A NOVEL HORTICULTURAL OIL, CIVITAS™, ALTERS TURFGRASS GROWTH AND PHYSIOLOGY

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A NOVEL HORTICULTURAL OIL, CIVITAS™, ALTERS TURFGRASS GROWTH AND PHYSIOLOGY

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For centuries, oil derivatives have been applied to plants for pest control. The novel horticultural oil called Civitas™ has been developed for use in the turfgrass industry. Incorporation of Civitas with low rates of fungicides provides excellent disease control reduced environmental impact. Civitas, like other horticultural spray oils, can be phytotoxic. Chlorosis, membrane disruption, and reduced photosynthesis, transpiration, and growth can result from horticultural oil phytotoxicity. Chlorosis is masked with a green pigment added to Civitas. Unsubstantiated claims by turfgrass managers suggest Civitas increases clipping yield, reduces ball roll, can cause stand decline.

The primarily goals of this work were to understand how Civitas affects turfgrass in the field and then understand how physiology is affected in the growth chamber. The objectives of this dissertation were to document how Civitas affects turfgrass growth, performance, and quality in the field, investigate factors known to induce spray oil phytotoxicity, measure turfgrass transpiration and photosynthesis, and finally monitor turfgrass carbon partitioning and nonstructural carbohydrate status.

Putting green clipping yield increased and golf ball roll distance declined for 10 days after application of Civitas. Ball roll was not correlated with clipping yield which indicates some other factor besides increased leaf elongation affected ball roll distance. Civitas application to a

mixed annual bluegrass (*Poa annua* L.) creeping bentgrass (*Agrostis stolonifera* Hud.) putting surface caused chlorosis, which was masked with the green pigment, and decline in stand density. Civitas also increased turfgrass canopy temperature 0 to 2°C depending on solar intensity.

The phytotoxicity induced by Civitas is consistent with chronic phytotoxicity described in the literature. While Civitas did not directly damage or alter the cuticle quantity or composition, oil deposition on the leaf reduced gas exchange. The decline in gas exchange reduced transpiration rate and radiation use efficiency (RUE) for several days after application. The decline in RUE was particularly dramatic at higher photosynthetic photon flux densities. Carboxylation efficiency was more sensitive to Civitas than transpiration, most likely due to a reduction in Rubisco activity. Increased clipping yield and reduced RUE reduced the content of storage carbohydrates, starch and fructans.

BIOGRAPHICAL SKETCH

Bill Kreuser was raised in West Allis, WI. He was always fascinated by science and enjoyed discovering how things worked. Bill became interested in turfgrass management and horticulture in high school after he installed a USGA putting green in his parents backyard. While Bill enjoys the game of golf on most days, he constructed the green mainly because family and friends told him high school student could not build and maintain a high quality putting green. After successful construction of the backyard putting green, and eventual fairway and bunkers, Bill began to work as a summer groundskeeper at Westmoor Country Club in Brookfield, WI. He worked for golf course manager Jerry Kershasky who challenged Bill work hard, have an eye for detail, and learn as much as he could about agronomy and turfgrass management.

Upon graduating from high school, Bill enrolled at the University of Wisconsin-Madison where he studied Turf and Grounds Management in the Department of Soil Science. During his B.S. at Madison, Bill actively pursued undergraduate research opportunities with Dr. Wayne Kussow and Dr. Doug Soldat. He also spent his first two summers as an intern at Whistling Straits Golf Course in Haven, WI. During the summer of his junior year, Bill was the field research manager for Dr. Soldat. That summer Bill conducted several experiments with plant growth regulators on golf course putting greens. That work continued through his graduation on May 2009 and led to his M.S. in Soil Science at UW-Madison in August 2010.

Bill began his Ph.D. studies at Cornell University in August 2010 under Dr. Frank Rossi in the Department of Horticulture. While in Ithaca, Bill learned how to fly and meet his fiancé Katie King. Upon graduation from Cornell University, Bill will begin a position as an assistant professor at the University of Nebraska-Lincoln as the state's turfgrass extension specialist in the Department of Agronomy and Horticulture.

Dedicated to all of those who have challenged me.

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ABSTRACT

Oil derivatives have been applied to plants to control insect and pathogen pests for centuries. Modern horticultural spray oils are primarily petroleum-derived mixtures of paraffinic hydrocarbons with up to 5 percent mass emulsifiers and pose minimal non-target environmental effects. Modern horticultural oils are free of phytotoxic aromatic and unsaturated hydrocarbons. A significant amount of evidence indicates horticultural spray oils kill insect pests via anoxia as a result of spiracle inhibition, but could also work via fumigant action, nervous system and cell disruption, and insect dissociation. Additionally horticultural spray oils have also proven to be fungistatic to many fungal diseases of horticultural crops.

Environmentally compatible pest management strategies such as integrated pest management have successfully integrated the use of horticultural spray oils to reduce reliance on traditional pest control products. Recently, a new petroleum derived spray oil (PDSO) Civitas has been developed for use in the turfgrass industry. The PDSO Civitas represents a new era of pest management products that induce innate plant defense mechanisms and is minimally fungistatic. Furthermore, the application of PDSO Civitas with low-labeled rates of traditional fungicides can maximize efficacy and minimize pesticide load rates.

Horticultural spray oils can cause acute or chronic phytotoxic response in plants. Acute toxicity is common in early, less refined, oil formulation and results from direct cell damage. Most modern paraffinic products cause a chronic phytotoxicity by inhibiting gas exchange. These products impede stomatal conductance that results in reduced transpiration, photosynthesis, and respiration. The degree and persistence of chronic toxicity of PDSO is proportional to dose. Plants treated with the PDSO Civitas exhibit a chronic phytotoxic response. The visible chlorosis that is masked by a phthalocyanine pigment Harmonizer in the commercially formulated products, Civitas Two Pack and Civitas One.

Leaf chlorosis is the most obvious symptom of spray oil of phytotoxicity. Yet, the chronic inhibition of gas exchange alters physiological processes that can induce significant oxidative stress. Increased oxidative stress often results in reduced plant growth that has negative implications where productivity and yield are important. Interestingly, plants treated with the PDSO Civitas exhibit increased growth and yield that directly contradict research with similar PDSO.

The goal of this research is to elucidate the physiological components such as transpiration and photosynthetic activity that are involved in the observed field response to Civitas application. Specifically, this project focuses on the response of turfgrass growth and visual quality, phytotoxicity, and ball roll distance, as well as carbon portioning, photosynthetic rates, and antioxidant activity.

INTRODUCTION AND LITERATURE REVIEW

Horticultural spray oils have been used to control plant pests for centuries. It was first reported by Goeze (1787) that application of oil to plant foliage and fruit helped control insect pests. Refinement of crude oil at the end of the 19th century increased oil availability and led to an increase in research on petroleum-derived spray oils. Early uses included a 10 to 25% kerosene emulsion applied to citrus plants for aphid and other insect control in the 1880's (Agnello, 2002). Interestingly, use of horticultural spray oils waned during the mid-20th century partially due to advances during the green revolution. Recently, there is a renewed interest in spray oils because of their benign environmental impact which is favored by an increasingly environmentally conscious public.

The resurgent use of horticultural spray oils can also be attributed to public pressure on growers to use more organic and sustainable practices. However the spray oils are used today for similar pests as over 200 years ago with expanded use to control insect and fungal pests in citrus, pome and stone fruits, vegetable, floricultural, and ornamental horticultural crops (Northover and Timmer, 2002). Additionally, recently identified synergistic effects of horticultural spray oils on traditional pest control products, especially at low spray volumes is reducing the number of pesticide applications and further enhancing the environmental innocuity (Rae, 2002).

Modern horticultural spray oils are highly refined to eliminate impurities that reduce risk to the environment and human health (Kuhlmann and Jacques, 2002). For example, purified white oil is commonly used in the food, cosmetic, and pharmaceutical industries (Oriana Persechini, personal communication, 2013). In fact, the US Food and Drug Administration (FDA) strictly defines properties of mineral oils in FDA 21CRD178.3620 to ensure safety for consumers consequently the Organic Research Material Institute (OMRI) commonly grants petroleum-derived mineral oils approval as certified organic product.

Recently, a Petroleum-Derived Spray Oil (PDSO), Civitas, has been developed for use as an OMRI-Certified product in the turfgrass industry (Petro-Canada, Mississauga, ON, CA). The PDSO Civitas is a mixture of food-grade isoparaffins ranging in size from 16 to 33 carbons in length (Hsiang et

al., 2013) and less than 5 percent by mass of emulsifiers. Applied alone Civitas can reduce incidence and severity of dollar spot (*Sclerotina homoeocarpa*) and gray leaf spot (*Pyricularia grisea*). In addition it is commonly tank-mixed with the low rates of traditional turfgrass fungicides to enhance control of a broad spectrum of fungal diseases (Popko et al., 2010; McCall and Focht, 2010; Aynardi et al., 2011; Popko and Jung; Aynardi and Uddin, 2013 a, b).

Spray Oils Physical Properties

The chemical composition of horticultural spray oils has evolved during a century of research. Early spray oils contained a diverse and often unplanned array of hydrocarbons. Common constituents included unsaturated hydrocarbons, aromatic and naphtheic ring compounds, and paraffinic hydrocarbons (alkanes) (Pearce et al., 1942; Riehla and LaDue 1952; Chapman, 1697 p.40). These early spray oils were problematic because they had the potential to damage the plants and could likely contained known carcinogens (Ebling, 1959; Chapman, 1967, and Riehl, 1967). Gray and de Ong (1926) described the potential phytotoxicity hazard posed by unsaturated hydrocarbons and aromatic rings which they removed by sulfuric acid treatment. Today, hydrocracking or solvent removal is commonly used to either break aromatic rings into saturated paraffins or remove aromatic rings (Jacques and Kuhlmann, 2002). The remaining saturated paraffin molecules are much less phytotoxic, relatively non-toxic to humans and the environment, yet still provide pesticidal properties (Chapman, 1947, 1967 p. 40). In fact, Chapman (1947, 1967) and Simanton and Trammel (1966 p40) found certain horticultural spray oils with greater than 60% paraffin content provide significant pest control.

Most modern horticultural spray oils are derived from petroleum-based mineral oil. Kuhlmann and Jacques (2002) proposed a classification for different spray oils based on the degree of refinement, composition, and physical properties. Mineral oil is the most fundamental level of classification and is defined by the FDA 21CFR178.3620. The minimum level of refinement required to meet this classification level assurances the oil is pure enough for contact with food according to the FDA. Mineral oils need to be from virgin petroleum distillate, have an initial boiling point greater than 232°C, and have an ASTM D 1500 color of less than or equal to 5.5. The regulations also specify strict UV absorbance

limits that reflect the content of carcinogenic aromatics to its photostability. While FDA mineral oils are safe for humans, they still have the potential to be phytotoxic to plants and may have limited insecticidal activity.

Agricultural mineral oils have stricter refinement criteria than FDA mineral oils. These oils meet the requirements of FDA 21CFFR178.3620 but also need to be of a paraffinic source, with minimum unsulfonated residue (UR) of 92%, and paraffin content greater than 60% (Kuhlmann and Jacques, 2002). Unsulfonated residue percentage is a measure of hydrogen saturation as defined by ASTM D 483. These minimum requirements of an agricultural mineral oil guarantee a higher level of saturated paraffins which have greater pesticidal activity than mixtures containing higher concentrations of contaminates such as naphthenes (Pearce and Chapman, 1948, 1952). Agricultural spray oils are generally safe enough for agronomic crops and are commonly used as spray adjuvants and inert matter in pesticide formulations. However, they still have the potential to cause unacceptable phytotoxicity on high value perennial crops. Civitas likely meets the definition of an agricultural mineral oil due to its wide range in paraffin molecules (Hsiang et al., 2013)

Horticultural mineral oils have the most stringent refinement criteria proposed by Kuhlmann and Jacques (2002). They have stringent tolerances for median-equivalent *n*-paraffin carbon number (median *n*C*y*) and an equivalent *n*-paraffin carbon range (*n*C*y* range) of 5 to 6. The authors proposed different median *n*C*y* to reflect difference in potential phytotoxicity across plant species. These narrow and specific ranges confirm research by Pearce and Chapman (1952), Riehl and Jeppson (1953), Simanton and Trammel (1966), and Furness and Maelzer (1981) that found paraffin *n*C*y* values less than *n*C18 and greater than *n*C25 increased the risk of phytotoxicity.

