrud et al. (2007) that found that maximum chlorophyll content for 'Winterboro' kale was observed in leaf tissue between 1 to 3 weeks of age. One characteristic of 'Red Russian' kale is a purpling of the leaves as they mature. It is also possible that the lower concentration of chlorophyll in the mature leaves was due to increases in anthocyanin pigments, which aided in the dilution effect. However, further research would be needed to confirm that hypothesis.

The ZAV concentration was higher for baby kale compared to mature plants. Again, this could be attributed to the overall decline in pigments in the mature kale compared to immature tissue. However, the ZA/ZAV ratio in mature kale exceeded immature plants by approximately 25%. Zeaxanthin together with lutein plays a role in the defense against macular degeneration (EDDC, 1993; Moeller et al., 2000) but is also viewed as the primary carotenoid responsible for the prevention of photoinhibitioin through NPQ (Demmig-Adams et al. 1999). Antheraxanthin has also been shown to function in photoprotection through NPQ when zeaxanthin is absent (Goss et al., 1998). Increases in the amount of zeaxanthin and antheraxanthin compared to the total xanthophyll cycle pigment concentrations (ZA/ZAV ratio) has been associated with increases in plant stress. The Car/Chl ratio was also higher in the mature kale. Even though the mature kale saw a decrease in all pigments, the total chlorophyll pigments declined more (-75%) than the total carotenoids did (-65%).

CONCLUSIONS

The effects of triazoles in stress protection in crops such as wheat and barley has been well documented. However no study has investigated the effects of the triazoles on creeping bentgrass or kale with two different maturation stages. Plant pigments, especially carotenoids, play many important roles in stress protection and when consumed in the diet can reduce the risk of cancer. Therefore, there is a great need to investigate ways to efficiently manage this group of compounds to maximize their efficiency.

Some minor changes in pigment concentrations were observed in creeping bentgrass, mainly with the xanthophyll cycle pigments. Because changes in the xanthophyll cycle can occur within minutes to hours (Demmig-Adams et al., 1996) further investigations should utilize different harvest times throughout the day to attempt to find a time when carotenoid pigments are maximized in the plant. Also, in this experiment, two applications of DMI fungicides were made, but an experiment applying just one application would be beneficial to determine if two applications are needed. This would greatly benefit the end user (i.e., golf course superintendent) as only one application of a given class of a fungicide is made on such a short interval.

Based on these data, the pigment concentrations in kale decrease as plants mature. Therefore, efforts should be focused on ways to maximize the nutritional content of carotenoids in younger kale plants. As with the creeping bentgrass, adding a harvest after the first fungicide application would be of interest to determine the changes in pigment concentrations between the first and second application.

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APPENDIX A

PRELIMINARY RESEARCH

Field Trial

A preliminary field trial was conducted during the summer of 2011 on a previously established 'Dominant Southern' (blend of SR1120 and SR1119) creeping bentgrass (*Agrostis stolonifera*) putting green constructed with a sand-based root zone built to United States Golf Association specifications. This experiment was located at the East Tennessee Research and Education Center (ETREC) in Knoxville, TN. Previous research (Chapter 1) indicated that applications of demethylation inhibiting (DMI) fungicides in the greenhouse could decrease zeaxanthin content in creeping bentgrass, potentially increasing summer stress tolerance. Therefore, the objective of this study was to determine if DMI fungicide applications in the field affect creeping bentgrass turf quality during summer stress.

The experimental design consisted of a randomized complete block with a split-plot treatment arrangement. Because nitrogen is often a limiting factor on putting greens, the effect of different nitrogen levels was added to investigate the effect of fertility combined with the fungicides. Treatments consisted of nitrogen applied to the whole plot at 24 and 48 kg N ha^{-1} $month^{-2}$. Propiconazole and tebuconazole were applied to the sub-plots at 0, 976, and 1952 g ha^{-1}, and 0, 1527, and 3054 g ha^{-1}, respectively. Two applications of each fungicide were made on a 7 day interval using a CO_2 pressurized hand-held boom sprayer with two flat-fan nozzles (TeeJet XR8004, Spraying Systems Co., Roswell, GA) calibrated to deliver a spray volume of 815 L ha^{-1}. Seven days after the second application, the irrigation on the putting green was turned off until the

volumetric soil moisture reached 6% to simulate a drought stress. Volumetric soil moisture content was measured with a TH_2O soil moisture meter (Dynamax Inc., Houston, TX). Once the volumetric soil moisture had been obtained, the irrigation was turned back on the plants were allowed to recover.