Spray Oil Modes of Action

There have been several proposed mechanisms regarding the pesticidal activity of horticultural spray oil. De Ong (1926) was the first to show lubricating oil entered the arthropod spiracles and suffocated red scale (*Aonidiella aurantii*). This mode of action has become the most favored theory for horticultural spray oil mode of action (Davidson, 1991). However, other potential insecticidal modes of

action include direct fumigation, nervous system disruption, cell disturbance, and direct arthropod desiccation (Taverner, 2002).

Diversity in spray oil formulation used in studies often confounds research. For example, different researchers have used different spray oils ranging from kerosene and other highly phytotoxic materials of the late 19th century to paraffinic spray oils more common today. Clearly additional research is required to understand how modern horticultural spray oils affect arthropod pests.

Horticultural oils can reduce plant disease. Paraffinic horticultural spray oils have been found to control powdery mildew (*Podosphaera* spp., *Uncinula* spp., *Sphaerotheca* spp., Microphaera penicillata (Wallr.:Fr.) Lév., *Erysiphe polygoni* DC, and *Lycopersicon esculentum* Mill.) and rust (*Uromyces* spp.) in many species of horticultural crops (Northover and Timmer, 2002). Horticultural spray oils are also important for control of greasy spot (*Mycosphaerella citri* Whiteside) in citrus fruit.

Despite moderate to good disease control, horticultural spray oils are rarely fungitoxic (Northover and Tummer, 2002), and have actually been used in precision disease inoculations (Rowell and Hayden, 1956; Rowell and Olien, 1957; p520). There have been reports of decreased conidial germination (Nikolov and Andreev, 1997; Kalliampur et al. 2002) and reduced germ tube growth (Stover and Dickson, 1968 p521) with horticultural oil. However, the short-lived fungistatic responses and good curative activity suggest horticultural oils may have additional biological activity at a more fundamental level. Specifically, Nothover and Timmer (2002) have hypothesized that horticultural spray oils may elicit host resistance.

The PDSO Civitas has been shown to prime plant defense with no fungicidal and very limited fungistatic activity (Cortes et al., 2010b). Cortes et al. (2010a, 2010b) demonstrated Civitas applied to both *Nicotiana benthamiana* and creeping bentgrass primed or upregulated genes involved in induced systemic resistance (ISR), including genes involved in the jasmonic acid pathway. Priming of the JA pathway with small elicitor molecules has been demonstrated in other plant systems including *Zea mays* (Djonovic et al., 2007). In creeping bentgrass, expression of allene oxide synthase (OPR3), the rate limiting enzyme in the JA pathway was dramatically enhanced shortly after inoculation with

Microdochium nivale following Civitas application (Cortes et al., 2010b). The control plants did not increase OPR3 expression post-inoculation. Jasmonic acid works in conjunction with other phytohormones, especially ethylene, to increase expression of various pathogenesis related (PR) genes which aid in plant defense against pathogens, as well as insect herbivory (Vallad and Goodman, 2004; Cho et al., 2007). Expression of genes associated with plant systemic acquired resistance (SAR) did not change as a result of Civitas application (Cortes et al., 2010a, 2010b).

Induced systemic resistance is a "state of enhanced defensive capacity" that increases plant's defense against pathogens (van Loon et al., 1998). In nature ISR typically arises from interactions with soil-borne microorganisms (van Loon et al., 1998). Inoculation with plant growth-promoting rhizobacteria (PGPR) or *Trichoderma* spp. can elicit ISR and enhance plant growth (Ryu et al., 2004; Vallad and Goodman, 2004; Shoresh et al., 2010). Transcriptome and proteome studies in *Arabidopsis thaliana* found pathways involved in mineral uptake, photosynthesis, and the TCA cycle were upregulated in response to a PGPR volatile, GB03, (Kwon et al., 2010). The authors concluded that these changes likely enhanced growth rate.

Plant growth promoting rhizobacteria often liberate volatile organic compound (2R,3R)-butanediol which elicits ISR in plants (Ryu et al., 2004). Cortes et al. (2010a and 2010b) found Civitas and (2R,3R)-butanediol had a similar effect on gene expression in creeping bentgrass and *Nicotiana bethaminana* and concluded that Civitas application also elicits ISR. While other known ISR inducing volatiles are typically small molecule, hexadecane, a C_{16} alkane, triggered a stronger ISR response than (2R,3R)-butanediol (Lee et al., 2013). Sixteen carbon paraffins are common in spray oils and are known to be in Civitas (Hsiang et al., 2013). This finding supports the hypothesis that paraffinic horticultural oils elicit host resistance (Nothover and Timmer, 2002) and is directly supported by the results of Cortes et al. (2010a, 2010b).

Spray Oil Phytotoxicity

A significant limitation to widespread use of horticultural spray oils continues to be the risk of plant phytotoxicity (Johnson and Hodgkinson, 2002). While research over the past 130 years has isolated

and removed particularly phytotoxic compounds in horticultural spray oils, the highly refined paraffinic products have a high risk of phytotoxicity. Susceptibility to horticultural spray oil phytotoxicity can be plant species dependent. The degree of phytotoxicity is also a function of existing biotic or abiotic stress (Hodgkinson et al., 2002). High levels of environmental stress such as drought stress can exacerbate phytotoxicity.

Horticultural spray oil phytotoxicity is commonly classified as either acute or chronic. A review of spray oil phytotoxicity by Hodgkinson et al. (2002) summarized the differences between acute and chronic phytotoxicity. Characteristic of acute phytotoxicity results in direct damage to the plant or plant fruit; leaf lesions, fruit sunburn, leaf abscission, fruit drop, and damage to meristematic tissue. Chronic phytotoxicity is a function of oil persistence on leaves or fruit which can inhibit gas exchange. This commonly results in photosynthesis, transpiration, respiration, and ultimately less growth. Hodgkinson (1999) theorized that membrane disruption and distillation temperature explain acute and chronic phytotoxicity. The degree of phytotoxicity also depends on application rate. Reihl (1981) found oil deposition of 100 to 300 µg oil cm^{-2} provided efficacy and minimized phytotoxicity in citrus.

Membrane disruption and acute phytotoxicity of is more common when the base of a spray oil contains aromatic and unsaturated hydrocarbons. These compounds are more reactive than saturated paraffins and can disrupt cellular membranes and lead to chlorophyll degradation (Crafts and Reiber, 1952; Orendevill and Warren, 1977; Brown, 1982). Damage is commonly quantified with measurement of electrolyte leakage and betracyanin efflux (Coupland et al., 1898; Manthey and Nalewaja, 1992; Matsui et al., 1992; Hodgkinson and Mackey, 1995). Measurement of electrolyte leakage is commonly used to measure plant response to drought and heat stress in turfgrass.

Chronic oil induced phytotoxicity is more common with modern paraffinic horticultural oils. Although Kuhlmann and Jacques (2002) proposed specifications narrow and specific paraffin ranges for horticultural spray oils, there remains the potential for phytotoxicity due by inhibition of gas exchange. There is a large body of literature that shows horticultural spray oils can reduce gas exchange and inhibit transpiration, photosynthesis, and respiration in many plant species (Wedding et al., 1952; Baker, 1970;

Riehl and Wedding, 1959a, 1959b, 1959c; Jones et al., 1983; Salyani et al., 1990; Rethwisch, 1992; Allison and McKenna; 2002; Finger et al., 2002). Infrared gas analyzers are commonly used to measure plant stomatal conductance, transpiration, respiration, and photosynthesis. Inhibition of fundamental plant processes can reduce plant growth and yield (Rethwisch, 1992; Finger et al., 2002). Inhibition of gas exchange also has the potential to increase photooxidative stress and increase photorespiration in C_3 plants as the photosynthetic apparatus becomes starved from its substrate, CO_2. Both processes reduce photosynthetic efficiency and lead to the production of damaging reactive oxygen species (ROS) which cause damage to plant lipids, proteins, pigments, and DNA (Polle, 1997). As with any horticultural spray oil, optimization of application rate is essential to the application will be efficacious and reduce the risk of phytotoxicity.

Civitas can be phytotoxic to turfgrass, especially creeping bentgrass at low height of cut (Michael Fefer, personal communication, 2011). It is not clear how Civitas causes phytotoxicity in turfgrass. Its physical properties suggest Civitas has the potential to cause chronic phytotoxicity (Hsiang et al., 2013). To minimize the decline in turfgrass quality, Petro-Canada has developed a green pigment called Harmonizer which is applied with Civitas. Harmonizer is a phthalocyanine pigment (Nash, 2011). Originally Civitas and Harmonizer needed to be mixed on-site, commonly referred to as the Civitas Two Pack. In 2013, Civitas One was released which contains both the Civitas oil and Harmonizer pigment in one product. It is not clear if the Harmonizer simply masks or mitigates phytotoxicity. Spray oil induced phytotoxicity is magnified during stress. Ervin et al. (2004) found that application of phthalocyanine pigment to turfgrass reduced UV-B stress. It is possible that Harmonizer masks and mitigates phytotoxicity by reducing plant stress.

Plant Growth Response

Crop growth is a function of photosynthesis where net dry matter production is equal to the rate of gross photosynthesis minus ‘dark’ and photorespiration (Hay and Porter, 2006). While photosynthate partitioning between vegetative growth, yield production, and storage carbohydrates can vary with species and time or year, any reduction in photosynthesis or increase in respiration diminishes available

photosynthate to drive growth. Horticultural oil induced phytotoxicity, regardless of cause, typically reduces net photosynthesis. As a result, horticultural spray oils have been found to reduce plant growth and yield. Application of spray oil to wine grapes reduced berry weight, cluster weight, the number of clusters per vine, total soluble solids in juice and delayed sugar accumulation by nearly two weeks (Nothover and Homeyer, 1998; Finger et al., 2002). Reduced growth and yield as a result of depressed photosynthesis from horticultural spray oils has been found in other crops (Wedding et al., 1952; Gudin et al., 1976; Wood and Pyane, 1986).

While quantitative measurement of turfgrass growth rate and photosynthetic rate following treatment with the horticultural spray oil Civitas is lacking, there has been anecdotal evidence from golf course managers that Civitas increases clipping yield and reduces golf putting green ball roll distance. Ball roll distance (BRD), more commonly referred to as green speed, is the distance a golf ball rolls across a putting surface (Hartwiger et al., 2001). It is an indirect measurement of the friction and surface uniformity of a putting green (Gaussoin et al., 1995). Many golf course superintendents try to maximize ball roll distance with a variety of cultural practices to satisfy high golf expectations. Alternatively, golf course superintendents typically avoid products or practices that may reduce ball roll distance.

The vast collection of ball roll literature clearly suggests practices that increase turfgrass clipping yield, the amount of dry matter removed during mowing, negatively affect ball roll distance. Many of these practices have been summarized by Nikolai (2005). The anecdotal increase in putting green clipping yield and decline in ball roll distance is therefore logical and worth further exploration.

Although the horticultural oil literature suggests an increase in turfgrass in clipping yield following Civitas application would be unlikely, molecules that found to elicit ISR have also been known to increase plant growth and yield (Ryu et al., 2004; Vallad and Goodman, 2004; Shoresh et al., 2010). This increase in growth has been attributed to increase photosynthesis (Kwon et al., 2010). Interestingly however, exogenous application JA to rice, soybean, and *Arabidopsis* significantly reduced shoot and root growth (Anderson, 1988; Swiatek et al., 2003; Cho et al., 2007). Additionally, increased clipping yield in turfgrass with plant growth regulators or shade is known to decrease levels of storage carbohydrates in

turfgrass (Schnyder and Nelson, 1989; Richie et al., 2001). There is a high likelihood that Civitas affects turfgrass photosynthesis and carbon partitioning in some capacity. Detail measurement of gas exchange, growth rate, and carbon partitioning is required to understand the potential changes in carbon dynamics.

Dissertation Objectives

Civitas use has continued to increase since it was first labeled in 2009; yet a clear understand of how Civitas affect turfgrass growth, performance, and physiology is lacking. Turfgrass managers have demanded a deeper understand of Civitas which has limited widespread adoption in the industry. The first core research goal was to carefully characterize turfgrass response to Civitas in the field. Clipping yield, ball roll distance, and phytotoxicity were measured in response to various combinations of Civitas and the Harmonizer pigment. A commonly applied plant growth regulator was also evaluated for its potential to mitigate changes in ball roll caused by Civitas. These fundamental studies were required to develop future hypothesizes regarding turfgrass physiological responses to Civitas.

The second core research goal was to understand how Civitas affects whole plant physiology under controlled growth chamber experiments. Specifically, common sources of spray oil phytotoxicity were evaluated. Turfgrass electrolyte leakage, cuticle morphology and composition, and gas exchange was measurements were used to understand the nature of Civitas induced phytotoxicity. Net photosynthetic, respiration, and transpiration rates were examined in further detail since these processes are fundamental to plant physiology. Finally, a growth chamber study of carbon partitioning was conducted to bridge turfgrass growth response with phytotoxicity. The objective of that study was to determine how plant carbon dynamics change in response to increased clipping yield and phytotoxicity. Ultimately, these diverse studies will illuminate future lines of Civitas research and be used to develop best management practices for Civitas on turfgrass.

Chapter Two: Civitas Formulation Affects Putting Green Clipping Yield and Ball Roll Performance

ABSTRACT

Civitas is a petroleum-derived horticultural oil used in turfgrass to induce plant systemic resistance and reduce reliance on traditional pesticides. Anecdotal field observations suggest Civitas enhanced clipping yield production and reduced ball roll distance (BRD) on golf putting surfaces. However, these claims have not been evaluated scientifically. Two field studies were conducted to assess the effect of Civitas formulation on clipping yield and BRD on mixed creeping bentgrass (*Agrostis stolonifera*) and annual bluegrass (*Poa annua*) putting surfaces. Clipping yield was measured daily in both experiments and BRD was measured after the surfaces was mowed, rolled, and 6 hours after rolling daily in six 10 day runs. The effect of the plant growth regulator, trinexapac-ethyl, was also evaluated. Application of Civitas increased clipping yield for 10 days after treatment. Addition of the green pigment Harmonizer did not affect clipping yield response. Civitas reduced BRD for a period of 9 days after treatment. Ball roll distance was always greatest after the surface was rolled and declined 6 hours after rolling within a day and was greatest 4 to 7 days after daily rolling began. The change in BRD from Civitas was great enough to be perceived by golfers. Trinexapac-ethyl increase BRD on half the days but the increase was not of practical significance. Ball roll distance was rarely correlated to clipping yield. The literature indicates practices, such as nitrogen fertilization, that stimulate clipping yield, also reduce BRD. However, the data presented in this study suggest that factors other than increased leaf elongation cause a decline in BRD.

INTRODUCTION

Ball roll distance (BRD), more commonly referred to as green speed by golfers and golf course superintendents alike, is the distance a golf ball rolls across a putting surface (Hartwiger et al., 2001). It is an indirect measurement of the friction and surface uniformity of a putting green (Gaussoin et al., 1995). For decades BRD has been measured with a grooved 91 cm inclined plane designed to release a golf ball at an angle of 20° called a Stimpmeter (USGA, 1979). Although the Stimpmeter was originally designed to help golf course superintendents provide consistent BRD among the putting surfaces, golfers today commonly use BRD as a proxy for the caliber of a golf course (Nikolai, 2005). Despite a myriad of trade and magazine articles describing the hazards of large BRD on course playability and plant stress, many golf course managers and superintendents still strive to maximize BRD.

The literature suggests maintenance practices that promote vigorously growing plants have a detrimental effect on BRD. For example, lowering mowing height, increasing mowing frequency, rolling the surface, and frequent sand topdressing can increase BRD (Salaiz et al., 1995; Hartwiger et al., 2001; McCullough et al., 2005a; Streich et al., 2005; Koeritz and Stier, 2009; Iguagiato et al., 2009; McDonald et al., 2013). Alternatively, BRD declined as N fertilizer rate and irrigation frequency as increased thereby increasing turfgrass visual quality and clipping yield (Christians et al., 1979; Streich et al., 2005; McCullough et al., 2006b; Schlossberg and Schmidt, 2007; Koeritz and Stier, 2009; Pease et al., 2011; Baldwin and Brede, 2012). Finally, plant growth regulators (PGRs), typically gibberellin biosynthesis inhibitors that reduce cell elongation and clipping production (a measure of growth), are commonly used to increase BRD or maintain distance over time (Fagerness et al., 2000; Streich et al., 2005; McCullough et al., 2005a, b, c; McCullough et al., 2006a, b; McCullough et al., 2007; McCarty et al., 2011;

McDonald et al., 2013). Clearly this vast body of research suggests BRD is inversely proportional to growth as measured by clipping yield.

Many of the methods employed to maximize BRD can lead to significant plant stress. For example, reducing mowing height from 4 to 3 mm reduced visual turfgrass quality, color, net photosynthetic rate, and leaf photochemical efficiency (Fv/Fm) on a creeping bentgrass (*Agrostis stolonifera*) research putting green (Liu and Huang, 2003). Howieson and Christians (2008) found that single or double-cutting the putting surface reduced leaf glucose and fructan concentrations compared to where mowing was withheld for the day. Similarly, withholding irrigation or N fertilizer to minimize growth in conjunction with stressful rolling and cultivation programs often leads to surface decline, especially during the most stressful periods of the growing season. Consequently, golf course superintendents face a conundrum where practices employed to maximize BRD often lead to detrimental effects on plant growth and development.

In an effort to compensate for immutable mechanical stressors designed to maximize BRD, golf course superintendents regularly integrate chemical technologies into management regimes considered supplemental to traditional nutrient and pest management programs. The chemical technology used by superintendents includes but is not limited to seaweed extracts, amino acids, plant hormones (Ervin, 2013). One such product currently labeled as a synthetic fungicide, Civitas (Suncor Energy, Calgary, AB, CA) is an OMRI-certified horticultural oil which is marketed to enhance plant defense against diseases. Specifically, it appears to prime plant defensive pathways which can reduce reliance on traditional synthetic fungicides (Cortes et al., 2010a, 2010b). Applied alone, Civitas has the potential to cause turf injury and therefore is mixed by the user prior to application with a copper phlalocyanate-based pigment, Harmonizer (Suncor Energy, Calgary, AB, CA), commonly referred to as the Two-Pack. The mineral oil is

also applied in a premixed formulation with the pigment (Civitas One, Suncor Energy, Calgary, AB, CA). There are anecdotal reports of increased clipping yield and reduced BRD following application of the Two-Pack, however, these claims have not been substantiated scientifically. The goal of this project was to evaluate the effect of Civitas on growth, as measured by putting clipping yield, and its subsequent influence on BRD. The first objective was to determine if growth increases following application of the components of Civitas applied alone and in combination. The additional objectives were to determine how Civitas affects ball roll distance within a day and over the course of 10 days after application. Finally interactive effects of PGRs applied with Civitas were evaluated for BRD and clipping yield.

MATERIALS AND METHODS

Experiment 1: Clipping Yield Assessment

Site Description

This two year study was conducted on a mature mixed stand of creeping bentgrass (*Agrostis stolonifera* L. cv. Penncross) and a perennial biotype of annual bluegrass (*Poa annua* var. *reptans*) putting green at the Cornell University Bluegrass Lane Turf and Landscape Research Center in Ithaca, NY initiated in 2011. The green was constructed from the on-site Arkport fine sandy loam soil (originally mixed, active, mesic lamellic hapludalf) and was topressed monthly to 1mm depth with sand meeting USGA specifications (USGA, 2004). Plots were mowed daily at 3.0 mm with a Toro Greensmaster 1000 walking greensmower (Toro Co., Bloomington, MN), irrigated to prevent drought stress, and trafficked daily with a modified traffic device fitted with golf spikes designed to simulate an annual playing rate of 30,000 rounds of golf. Liquid ammonium sulfate fertilizer was applied to the green weekly at the rate of 10 kg

N ha^{-1} which was lightly watered into the root zone during the growing season. Disease outbreaks were controlled with chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) as needed to avoid potential growth regulating properties of certain classes of fungicides.

Experimental Design

The experiment was a randomized complete block design with four replicate blocks. Plots measured 2.4 by 1.2 m. Treatments include a non-treated control, the Civitas oil alone (51 L ha^{-1}), Harmonizer pigment alone (3.1 L ha^{-1}), Civitas and Harmonizer (Two-Pack) mixed on-site (51 and 3 L ha^{-1}, respectively), and Civitas One (55 L ha^{-1}). The Civitas One product is a mixture of Civitas oil and Hamonizer pigment mixed in the factory. Treatments were applied on 27 May, 20 June, 21 July 2011 and 21 June, 24 Aug, and 18 Sept 2012. All applications were made with a CO_2 powered backpack sprayer equipped with two TeeJet AI 8004 nozzles (TeeJet Technologies, Wheaton, IL) calibrated to deliver 810 L ha^{-1} at 275kPa.

Data Collection

Clippings were collected daily for a period 10 d after each application unless weather conditions were not conducive for collection. Each 10 d run constituted one of six experimental runs. Clippings were collected by mowing one 2.4 m pass down the center of each plot after 25 cm alleys were mowed at top and bottom of each plot to minimize variation from starting and stopping the mower. Clippings were then brushed from the mower collection bucket into a paper bag, dried for 24h at 65°F, and cleaned of sand debris with the vibratory pan method described by Kreuser et al. (2011), and weighed.

Statistical Analysis

The JMP (version 10.0.1, SAS Institute, Cary, NC) software package was used to analyze the clipping yield data. Repeated measures analysis was conducted with the random term of

Plot[TMT]. Run was treated as a random term to more clearly assess changes in clipping yield as result of treatments. Logarithmic transformation was used to satisfy the assumption of constant variance. Main effects tested included Treatment, Days After Treatment (DAT), Block, and all interaction terms. Non-significant interaction terms were then systematically removed from the model starting with the highest order interactions. Means were separated with Fisher's protected LSD ($\alpha = 0.05$).

Experiment 2: Ball Roll Assessment

Site Description

A two-year study conducted on a mature creeping bentgrass cv 'Princeville' putting surface at the Cornell University Bluegrass Lane Turf and Landscape Research Center in Ithaca, NY as initiated in 2011. The sand-based putting surface was constructed with 75cm of medium textured sand without amendment placed over top of 10 cm gravel drainage bed. Standard of care is similar to the methods outlined in Experiment 1.

Experimental Design

The experiment was conducted as a completely randomized factorial design with four replicates consisting of three Civitas formulations (non-treated control, Two-Pack, and Civitas One) with or without the plant growth regulator trinexapac-ethyl (TE; Primo Maxx, Syngenta Co., Greensboro, NC). Plots measured 4.5 by 1.2 meters. Trinexapac-ethyl applications were made following the 200 (base 0°C) GDD model developed by Kreuser and Soldat (2011) starting 2 June 2012 and 30 May 2013 at the rate of 0.10 kg a.i. ha^{-1}. Three Civitas applications were made in each growing season. The ten day period following each Civitas application constituted a ten day run for a total of six runs during the two growing seasons. Runs began on 21 June, 21 July, and 8 August 2011 and 14 June, 7 July, and 30 July 2012. The Civitas Two-Pack

formulation was produced on site by mixing the Civitas oil with Harmonizer pigment at rates of 25 and 1.6 L ha^{-1}, respectively and Civitas One was applied at 28 L ha^{-1}. Treatments were applied with the CO_2 powered backpack sprayer described in Experiment 1. The plot area was rolled with a light-weight greens roller (Tru-Turf Pty. Ltd, Arundel, QLD, Australia) following mowing and data collection during each ten day run.

Data Collection

Ball roll distance (BRD) was measured three times per day (immediately after mowing, immediately after rolling, and six hours after rolling) to determine how BRD changed during the day. Ball roll distance was calculated as the average distance traveled by three golf balls when released from a Pelzmeter (PelzGolf, Spicewood, TX) in two opposing directions. Clipping yield was measured during the final run in 2011 and all three runs in 2012. Clippings were collected, cleaned, and analyzed as described in Experiment 1.