Field Data Collection

Clippings were collected four times during the experiment: 1) seven days after the first fungicide application; 2) seven days after the second fungicide application; 3) once drought stress had been reached (i.e., 6% VMC); and 4) when turf had fully recovered from drought stress symptoms.. Clippings were harvested using a Jocobsen TC-22 walking greens mower and analyzed for carotenoid and chlorophyll concentrations using procedures cited in Chapter 1. After clipping collection, cores (17.78 cm diameter) were removed using a cup cutter (Par Aide, Lino Lakes, MN) and transported back to the lab to measure chlorophyll fluorescence. Measurements were obtained using an Open Fluorcam FC (Photon Systems Instruments, Brno, Czech Republic). Parameters of Fv/Fm and NPQ were of specific interest since their measurements provide a direct measure of photochemical efficiency and plant stress. Analysis of variance was conducted using SAS 9.2 (Statistical Analysis Software, Cary, NC) and means were separated using the Fisher's protected least significant difference test (P= 0.05).

Results

Analysis of variance of data collected during the second harvest (i.e., 7 days after the second fungicide application) indicated that plots receiving propiconazole and 48 kg N ha^{-1} month had a significantly (P=0.009) lower zeaxanthin concentration than plots receiving no fungicide and the same rate of N or plots receiving tebuconazole and 24 kg

N ha^{-1} month (Table 10). Antheraxanthin concentrations for harvest two, showed that propiconazole treated plots with high fertility had significantly (P=0.01) lower concentrations than plots with 48 kg N ha-1 month. No significant differences in violaxanthin concentration were detected between treatments.

All treatment combinations increased in zeaxanthin concentrations when exposed to drought stress in harvest three. The biggest increase in zeaxanthin concentration occurred with propiconazole (+112%) and tebuconazole (+94%) applied in conjunction with the 24 kg ha^{-1} rate of N (Table 10). Interestingly, these two treatments also had the lowest zeaxanthin concentrations during harvest two. When receiving 24 kg N ha^{-1}, the untreated control had lower concentrations of antheraxanthin than plots receiving the same rate of N and DMI fungicide (Table 10).

No differences were found among any of the harvests for chlorophyll *a*, chlorophyll *b*, or total chlorophyll concentration (Table 11). All chlorophyll pigments were the highest during harvest two prior to the drought stress being initiated. After the drought stress was imposed, there was approximately a 10% decline in concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll. When the plants were allowed to recover, plots treated with tebuconazole at the high nitrogen rate and the untreated at the high nitrogen rate were the only treatments that saw an increase in chlorophyll pigments.

The response of fungicide and fertility treatments in this study indicated that it is possible to lower the concentration of zeaxanthin in creeping bentgrass leaf tissue prior to drought stress. However, our results indicated that to recover from a stress, fertility might play a bigger role than fungicides.

This study was also conducted in the absence of disease, so it is purported that a bigger response could possibly be seen when disease is present and the fungicides are used as their labels currently designate.

APPENDIX B

TABLES AND FIGURES

Table 1. Mean fresh mean values ± standard deviation for pigment concentrations in creeping bentgrass (*Agrostis stolonifera*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment concentrations (mg 100 g^{-1} FW)						
	ZEA[a]	ANT	VIO	NEO	LUT	BC	Total
Propiconazole	1.25 ± 0.35	5.11 ± 1.22	4.33 ± 1.45	5.34 ± 1.13	15.35 ± 3.25	3.81 ± 1.20	35.21 ± 7.89
Tebuconazole	1.34 ± 0.39	5.05 ± 1.04	4.30 ± 1.08	5.16 ± 0.74	14.92 ± 2.56	3.70 ± 1.31	34.47 ± 6.36
Untreated	1.77 ± 0.55	5.31 ± 0.96	3.87 ± 1.22	4.91 ± 0.97	14.90 ± 3.29	4.05 ± 1.50	34.81 ± 7.77
$LSD_{0.05}$[b]	0.25	0.65	0.71	0.55	1.78	0.74	4.29
Contrast							
Fungicide vs Untreated	***	NS	NS	NS	NS	NS	NS