Statistical Analysis

The JMP software package was used for data analysis (version 10.0.0; SAS Institute, Cary, NC). Clipping yield and BRD were subject to repeated measures analysis with the random term of Plot[Civitas x TE]. Clipping yield values were logarithmically transformed to satisfy the assumption of constant variance. The terms Run and Days After Treatment (DAT) nested within Run for the BRD model and Run in the clipping yield model were treated as random variables to more clearly assess the role of Civitas formulation and TE on yield and BRD. Main effects included Civitas formulation, TE, DAT, time of day, and the continuous term of clipping yield from the following day for the ball roll data. All possible interactions of the main effects were also evaluated. The main effect in the clipping yield ANOVA model included Civitas formulation, TE, and DAT. Non-significant terms were systematically removed from each

model starting with highest order non-significant interactions. Means were separated with Fisher's protected LSD ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Weather

The 2011 growing season was warmer than the 2012 season (Fig. 2.1). The 2012 season was warmest at the beginning for the growing season and cooled at the season progressed. The 2011 growing season was had more consistent air temperatures. The 2011 season was unseasonable dry while 2012 was unseasonable wet (Fig 2.1).

Experiment 1: Clipping Yield Assessment

Civitas formulation and DAT both had a significant effect on clipping yield ($p<0.0001$). Run accounted for 42.9% of the variance in the model. The Civitas One, Civitas Two-Pack, and Civitas oil by itself increased clipping yield compared to the non-treated control (Table 2.1). Average clipping yield fluctuated during the course of each run (Table 2.1). Clipping yields slightly declined as each run progressed; this is likely the result of mowing the plots in the same direction for 10 consecutive days. Although the direction the mower traveled up and down the plots was alternated daily, a mower line down the center of each plot was obvious at the end of each run. The Civitas formulation x DAT interaction was not significant which indicates all treatments containing the Civitas oil enhanced clipping yield for the duration of each run.
The Harmonizer pigment applied alone did not significantly alter clipping yield with respect to the control. There has been speculation that Harmonizer may alter the light quality reaching the leaf surface. Harmonizer, like other copper phlalocyanate-based pigments, selectively absorbs light in both the UV and red spectrum. It therefore, has the potential to reduce the ratio of

red:far-red light reaching the leaf. A similar effect occurs under dense deciduous shade trees (McBee, 1969). Enrichment in far red light in grass can inactivate the photoreceptor phytochrome which increases production of the phytohormone gibberellin and increases clipping yield (Cooke, 1975; and Rood et al., 1986). Lack of a yield response to Harmonizer alone precludes this hypothesis.

Increased clipping yield from the Civitas oil is a novel response since studies have shown other horticultural oils lead to a reduction in yield (Hodgkinson et al., 2002). Horticultural oils are believed to reduce yield in other horticultural crops as a result of direct tissue damage, i.e. membrane disruption, or through inhibition of stomatal conductance which limits photosynthesis. In grape vines, photosynthetic capacity and flower clusters per shoot were reduced and accumulation of soluble sugars was delayed following application of horticultural oils (Finger et al., 2002).

There is a key difference between yield in most horticultural crops and turfgrass. In most horticultural crops yield refers to the amount of storage organ or fruit produced per unit area, whereas yield on a putting green refers to the dry biomass of leaves mowed off between mowings per unit area. As a result, other factors such as the rate of cell division and elongation, new leaf initiation, and tiller density may have more of a direct effect on clipping yield. Alternatively, the mass of the oil added to the leaves likely increased the total clipping yield mass. The Civitas application rate contributes approximately 0.49 g of mass to the canopy m^{-2}. While this increase in mass certainly could increase clipping mass, time-lapse photography and measurement of canopy height of annual bluegrass in a growth chamber showed increased rates of turfgrass leaf elongation (data not shown). Clearly, more research is required to understand why field clipping yield is enhanced following application of Civitas.

The 16 to 21% increase in clipping yield following Civitas application is substantial; similar to doubling N application rate (Schlossberg and Schmidt, 2007; Kreuser and Soldat, 2012). Dramatic increases in clipping yield are known to reduce levels of storage carbohydrates and can reduce BRD (Schnyder and Nelson, 1989; Salaiz, 1995; Han et al., 1998; Richie et al., 2001; Johnson et al., 2003; Han et al., 2004; Streich et al., 2005; McCullough et al., 2006b; Koeritz and Stier, 2009). Plant growth regulators, such as trinexapac-ethyl, may mitigate the growth surge following application and sustain BRD.

Experiment 2: Ball Roll Assessment

Clipping Yield

The random term Run accounted for 61% of the model variance which is likely the result of changes in growing environment over the course of two field seasons. When averaged across all runs, clipping yield declined from 41% first day to the last of each run (Table 2.2). A linear contrast found this decline to be highly significant ($p<0.0001$). The Civitas Two-Pack treatment had greater clipping yield, 3.64 kg dry tissue ha^{-1}, than the Civitas One and the control which were statistically similar, 3.42 and 3.33 kg dry tissue ha^{-1} ($p=0.0119$). Application of TE every 200 GDD reduced clipping yield 0.47 kg dry tissue ha^{-1} ($p<0.0001$).

The interaction terms were not significant which indicates the effect of Civitas formulation and TE on clipping yield was consistent during the entire 10 day run. Trinexapac-ethyl reduced putting green clipping yield by an average of 13% which is similar to values found in the literature (McCullough et al., 2006; McCullough et al., 2007; Kreuser and Soldat 2011, Kreuser and Soldat 2012). Application of TE every 200 GDD sustained clipping yield suppression for the duration of the study and further validated the GDD model developed in Wisconsin (Kreuser and Soldat, 2011).

It is unclear why the Civitas Two-Pack performed differently than the Civitas One in this study. Both the premixed Civitas One and Civitas Two-Pack contain equal quantities of the Civitas oil and the Harmonizer pigment. It is likely that something in the formulation, such as an emulsifier, was changed to keep the pigment and oil in suspension during storage. If so, the Civitas oil or Harmonizer pigment are not the likely cause of enhanced clipping yield production with the Civitas Two-Pack. Since the Civitas oil is a complex mixture of proprietary mixture of hydrocarbons and spray adjuvants, identification of an active growth promoting compound cannot easily be determined until all the individual components have been assayed.

Ball Roll Distance

Ball roll distance is very dynamic. It typically changed within a rating day, across rating days, and between runs (Table 2.3). The random terms Run, DAT[Run], and Plot[Civitas x TE] accounted for 59, 13, and 2% of the model variance, respectively. There was a significant DAT x Time of Day interaction (Fig. 2.2). Ball roll distances were shortest following mowing and greatest post rolling. Ball roll distance declined six hours after rolling on every day except on the third day when it remained at the post roll level. The afternoon BRD declined towards the end of each run. Ten DAT the afternoon BRD was statistically similar to post mow distance. In all cases, the greatest BRD occurred four to seven DAT (and daily rolling) before they declined eight to ten DAT.

Very consistently, and regardless of TE or Civitas treatment, the greatest BRD occurred several days after the start of daily mowing and rolling one DAT. The surface was not rolled between runs but was mowed daily. Rolling most likely caused BRD to increase over a period of five or six days before it plateaued and eventually declined towards the end of each run. The decline in both BRD and clipping yield was likely due to the decline in turfgrass quality and

stand density that was visually apparent nine and ten days after application. Rolling is known to increase putting green BRD (Inguagiato et al., 2009; McDonald et al., 2013) and excessive rolling can reduce putting green quality due to mechanical stress (Hartwiger et al., 2001). Clearly, maintaining a high quality, dense, and uniform putting surface is equally as important as frequent mowing and rolling to maximize BRD during an entire season.

Although many golfers believe BRD increases as putting surface drys down, this data clearly illustrates how ball roll distance declines during the day. This is consistent with the results of McDonald et al. (2013), McCullough et al. (2006b), and Fagerness et al. (2000). It is still possible that BRD may increase as soil water declines and grass enters moderate to extreme water stress. However this data and previous data indicate that ball roll declines into the afternoon under typical irrigation management. It is still unclear, however, why BRD declines during the day in this study. It is commonly thought that non-uniform growth during the day increases the degree of friction on the golf ball. Alternatively, plant leaves may change their confirmation as they recover from the compression of mowing and rolling in the morning. This may increase surface friction.

Civitas formulation and TE had a significant effect on putting surface BRD (Table 2.3). Both the Civitas One and Civitas Two-Pack reduced BRD compared to the control except for the last day of the run when all treatments had statistically similar BRD (Fig. 2.3). The Civitas One reduced BRD more than Civitas Two-Pack on five occasions during the middle of each run. Trinexapac-ethyl significantly increased BRD three, five, seven, eight, and nine days after treatment application (Figure 2.4). On those days the average increase from TE was only 7.2 cm (2.0%) which is consistent with the literature for cool-season putting surfaces (McCullough et al., 2005a, 2005b, 2005c; McDonald et al., 2013). The Civitas formulation x TE x DAT

interaction was not significant; TE was not able to mitigate the decline in BRD that resulted from Civitas. Additionally, TE did not help sustain BRD during the day (Time x PGR interaction was not significant). That result is consistent with the work by McDonald et al. (2013) but contrary to Fagerness et al. (2000).

Golfers cannot differentiate BRD's less than 15cm (Karcher, 1996). Both Civitas One and Civitas Two-Pack reduced BRD in excess of 30cm which may limit their use on cool-season putting greens. Trinexapac-ethyl did increase BDR to a level perceivable by golfers despite frequent re-applications.

Clipping Yield and Ball Roll Distance

There was no obvious relationship between clipping yield and BRD in these experiments (Fig. 2.5). However, the three way interaction of Time x DAT x Yield was statistically significant (Table 2.3). On three of the possible 30 occasions BRD was correlated with clipping yield. On two of those occasions BRD was positively correlated with clipping yield and on the other occasion BRD increased as clipping yield declined. On all other rating times and days BRD was not correlated with clipping yield. Similarly, BRD was not correlated to clipping yield for a majority of model parameters in the Civitas x DAT x Yield interaction. The three exceptions included the no Civitas control treatment two DAT and Civitas ONE treatment nine DAT where ball roll declined as yield increased and the control treatment four DAT where BRD increased with clipping yield. All other model effects and parameters involving clipping yield were not significant.

The lack of a relationship between clipping yield and BRD was a surprise. It is well established that practices that increase clipping yield, such as increased N fertilization, decrease BRD (Christians et al., 1979; Streich et al., 2005; McCullough et al., 2006b; Schlossberg and

Schmidt, 2007; Koeritz and Stier, 2009; Pease et al., 2011; Baldwin and Brede, 2012). Conversely, practices that reduce clipping yield, such as application of PGRs, enhanced BRD (Fagerness et al., 2000; Streich et al., 2005; McCullough et al., 2005a, b, c; McCullough et al., 2006a, b; McCullough et al., 2007; McCarty et al., 2011; McDonald et al., 2013). These studies illustrate an obvious cause and effect; more clipping yield reduces BRD. However the two Civitas formulations challenge this causality. Both Civitas One and Civitas Two-Pack drastically reduce ball roll distance yet only the Civitas Two-Pack increased clipping yield in the ball roll study. The Civitas Two-Pack BRD was consistently less than the control at any given clipping yield (Figure 2.5). It is possible that other factors, such as grass leaf plasticity, may play a larger role on BRD. There is potential that practices such as increased irrigation frequency or nitrogen applications affect BRD in ways other than increased leaf elongation rate. Factors such as leaf relative water content or increased stand density may cause the decline in BRD. Despite the high concern of BRD by golf course superintendents, it is still unclear how different practices actually affect friction of a putting surface. High resolution research needs to be conducted to understand the nuance of BRD in response to different cultural practices and would improve best management practices for ball roll.

CONCLUSIONS

Both Civitas One and the Civitas Two-Pack dramatically reduced creeping bentgrass putting green BRD for a period of ten days. The decline in BRD is great enough to be noticed by the average golfer. Although both Civitas One and the Civitas Two-Pack have the potential to increase clipping yield, some unknown factor has a more direct effect on BRD than clipping enhancement. The Harmonizer pigment alone had no effect on clipping yield. Use of TE nominally increased BRD but did not mitigate the decline caused by the Civitas One and Two-Pack. Peak ball roll occurred four to five days after daily rolling commenced independent of chemical treatment. Constant daily rolling did result in plant stress, which was apparent as clipping yield and visual quality declined at the end of ten day run. It would be prudent to avoid application of Civitas to cool-season golf putting greens and maintain growth at a level to provide a recuperative potential from practices such as rolling to maximize BRD over the course of the entire growing season.