[a]Abbreviations: Zea, Zeaxanthin; ANT, Antheraxanthin; VIO, Violaxanthin; NEO, Neoxanthin; LUT, Lutein; BC, β-Carotene; Total, Total Carotenoids; FW, Fresh Weight.
[b]Means separated using Fisher's least significant difference test.
*** Significant at $P \leq 0.001$, NS Not Significant

Table 2. Mean fresh values ± standard deviation for pigment concentrations in creeping bentgrass (*Agrostis stolonifera*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment concentrations (mg 100 g^{-1} FW)		
	Chl *a*	Chl *b*	Total Chl
Propiconazole	179.61 ± 44.83	64.81 ± 11.72	244.42 ± 55.38
Tebuconazole	174.54 ± 31.24	62.90 ± 8.18	237.45 ± 37.17
Untreated	165.10 ± 40.32	59.02 ± 9.49	224.12 ± 48.93
$LSD_{0.05}$	22.38	5.24	26.83
Contrast			
Fungicide vs Untreated	NS	*	NS

[a]Abbreviations: Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b*; Total Chl, Total Chlorophyll; FW, Fresh Weight.
[b]Means separated using Fisher's least significant difference test.
*Significant at P≤ 0.05, NS Not Significant

Table 3. Mean fresh values ± standard deviation for pigment ratios in creeping bentgrass (*Agrostis stolonifera*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment concentrations (mg 100 g^{-1} FW)		
	ZAV[a]	ZA/ZAV[b]	Car/Chl[c]
Propiconazole	10.68 ± 2.61	0.60 ± 0.06	0.15 ± 0.01
Tebuconazole	10.69 ± 2.06	0.60 ± 0.05	0.14 ± 0.01
Untreated	10.96 ± 2.20	0.65 ± 0.06	0.16 ± 0.01
$LSD_{0.05}$ Contrast	1.36	0.03	0.01
Fungicide vs Untreated	NS	***	***

[a]Combined concentrations of zeaxanthin, antheraxanthin and violaxanthin.
[b]Ratio of combined concentrations of zeaxanthin and antheraxanthin to zeaxanthin, antheraxanthin and violaxanthin.
[c]Ratio of carotenoid to chlorophyll concentrations
*** Significant at $P \leq 0.001$, NS Not Significant

Table 4. Mean dry values ± standard deviation for pigment concentrations in baby kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

	Leaf tissue pigment concentrations (mg 100 g^{-1} DW)						
Treatment	ZEA	ANT	VIO	NEO	LUT	BC	Total
Propiconazole	3.57 ± 1.82	15.37 ± 3.27	23.44 ± 5.58	27.07 ± 3.44	80.28 ± 12.92	44.87 ± 9.86	194.60 ± 28.03
Tebuconazole	3.31 ±1.62	14.58 ± 3.31	22.99 ± 5.13	26.17 ± 3.29	77.22 ± 11.32	43.39 ± 10.38	188.10 ± 28.32
Untreated	3.64 ±1.71	14.31 ± 3.57	20.51 ± 7.59	23.23 ± 6.13	70.64 ± 20.30	40.00 ± 14.18	172.34 ± 47.92
$LSD_{0.05}$	0.99	2.30	3.58	2.63	9.15	6.89	21.14
Contrast							
Fungicide vs Untreated	NS	NS	NS	**	*	NS	*

[a]Abbreviations: Zea, Zeaxanthin; ANT, Antheraxanthin; VIO, Violaxanthin; NEO, Neoxanthin; LUT, Lutein; BC, β-Carotene; Total, Total Carotenoids; DW, Dry Weight.
[b]Means separated using Fisher's least significant difference test.
*** Significant at P≤ 0.001, NS Not Significant

Table 5. Mean dry values ± standard deviation for pigment concentrations in mature kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