Table 2.1. The effect of Civitas components on clipping yield during experiment 1.

Product	Dry clipping yield
	------ g m^{-2} ------
Civitas One	1.83a[†]
Civitas Two-Pack	1.75a
Civitas Oil	1.75a
Harmonizer	1.56b
Control	1.52b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 2.2. The effect of Civitas components on clipping yield during experiment 1.

Days after application	Dry clipping yield
	------ g m^{-2} ------
1	2.54a†
2	2.21b
3	2.01b
4	1.32e
5	1.35e
6	1.51de
7	1.70cd
8	2.00bc
9	1.42e
10	1.11f

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 2.3. Ball Roll ANCOVA table. Other non-significant sources were removed from the model when permissible.

Source	df	F Ratio
Civitas (Civ)	2	15.2 ***
Trinexapac-ethyl (TE)	1	6.0*
Time	2	265.4***
Days after treatment (DAT)	9	3.4*
Clipping yield	1	0.8
Civ*DAT	18	2.6***
TE*DAT	9	2.3*
Time*DAT	18	2.9***
Civ*Yield	2	0.2
Time*Yield	2	0.9
DAT* Yield	9	1.6
Civ*DAT* Yield	18	2.7***
Time*DAT* Yield	18	2.3**

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 2.4. The daily average clipping yield during the six runs of experiment 2.

Days after application	Dry clipping yield
	------ g m^{-2} ------
1	1.91a[†]
2	1.71ab
3	1.39cd
4	1.34de
5	1.19ef
6	1.23def
7	1.59bc
8	1.33de
9	1.27def
10	1.11f

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

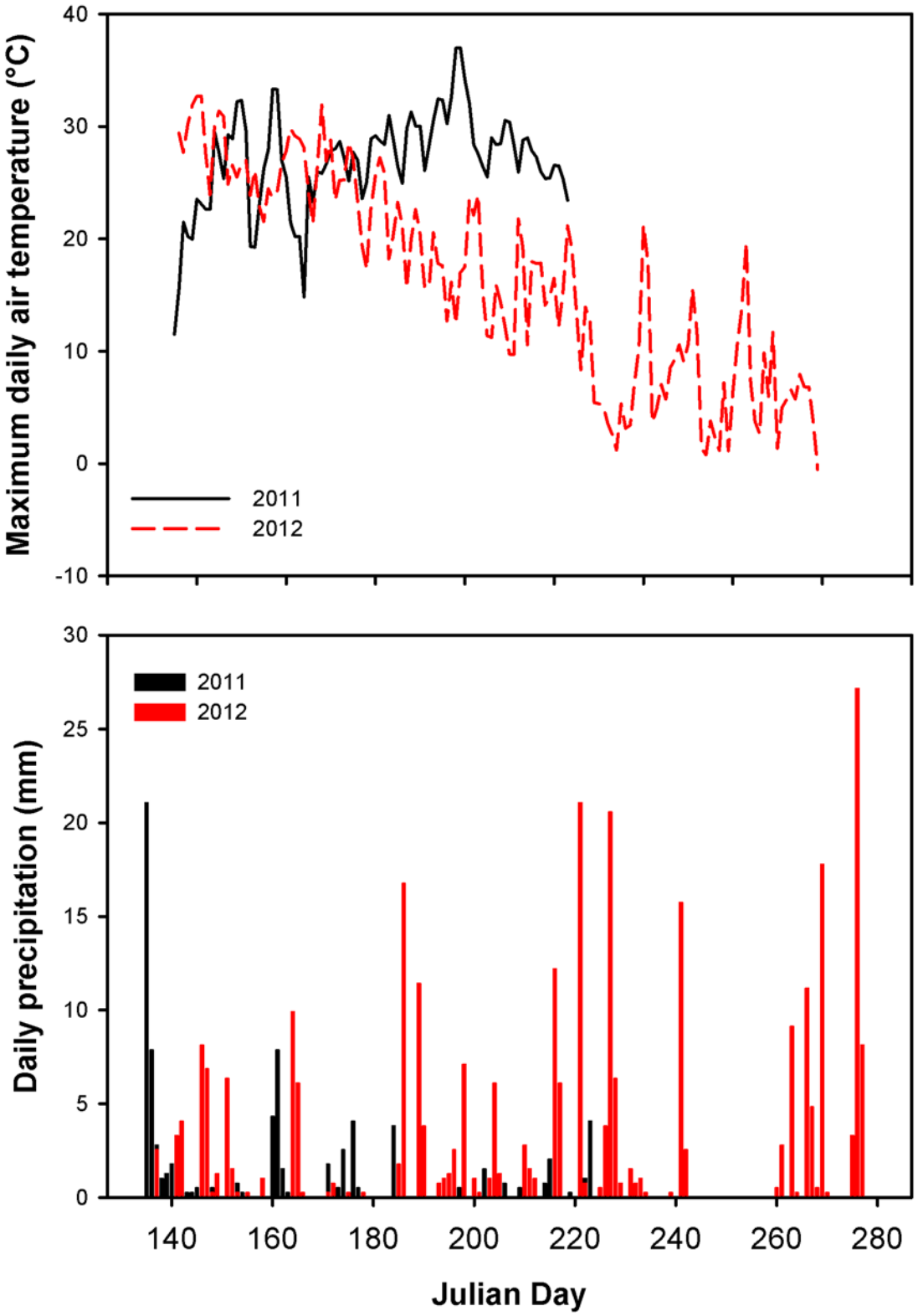

Figure 2.1. The daily maximum air temperature and precipitation during the 2011 and 2012 growing seasons.

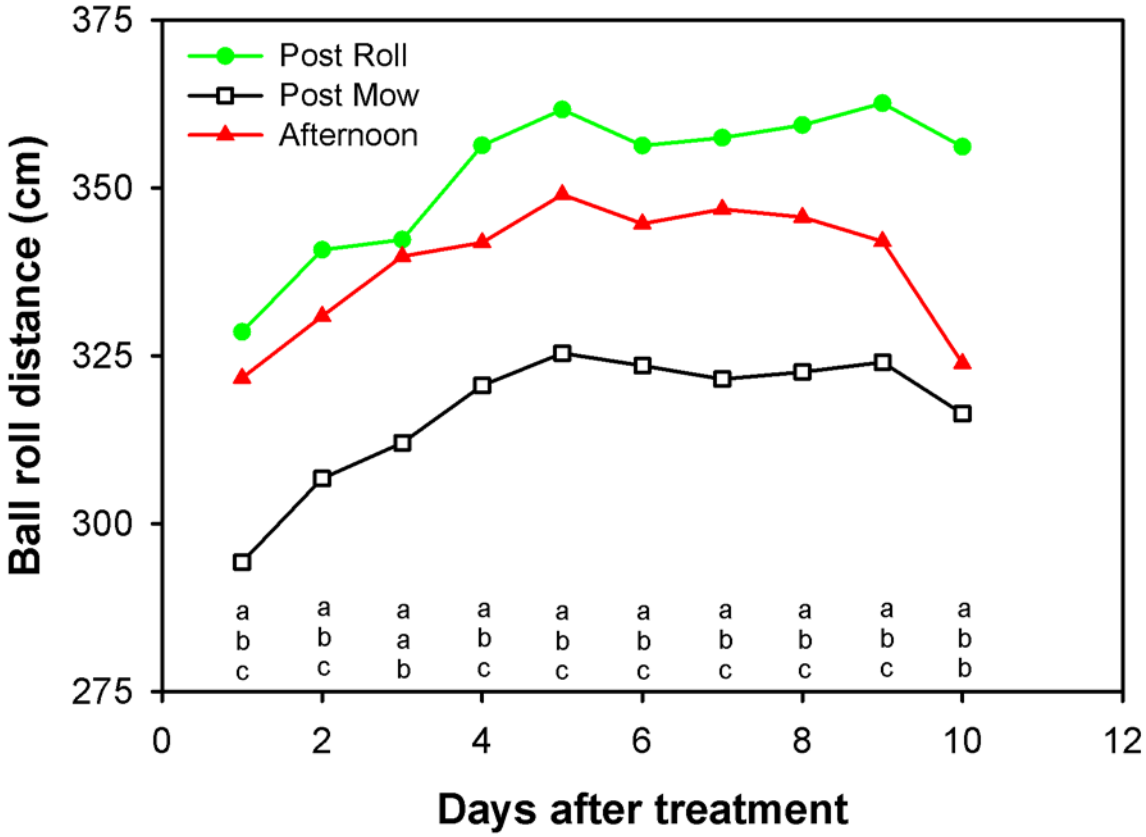

Figure 2.2. The average change in ball roll distance during the course of a day and course of the each run. Different letters reflect significant treatment differences within a rating day.

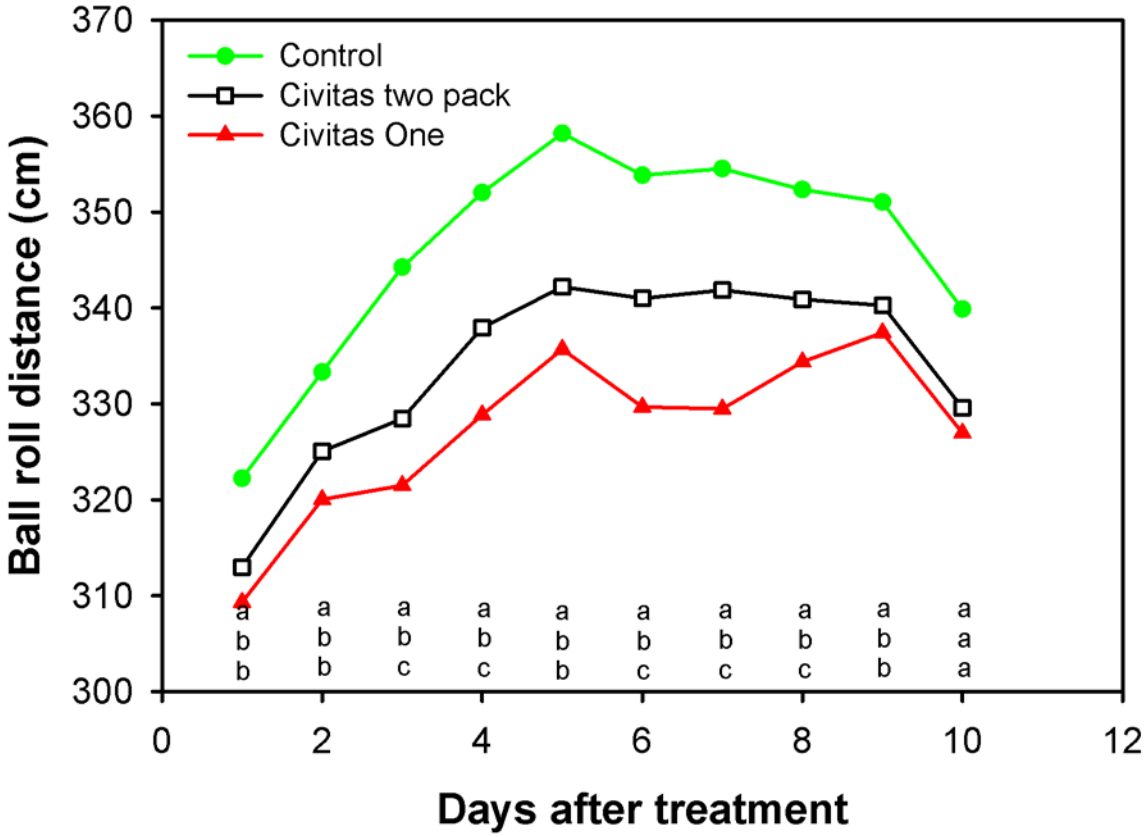

Figure 2.3. The average effect of Civitas formulation on ball roll distance over the course of the 10 day run. Different letters reflect significant treatment differences within a rating day.