	Leaf tissue pigment concentrations (mg 100 g^{-1} DW)						
Treatment	ZEA	ANT	VIO	NEO	LUT	BC	Total
Propiconazole	2.91 ± 1.36	6.35 ± 1.41	5.98 ± 2.53	8.22 ± 2.63	29.59 ± 8.77	14.52 ± 4.71	67.56 ± 18.25
Tebuconazole	3.34 ± 1.48	6.89 ± 1.36	5.81 ± 2.72	8.02 ± 2.96	28.56 ± 9.75	14.59 ± 5.45	67.20 ± 21.14
Untreated	3.30 ± 1.73	6.26 ± 1.20	5.60 ± 2.22	7.32 ± 2.08	26.48 ± 7.16	13.80 ± 4.38	62.76 ± 15.80
$LSD_{0.05}$	0.88	0.73	1.46	1.49	5.01	2.87	10.74
Contrast							
Fungicide vs Untreated	NS	NS	NS	NS	NS	NS	NS

[a]Abbreviations: Zea, Zeaxanthin; ANT, Antheraxanthin; VIO, Violaxanthin; NEO, Neoxanthin; LUT, Lutein; BC, β-Carotene; Total, Total Carotenoids; DW, Dry Weight.
[b]Means separated using Fisher's least significant difference test.
NS Not Significant

Table 6. Mean dry values ± standard deviation for pigment concentrations in baby kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment concentrations (mg 100 g^{-1} DW)		
	Chl *a*	Chl *b*	Total Chl
Propiconazole	722.63 ± 185.56	273.88 ± 33.91	996.51 ± 204.62
Tebuconazole	677.68 ± 165.01	261.43 ± 31.58	939.12 ± 182.44
Untreated	598.04 ± 235.49	233.70 ± 57.16	831.74 ± 283.32
$LSD_{0.05}$	118.41	25.25	136.09
Contrast			
Fungicide vs Untreated	*	**	*

[a]Abbreviations: Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b;* Total Chl, Total Chlorophyll; DW, Dry Weight.
[b]Means separated using Fisher's least significant difference test.
*Significant at P≤ 0.05, ** Significant at P≤ 0.01

Table 7. Mean dry values ± standard deviation for pigment concentrations in mature kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment concentrations (mg 100 g^{-1} DW)		
	Chl a	Chl b	Total Chl
Propiconazole	164.83 ± 84.90	85.18 ± 20.28	250.01 ± 103.63
Tebuconazole	168.40 ± 89.70	80.91 ± 23.02	249.31 ± 111.64
Untreated	154.84 ± 73.08	75.62 ± 16.87	230.46 ± 88.26
$LSD_{0.05}$	49.45	11.36	60.15
Contrast			
Fungicide vs Untreated	NS	NS	NS

[a]Abbreviations: Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b*; Total Chl, Total Chlorophyll; DW, Dry Weight.
[b]Means separated using Fisher's least significant difference test.
NS Not Significant

Table 8. Mean dry values ± standard deviation for pigment ratios in baby kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications

Treatment	Leaf tissue pigment ratios (mg 100 g^{-1} DW)		
	Z AV	ZA/ZAV	Car/Chl
Propiconazole	42.37 ± 4.66	0.49 ± 0.11	0.20 ± 0.02
Tebuconazole	41.32 ± 7.06	0.44 ± 0.11	0.20 ± 0.02
Untreated	38.47 ± 8.75	0.47 ± 0.11	0.21 ± 0.02
$LSD_{0.05}$ Contrast	3.96	0.07	0.01
Fungicide vs Untreated	*	NS	*

[a]Combined concentrations of zeaxanthin, antheraxanthin and violaxanthin.
[b]Ratio of combined concentrations of zeaxanthin and antheraxanthin to zeaxanthin, antheraxanthin and violaxanthin.
[c]Ratio of carotenoid to chlorophyll concentrations
* Significant at $P \leq 0.05$, NS Not Significant

Table 9. Mean dry values ± standard deviation for pigment ratios in mature kale (*Brassica oleracea*) grown in a greenhouse. Fungicide values are pooled over two application rates and are the mean of two experiments performed in 2010 and 2012 with six replications each.