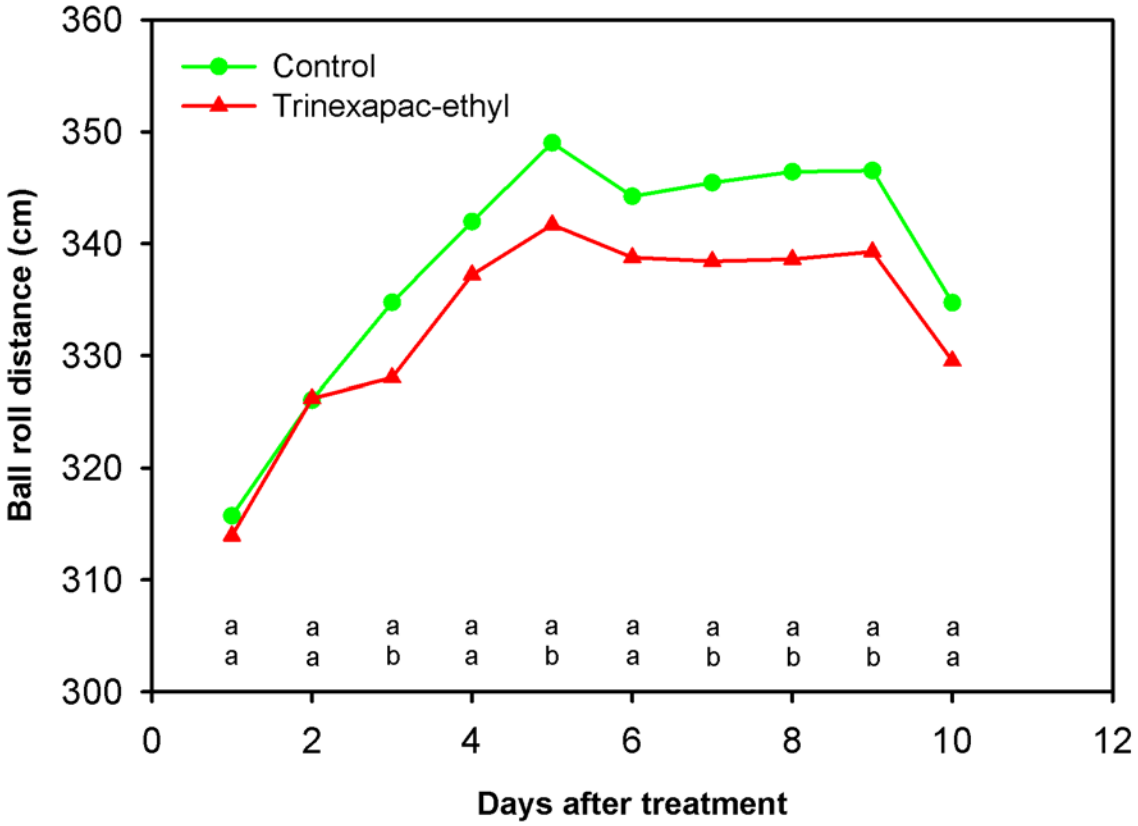

Figure 2.4. The average effect of the plant growth regulator trinexapac-ethyl on ball roll distance over the course of the 10 day run. Different letters reflect significant treatment differences within a rating day.

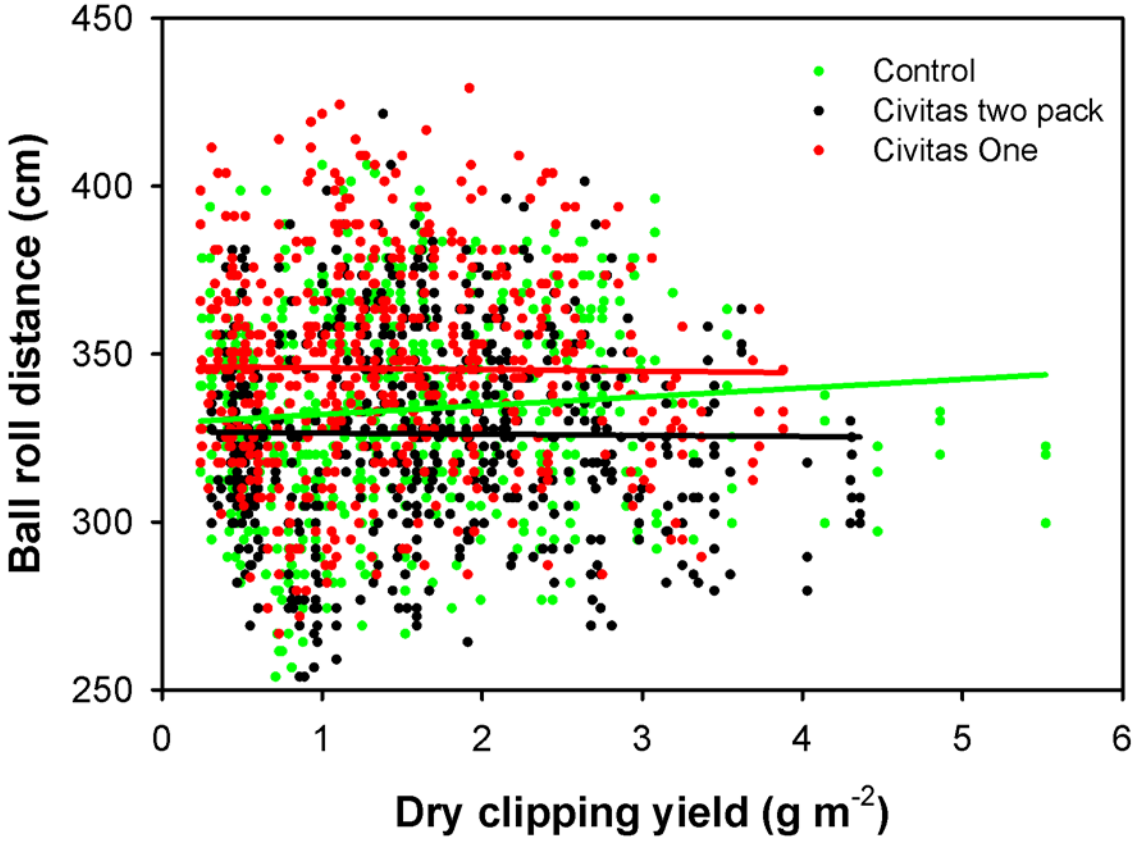

Figure 2.5. The effect of dry clipping yield on ball roll distance. All measurements were pooled.

Chapter 3: Civitas Causes Chronic Phytotoxicity in Turfgrass

ABSTRACT

Petroleum-derived spray oils (PDSO) have been used for pest management in horticulture and agronomy for over a century. Civitas is a PDSO designed for use in the turfgrass industry. It is commonly mixed with low rates of pesticides to reduce environmental impact. However, PDSO Civitas can cause phytotoxicity which has limited its acceptance in the turfgrass industry. A field study and growth chamber study was designed to quantify phytotoxicity and isolate the cause of Civitas-induced phytotoxicity. Civitas was applied to a research putting surface in Ithaca, NY. Visual turfgrass quality rating and canopy temperature was quantified throughout both growing seasons. PDSO Civitas caused a decline in turfgrass quality both years. Addition of a pigment called Harmonizer masked chlorosis but did not prevent the decline in stand density during the second field season. Growth chamber experiments found that cell membranes were not disrupted with PDSO Civitas. Scanning electron micrographs showed PDSO Civitas changed the morphology of epicuticular wax on the leaves. Epicuticular wax composition and quantity was not affected by Civitas as determined with GC/MS. There were signs of oil persistence around stomata. Transpiration rate and radiation use efficiency were reduced with Civitas, likely from stomatal impedance. PSDO Civitas causes chronic phytotoxicity in turfgrass as a result of reduced gas exchange.

INTRODUCTION

Petroleum-derived spray oils, commonly referred to as horticultural spray oils, have been used to control insect and fungal pests in horticultural crops since the 1800s (Agnello, 2002). There has been renewed interest in PDSO because these products, when properly refined, have minimal human toxicity and environmental impact (Ebbon, 2002). While there is debate as to how PDSO affect plant pests, they can be lethal to plant pests and have the potential to elicit innate plant defenses (Taverner, 2002; Nothover and Timmer, 2002; Cortes et al., 2010a).

Civitas™ is a novel PDSO that contains a mixture of food-grade isoparaffins (alkanes) ranging in size from 16 to 33 carbons in length and an emulsifier (Hsiang, 2013). It is currently produced and marketed by Petro-Canada for disease and insect control in golf turf management. Civitas represents a novel approach to turf pest management because it primes plant defense activation and has no fungicidal and limited fungistatic properties (Cortes et al., 2010a). Specifically, Cortes et al. (2010a, 2010b) demonstrated Civitas applied to both *Nicotiana bethamiana* and creeping bentgrass (*Agrostis stolonifera* Hud.) primed or activated genes involved in induced systemic resistance, including genes involved in the jasmonic acid pathway. Consequently, several studies have shown reduced pesticide requirements when using Civitas in a disease management program (Popko et al., 2010; McCall and Focht, 2010; Aynardi et al., 2011; Popko and Jung; Aynardi and Uddin, 2013 a, b).

Phytotoxicity is a known limitation to the PDSO and is categorized as acute or chronic (Hodgkinson et al., 2002). Acute phytotoxicity results from direct damage to plant tissue including, leaf lesions, fruit sunburn, and membrane disruption. Chronic phytotoxicity arises from inhibition of stomatal conductance causing reductions in transpiration, photosynthesis, and respiration and an increase in photorespiration, photooxidation and oxidative stress. For

example, the paraffinic PDSO JMS Stylet Oil™ has been shown to reduce stomatal conductance in grapes and alter carbon partitioning (Finger et al., 2002). Similar results have been demonstrated in other horticultural crops (Rethwisch et al., 1992).

Most modern PDSO are highly paraffinic and are considered safe at concentrations less than 2% by weight when applied to citrus (Beattie; 1990). Currently, Civitas is applied at 2.5 to 5% concentrations and results in chlorotic turf within hours of application. To mask the chlorosis, Civitas is applied with a green phthalocyanine pigment Harmonizer (Nash, 2011). These pigments have been shown to mask injury and mitigate UV stress (Ervin et al., 2004). The original commercial formulation consisted of the PDSO Civitas and the pigment Harmonizer packaged separately Civitas Two-Pack and required on-site mixing. A new pre-mixed single product formulation was introduced in 2013 that contains both the Civitas oil and Harmonizer pigment Civitas One.

The objectives of this project were to characterize the phytotoxic response of turfgrass treated with the components and combinations of the PDSO Civitas and pigment Harmonizer and determine how phytotoxicity is elicited. Our hypothesis is that the PDSO, Civitas, causes chronic phytotoxicity due to oil persistence that reduces stomatal conductance.

MATERIALS AND METHODS

Civitas Phytotoxicity Field Assessment

This study was conducted over two field seasons on an mature mixed stand of annual bluegrass (*Poa annua*, var. *reptans*) and creeping bentgrass (*Agrostis stolonifera* Hud. cv. L-93) managed as a putting surface at the Cornell University Turf and Landscape Research Center in Ithaca, NY. The green was constructed from the on-site Arkport fine sandy loam soil (originally mixed, active, mesic lamellic hapludalf) and topdressed regularly with sand meeting United State Golf Association specifications regularly that resulted in a 7.5 cm layer of greater than 80 percent sand-based growing medium.

The experimental putting surface is managed for high performance by mowing six days wk^{-1} at 3 mm with a fixed-head walking greensmower (Toro Greenmaster 1000, Toro Co., Bloomington, MN). Plots are irrigated to prevent drought stress, and trafficked daily with a modified traffic devices fitted with golf spikes designed to simulate 30,000 rounds of golf yr^{-1}. Liquid ammonium sulfate fertilizer was applied weekly at 10 kg N ha^{-1} throughout the growing season. Fungal disease outbreaks were controlled with chlorothalonil as needed. Treatments consisted of a 2x2 factorial of Civitas oil (with or without PDSO Civitas) and pigment (with or without Harmonizer pigment) arranged in a randomized complete block design with four replicates. Civitas and Harmonizer were applied every two weeks at the rates of 50 L ha^{-1} and 3 L ha^{-1}, respectively with a CO_2 powered backpack sprayer equipped with two TeeJet AI 8004 nozzles (TeeJet Technologies, Wheaton, IL) calibrated to deliver 810 L ha^{-1} at 275kPa. Applications began on 22 Aug 2012 and 31 May 2013 and with final measurements occurring on 30 Sept 2012 and 30 Aug 2013.

Visual turfgrass quality rating, and canopy temperature were recorded several times wk^{-1} during each year. Visual turfgrass quality rating is a composite rating accounting for factors such as color, canopy density, and surface uniformity. It was rated on a 1 to 9 scale where 1 represents completely dead turf and 9 represents perfect putting surface quality. Values greater than 6 are deemed minimally acceptable turfgrass quality (Skogley and Sawyer, 1992). Surface temperature was measured with a FLIR i7 infrared camera (FLIR Systems, Inc, Portland, OR). The camera was consistently positioned approximately seven meters above the putting surface and approximately 10 meters south of the first block. The FLIR allowed for all 20 plots to be measured simultaneously and reduced variability associated with changing sky conditions. Plot images are imported into the FLIR Tools software package (FLIR systems, Inc., Portland, OR) to determine the average temperature. At the end of the 2013 growing season, six 20mm plugs were taken from each plot. Plugs were refrigerated at 5°C until tillers were counted. Tiller density counts were specific to 2013 treatments because the entire plot area was moved to a different location on the putting surface between 2012 and 2013. Changes in tiller density, therefore, resulted from application during 2013.

Visual turfgrass quality and surface temperature data was subjected to repeated measures analysis in JMP 10 (version 10.0.1, SAS Institute, Cary, NC). The random term in the model was Civitas x Harmonizer nested within plot. The main effect of PDSO Civitas, Harmonizer, Date, Block, and all interactions were examined. Tiller density data were subjected to ANOVA in JMP10. Means were separated with Fisher's LSD (α=0.05) when appropriate.

Civitas Phytotoxicity Growth Chamber Assessment

A growth chamber study was conducted to more thoroughly assess the phytotoxic response of Civitas and Harmonizer on membrane integrity, gas exchange, and cuticle composition and structure. Annual bluegrass (*Poa annua* L. cv. DW-184) was established was established in a Percival WE-1012 growth chamber fitted with a mixed incandescent & fluorescent light source (Percival Scientific, Perry, IA) at Cornell University in Ithaca, NY. Conditions were designed to simulate conditions similar to natural weather conditions in Ithaca, NY during early summer. Specifically, plants received approximately 450 µmol PAR m^{-2} s^{-1} for 16 h at 30°C and 50% relative humidity and 8 h of darkness at 20°C and 70% relative humidity.

Ray Leach SC10 3.8 x 210 cm cone-tainers (Stuewe & Sons, Tangent, OR) were filled and packed with non-amended medium textured sand meeting USGA specifications for putting green construction (USGA, 2004). Approximately 10 seeds of annual bluegrass were placed in cones that were filled with sand to 15mm below top of cone to allow for uniform clipping. Cones were fertilized weekly with 10 mL of a fertilizer solution containing 200, 50, 200, and 1 ppm N, P, K, and chelated Fe, respectively for the first ten weeks of establishment. After establishment, cones received 8 mL of the fertilizer mix every two weeks. Cones were clipped several times wk^{-1} and were watered daily with tap water to prevent drought stress. Plants were fully established and deemed ready for experiments twelve weeks after seeding.

The study was conducted twice as a randomized complete block design with four replicates. Treatments included PDSO Civitas alone, Harmonizer alone, Civitas One, Civitas Two-Pack, and a non-treated control. The PDSO Civitas and the pigment Harmonizer were applied alone at the rates of 5.0 and 0.3 mL m^{-2}, respectively or combined on-site to create Two-

Pack. Civitas One was applied at the rate 5.5 mL m^{-2} which is equivalent to the Civitas Two-Pack according to the manufacturer.

Applications were made with a CO_2 powered backpack sprayer equipped with a TeeJet AI 8004 nozzle (TeeJet Technologies, Wheaton, IL) calibrated to deliver 81 mL m^{-2} at 275kPa. All cones were arranged into a single line to ensure each cone was 50 cm beneath the nozzle tip. In an effort to determine changes in the cuticle morphology and to visualize potential stomatal obstruction after successive application, an additional application of Civitas Two-Pack and Civitas One was made to select cones 10 d after the first application.

All measured observations occurred 48 h after application of treatments except for the scanning electron micrographs that occurred 48 h after the first and second application. Membrane integrity was determined by measurement of electrolyte leakage (EL) as based on the method described by Liu et al. (2000). Fifty mg of wet green leaf tissue was rinsed with Milli-Q ultrapure H_2O (EMD Millipore, Billerica, MA), dried with a Kimwipe (Kimberly-Clark, Irving, TX), and placed in a 50mL volumetric flask which contained 40 mL of Milli-Q H_2O. Tissue was gently shaken in darkness for 24h prior to initial measurement of electrical conductivity with an Orion Star conductivity meter (Thermo Fisher, Waltham, MA). Samples were then autoclaved for 20 min to release total plant electrolytes, allowed to cool to approximately 20°C, and conductivity was re-measured. Electrolyte leakage was expressed as the percent of initial conductivity divided by the total conductivity.

A hexane extraction method was used to quantify epicuticular wax content. The method was based on the method described by Jenks et al., (2001) and Bethea (2012). Approximately 500 mg of fresh green tissue was massed and submerged in 25mL of hexane for 50 s to remove the cuticle. Ten µg of tetracosane (C_{24} alkane, Sigma Aldrich, St. Louis, MO) was added to each

extraction which served as the internal standard. The extract was poured into a 50 mL beaker, evaporated to less than 4 mL in a fume hood, and stored in 4mL glass vials with TFE-lined caps (Wheaton Science Products, Millville, NJ) at -23°C. Prior to analysis, samples were transfered into 5mL Weaton™ V Vials™ (Wheaton Science Products, Millville, NJ) and evaporated to dryness under a N_2 stream in a fume hood. Dried samples were derivatized with 350 µl of bis(tismethylsilyl)-acetimeide (BSTFA, Sigma-Aldrich; St. Louis, MO) at 85°C for 25 min.

Compounds were separated and identified by gas chromatography/mass spectrometry using an Agilent 6890N GC (Santa Clara, CA) equipped with split/splitless injector and a J&W Scientific DuraGuard DB-5ms (30 m x 0.25 mm x 0.25 um film with 10 m guard column, Folsom, CA) coupled to a JEOL-GCMate II mass spectrometer (Akishima-Shi, TKY). One µl injection volumes were used with both split mode (1:150 split ratio) to quantify large components and splitless mode with an inlet purge wait time of 1 minute to quantify minor components. Gas chromatographic separation was performed with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The initial oven temperature of 50°C was held for two minutes followed by heating to 200°C at a rate of 40°C/min and to 300°C at a rate of 4°C /min. The final temperature was held for 14.25 minutes for a total analysis time of 45 minutes. Injector, interface, and ion-source temperatures were kept at 250°C, 280°C and 250°C, respectively. MS were acquired in positive-ion mode using electron-impact ionization (EI) with electron energy of 70 eV and filament current of 0.3 mA. Preamp gain was set to X1 and detector voltage to 350 V. Masses were measured with linear magnetic-field scans from m/z 35–600 with 0.2 scan s^{-1} scan speed and 0.1 s interscan delay using external calibration. Nominal mass resolution (10% valley) was 500, actual resolution on PFK calibrant prior to acquisition was ~650.

Carbon$_{16}$ and C_{18} fatty acids and $C_{18,}$ C_{22}, C_{24}, C_{26}, and C_{28}, primary alcohols were the prominent cuticle species in all samples. They were identified from m/z peak data described in the Wiley NIST database summarized by Bethea (2012). Area under each peak was integrated in TSS Pro 3.0 (Shader Analytical and Consulting Laboratories, Inc, Detroit, MI). Due to electron impact ionization, the peak area of each compound was compared to the internal standard on a mole-to-mole ratio to calculate the quantity in µg g^{-1}fresh weight.

Epicuticular wax morphological structure and stomata were examined with a Hitachi SU-70 Ultra-High Resolution Analytical Field Emission Scanning Electron Microscope (Hitachi, Chiyoda, TKY) at Suncor Petro-Canada Research Lab in Mississauga, ON. Treatments examined included a non-treated control, Civitas oil and Civitas One 24 and 48 h after the first application, and Civitas oil and Civitas One 24 and 48 h after the second application. One mm sections of leaves were fixed to the aluminum stage with carbon tape. The microscope was set at 1kV and 9.5mm working distance. Micrographs were taken at 0.5, 2.5, and 5.0k magnification.

Carbon exchange rate (CER) and transpiration were measured with a Li-Cor 6400XT photosynthesis meter with 6400-17L Lighted Whole Plant *Arabidopsis* Chamber (Li-Cor, Lincoln, NE). The chamber was configured to provide constant flow rate at 500 µmol air s^{-1} and 400ppm CO_2 concentration. Light response curves were created with an AutoProgram that adjusted photosynthetic photon flux density (PPFD) from 2000 to 0 µmol of photosynthetically active radiation (PAR) m^{-2} s^{-1}. Carbon exchange rate and transpiration where measured automatically after CO_2 and H_2O concentrations of the sample had stabilized, CV values less than 5%. The stability values were optimized for time and measurement precision. Photon flux density was then automatically reduced to the next lowest intensity. Light response curves were created 48 h after application at PPFD values of 2000, 1500, 1000, 750, 400, 200, and 0 PPFD.

Electrolyte leakage, cuticle components, and CER at each light level were subject to ANOVA in JMP 10 (version 10.0.1, SAS Institute, Cary, NC). Multiple regression was used for the transpiration data due to the linear response to PPFD. The main effects of Civitas Component, Block, Run, and all interactions were examined and non-significant effects and interactions were removed from the final statistical model. Means were separated with Fisher's LSD (α=0.05) when appropriate.

RESULTS AND DISCUSSION

Civitas Phytotoxicity Field Assessment

The maximum daily air temperatures were above average for Ithaca, NY in both 2012 and 2013. Maximum daily air temperatures were greater 2013 than 2012 (Fig. 3.1). The total daily light integral was more variable in 2013 than 2012. There were more clear days in 2013 than 2012 which caused the higher light integral during 2013. The weather station was struck by lightning on 8 Aug 2013 and stopped logging data after than event.

The PDSO Civitas and the pigment Harmonizer had a significant effect on putting surface visual quality and canopy temperature (Table 3.1). The PDSO Civitas applied by itself reduced visual quality rating below acceptable limits (less than 6 on the 1 to 9 scale) due to chlorosis on 44 of the 47 rating dates (Fig. 3.2). Comparatively, the non-treated plot had acceptable quality on a majority of the rating dates in both years. Addition of the pigment Harmonizer increased the turfgrass visual quality ratings an average of 0.68 units compared to the control which is of practical significance.

There was a significant Civitas x Harmonizer x Date interaction (Table 3.1). In 2012, plots that received pigment Harmonizer alone, Civitas Two-Pack, or the non-treated control had

similar turfgrass quality ratings throughout the season (Fig. 3.2). Turfgrass visual quality declined with PDSO Civitas alone as the season progressed in 2012. In 2013, visual quality rating of plots receiving only the pigment Harmonizer and the non-treated control was consistently acceptable. In contrast the PDSO Civitas alone produced unacceptable turfgrass quality ratings on all days in 2013. Addition of the pigment Harmonizer to Civitas mitigated the decline and masked phytotoxicity from May until mid-July, however, visual quality rapidly declined beginning 24 July 2013 and turfgrass quality rating was unacceptable by the end of the study. Canopy tiller density declined with PDSO Civitas despite application of the pigment Harmonizer (Table. 3.2). The decline in quality resulted from thinning of the turfgrass canopy and pigment staining of voids and sand root zone (Fig. 3.3).

It was anecdotally observed that turf treated with the pigment Harmonizer was slow to recover from traffic and pest damage. Bristow et al. (2013) showed application of the heavy metal Zn to newly established cool-season turfgrass resulted in decline and death. However, mature turfgrass stands were less affected by Zn application. It is possible that routine application of mineral-based pigments such as Harmonizer that contain chelated copper may inhibit regrowth observed in this study once voids are created for new seedlings to emerge.