Treatment	Leaf tissue pigment ratios (mg 100 g^{-1} DW)		
	Z AV	ZA/ZAV	Car/Chl
Propiconazole	15.24 ± 2.65	0.61 ± 0.14	0.29 ± 0.05
Tebuconazole	16.04 ± 3.43	0.64 ± 0.14	0.29 ± 0.05
Untreated	15.17 ± 2.86	0.63 ± 0.12	0.29 ± 0.05
$LSD_{0.05}$ Contrast	1.70	0.08	0.03
Fungicide vs Untreated	NS	NS	NS

[a]Combined concentrations of zeaxanthin, antheraxanthin and violaxanthin.
[b]Ratio of combined concentrations of zeaxanthin and antheraxanthin to zeaxanthin, antheraxanthin and violaxanthin.
[c]Ratio of carotenoid to chlorophyll concentrations
NS Not Significant

Table 10. First experimental run (2011) for xanthophylls cycle pigments harvested on a creeping bentgrass (*Agrostis stolonifera*) putting green.

		ZEA			ANTH			VIOL		
		mg 100 g^{-1} FW								
Treatment	Fertility	Harvest								
		2	3	4	2	3	4	2	3	4
Propiconazole	high	1.22 b	2.59 a	1.91 a	6.84 ac	8.23 a	6.05 a	10.07 a	7.65 a	8.41 a
Propiconazole	low	2.55 a	3.12 a	2.13 a	8.63 cd	8.91 a	5.73 a	10.66 a	7.57 a	6.71 a
Tebuconazole	high	1.84 ab	2.82 a	2.41 a	7.64 abc	8.44 a	6.84 a	9.95 a	7.45 a	8.80 a
Tebuconazole	low	1.41 b	2.77 a	1.96 a	7.13 ab	7.96 a	6.08 a	9.87 a	6.77 a	7.98 a
Untreated	high	2.64 a	3.09 a	2.33 a	8.44 bd	9.01 a	7.11 a	10.16 a	7.10 a	8.06 a
Untreated	low	1.76 ab	2.56 a	1.99 a	6.99 ab	7.94 a	6.21 a	9.33 a	6.55 a	7.40 a
LSD (0.05)		1.12	0.78	0.86	1.85	2.11	1.68	2.46	1.89	1.29

[a]Abbreviations: Zea, Zeaxanthin; ANT, Antheraxanthin; VIO, Violaxanthin; FW, Fresh Weight.
[b]Means separated using Fisher's least significant difference test.

Table 11. First experimental run (2011) for chlorophyll pigments harvested on a creeping bentgrass (*Agrostis stolonifera*) putting green.

Treatment	Fertility	Chl *a*			Chl *b*			Total Chl		
		mg 100 g^{-1} FW								
		Harvest								
		2	3	4	2	3	4	2	3	4
Propiconazole	high	250.85	223.70	213.11	90.12	78.44	68.45	340.97	302.14	281.56
Propiconazole	low	269.99	235.44	196.37	96.90	84.92	65.51	366.89	320.36	261.88
Tebuconazole	high	243.80	215.04	234.89	86.72	75.94	74.56	330.52	290.98	309.45
Tebuconazole	low	241.53	208.00	200.94	84.68	73.20	64.89	326.21	281.19	265.83
Untreated	high	243.87	206.55	232.12	85.99	71.17	76.33	329.86	277.71	308.45
Untreated	low	229.58	198.35	201.99	80.74	70.77	67.05	310.32	269.11	269.04
LSD (0.05)		32.96	49.82	31.41	11.23	16.76	10.31	44.11	66.54	41.54

[a]Abbreviations: Chl *a*, Chlorophyll *a;* Chl *b*, Chlorophyll *b*; Total Chl, Total Chlorophyll; FW, Fresh Weight.

[b]Means separated using Fisher's least significant difference test.
Means followed by the same letter do not differ significantly at $P \leq 0.05$.

VITA

David Paul Shell was born on April 27, 1982 in Kokomo, IN to Dean and Elizabeth Shell. He attended Kokomo High School and graduated in 2000. After high school, he enrolled in the Criminal Justice program at Indiana University. After his first year, he transferred to the Turfgrass Science program at Purdue University where he obtained his Bachelors of Science degree in 2005. After completing his Bachelors degree, David took a job as the assistant superintendent at Chippendale Golf Course in Kokomo, IN, where he worked for three years. He began his work toward his Masters of Science degree in 2010 under the direction of Dr. Brandon Horvath. David plans to enroll as a Ph.D. student at The University of Tennessee after graduation.