There was a significant Civitas x Harmonizer x Date interaction for putting surface canopy temperature (Table 3.1). On days with high solar radiation and low cloud cover, the pigment Harmonizer and PDSO Civitas applied alone increased canopy temperatures between 0 to 1.6°C. Combination of the PDSO Civitas and pigment Harmonizer had an additive effect on surface canopy temperature often increasing temperatures by 0-2°C (Fig. 3.4). McCarty et al., (2013) found that application of phthalocyanine pigments to turfgrass did not affect transpiration rate. Therefore, the pigment Harmonizer seems to increase canopy temperature because of color

and ability to absorb light energy. The increase in canopy temperature did not have an obvious effect on turfgrass health as the pigment Harmonizer consistently had the best quality rating while PDSO Civitas had the worst quality despite similar canopy temperatures. The increase in canopy temperature with PDSO Civitas suggests that stomatal conductance and transpiration could be involved as is expected based on previous research with PDSO's (Hodgkinson et al., 2002).

Civitas Phytotoxicity Growth Chamber Assessment

There was no evidence of membrane damage resulting from application of PDSO Civitas (Table 3.3). Previous research has shown that turfgrass subjected to heat or drought stress can have EL values from 40 to 85% (Liu and Huang, 2000; Yang et al., 2013). In this study, EL of was never greater than 20% for any treatment. The PDSO Civitas treated plants were between 8 and 14%. Plants that received pigment Harmonizer, either alone or in combination with PDSO Civitas, had lower levels of EL compared to the non-treated PDSO Civitas

The pigment Harmonizer is composed of a polychlorinated copper (Cu) II phthalocyanine pigment (Nash, 2011). Phthalocyanines strongly chelate Cu^{2+} and result in a stable blue-green colored pigment. Reduced EL with Harmonizer is likely an artifact of autoclaving the pigment which causes damage to the chelating agent. This release of Cu^{2+} into solution would increase the solution electrical conductivity, the denominator in the EL calculation, and thereby reduce % EL. Alternatively, the phthalocyanine may have chelated electrolytes that were liberated before the sample is autoclaved which would reduce the solution electrical conductivity and % EL. Despite the uncertainty as to why the pigment Harmonizer reduced EL, it is clear that the PDSO Civitas does not damage membranes and therefore explains why the PDSO is not acutely phytotoxic to the turfgrass (Hodgkinson et al. 2002).

The treatments did not alter the quantity or composition of the plant's epicuticular wax (Table 3.4). There were seven principle components of annual bluegrass epicuticular wax identified via GS/MS (Table 3.5). There were other minor peaks that represented less than 1% of the total epicuticular wax and were ignored (Fig. 3.5). Primary alcohols comprised 96.1% of the epicuticular wax while the primary C_{16} and C_{18} saturated fatty acids comprised 2.4 and 1.5%, respectively. The primary alcohol 1-hexacosanol was the predominant species at 86% of the total epicuticular wax or 51.1 µg g^{-1} fwt. Total epicuticular wax load averaged 59.4 µg g^{-1} fwt.

Application of PDSO Civitas, Civitas One Pack, or the Civitas Two Pack resulted in a broad peak on the total ion current chromatogram (TIC) from 9 to 24 min into the recording (Fig. 3.5). The PDSO Civitas TIC signature was absent from the pigment Harmonizer treatment (Fig. 3.5). Electron-impact ionization allowed for integration of the entire PDSO Civitas peak. The PDSO Civitas peak was compared to the C_{26} primary alcohol peak on a mole to mole basis after corrected with the internal standard. On average, there was 12.2 times more PDSO Civitas on treated leaves than C_{26} 1-hexacosanol, the dominant natural component of annual bluegrass epicuticular wax (Table 3.6). There was no statistical difference between the PDSO applied Civitas alone, Civitas One Pack, and Civitas Two Pack treatments.

Water-treated annual bluegrass leaves have a uniform cuticle with dense primary alcohol platelets (Barthlott et al., 1998; and Jeffree, 2006). These crystal structures are known to be very regular and dependent upon their chemical composition. The platelet crystal structure is common when epicuticular wax is composed mainly of primary alcohols (Barthlott et al., 1998; and Jeffree, 2006). Scanning electron microscopy clearly shows that both the Civitas One and Civitas Two-Pack alter the morphology of the epicuticular wax (Fig. 3.6) most notably 48 h after the second application of PDSO Civitas.

Dissolved epicuticular waxes are known to recrystallize after they have been removed from the plant surface with a solvent (Jetter and Riederer, 1994; Neinhuis et al., 2001; Koch et al., 2006). However, this recrystallization did not occur on turfgrass leaves treated with Civitas and it is plausible that Civitas disrupts the normal crystal structure and inhibits recrystallization. The alteration in cuticle morphology could reduce boundary layer affects and leaf surface wettability by altering surface tension that could have significant implications for water management (Yoshimitsu et al., 2002; Fernandez and Eichert, 2009). It is well known the PDSO act as excellent spray adjuvants because they increase pesticide absorption, this it likely the result of the modified epicuticular structure demonstrated in the electron micrographs.

In addition to the altered crystalline structure of the cuticle, the stomata appear occluded (Fig. 3.6). It is unclear if the substance is the PDSO Civitas, redistributed plant epicuticular wax, or both. In any event, the level of additional wax on the leaves resulting from Civitas was rather significant. It is very likely that both redistribution of natural epicuticular waxes and the addition of Civitas lead to the inhibition stomatal conductance and reduced transpiration that would lead to increases in canopy temperature measured in the field studies. This supports findings from work with other PDSOs that found chronic phytotoxicity the result of oil persistence and stomatal impedance (Hodgkinson et al., 2002).

Transpiration rates increased linearly as light level increased from 0 to 2000 µmol PAR m^{-2} s^{-1}. The PDSO Civitas treated plants had lower transpiration rates than plants treated with the pigment Harmonizer alone and non-treated plants (Table 3.7). There was also a significant run and Treatment x Run interaction. The Li-Cor 6400 was configured to have a constant air flow rate through the chamber. This led to small differences in relative humidity and vapor pressure deficit (VPD) over the course of the 20 measurements during each run (approximately seven

hours) and between the two runs. The Li-Cor 6400 does not actively add water vapor to the chamber, instead it reduces flow rate through the chamber to allow the mole fraction of water to increase during evapotranspiration. Treatments such as the PDSO Civitas reduce transpiration which would further limit additional water vapor into the chamber which reduces VPD, the driving force behind transpiration (Farquhar and Sharkey, 1982).

In contrast to the pigment Harmonizer, the PDSO Civitas significantly reduced CER and radiation use efficiency (RUE) as PPFD intensity increased (Table 3.8; Fig. 3.7). A formulation effect was noted as the Civitas One reduced CER and RUE more than the PDSO Civitas and Civitas Two Pack. Treatment differences were not statistically different below a PPFD of 750 µmol PAR m^{-2} s^{-1}. There were no differences in CER in darkness, which represents maximum dark respiration rate (Hay and Porter, 2006). This suggests the Civitas treatments did not affect respiration rate.

Reduction in maximum photosynthetic rate at higher PPFD is commonly the result of substrate (CO_2) limitation that can occur when stomata are closed or obstructed (Hay and Porter, 2006). Further study is required to clearly understand how long reduced CER and transpiration rate following application of Civitas. Measurements need to be taken over a time course to determine the duration of reduced CER/RUE and transpiration. Long term reduction in RUE has the potential to reduce nonstructural carbohydrates and increase photooxidation is photosynthesis is substrate limited. Future experiments also need to be conducted at constant VPD to more accurately measure transpiration rate. The pigment Harmonizer and Civitas Two-Pack could safely be eliminated from future experiments because they did not change CER and transpiration rate compared to the control and PDSO Civitas treatments, respectively. This change offers substantial time savings.

CONCLUSION

Application of the PDSO Civitas to a mixed annual bluegrass and creeping bentgrass golf putting surface resulted in chlorosis, canopy thinning, and elevated surface temperatures. Addition of the phthalocyanine pigment Harmonizer to PDSO Civitas masked the chlorosis in both years; however, the pigment Harmonizer was unable to mitigate the decline in turfgrass visual quality ratings as stand density declined in year two.

The chlorosis associated with PDSO Civitas phytotoxicity can be described as chronic because there was significant evidence of oil persistence and stomatal impedance. There was no evidence of membrane disruption or alteration of quantity or composition of epicuticular wax, although wax morphology was altered. Further research is required to understand how Civitas affects carbon partitioning, photoinhibition, water use efficiency, and pesticide absorption.

TABLES AND FIGURES

Table 3.1. Summary of repeated measures ANOVA table of main effects and their interactions on a creeping bentgrass putting green performance.

Source	Visual Quality		Chlorophyll Index		Surface Temperature	
	df	F-ratio	df	F-ratio	df	F-ratio
Civitas (C)	1	69.16***	1	56.94***	1	155.44***
Pigment (P)	1	143.98***	1	72.77***	1	127.45***
Date	47	8.81***	47	79.91***	35	11648.38***
Block	3	15.85	3	1.60	3	1.89
C x P	1	35.74***	1	14.76**	1	1.85
C x Date	47	11.88***	47	13.88***	35	8.65***
P x Date	47	5.80***	47	7.47***	35	6.65***
Block x Date	141	1.51**	141	1.73***	105	3.81
C x P x Date	47	6.12***	47	2.53***	35	1.47*

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 3.2. Putting green tiller density at the end of 2013 as affected by Civitas and Harmonizer.

Treatment	df	Tiller Density
		--- # cm^{-2} ---
Harmonizer		14.2a
Control		13.3ab
Civitas		12.4bc
Two Pack		11.6c
	ANOVA	
Civitas	1	***
Harmonizer	1	***
Civitas x Harmonizer	1	***

*** Significant at the 0.001 probability level.

Table 3.3. Electrolyte leakage of turfgrass leaves treated with different Civitas formulations.

Treatment	df	Electrolyte leakage
		%
Civitas		13.5a
Control		10.5ab
Two Pack		9.4b
Harmonizer		8.6b
Civitas One		8.5b
	ANOVA	
Treatment (T)	4	*
Run (R)	1	ns
T x R	4	ns

* Significant at the 0.05 probability level.

Table 3.4. ANOVA of turfgrass epicuticular wax components as affected by treatment and run.

Source	df	F-ratio							
		Fatty acids		Primary alcohols					Civitas:C_{26} primary alcohol
		C_{16}†	C_{18}	C_{18}	C_{22}	C_{24}	C_{26}	C_{28}	
Treatment (T)	4	1.23	2.05	0.76	2.08	0.73	1.25	0.71	9.14***
Run (R)	1	0.88	1.41	5.26*	14.45**	7.71*	0.68	7.20*	1.07
T x R	4	0.19	0.43	0.53	2.13	1.94	2.03	1.97	1.29

† Represents the size of the fatty acid or primary alcohol components in number of C atoms.

Table 3.5. Major epicuticular wax components of leaves in run 1 and 2.

Run	C_{16} FA[†]	C_{18} FA	PA C_{18}	PA C_{22}	C_{24} PA	C_{26} PA	C_{28} PA
	----------	----------	----------	µg g^{-1} fwt	----------	----------	----------
1	1.26a[‡]	0.97a	0.59b	1.36b	0.88b	46.33a	1.69b
2	1.59a	0.78a	0.96a	2.39a	1.37a	55.37a	2.33a

† Represents the size of the fatty acid (FA) or primary alcohol (PA) in number of C atoms.

‡ Means followed by the same letter are not significantly different according to LSD (0.05).

Table 3.6. Molar ratio of Civitas relative to C_{26} 1-hexacosanol extracted from turfgrass leaves.

Treatment	Civitas to C_{26} primary alcohol ratio
	mol:mol
Civitas	17.0a†
Civitas One	10.4a
Two Pack	8.7a
Control	0.3b
Harmonizer	0.2b

† Means followed by the same letter are not significantly different according to LSD (0.05).

Table 3.7. Transpiration rate as affected by treatment, photon flux density, and run.

Run	Treatment	df	Transpiration rate
			unit
1	Control		13.62cd†
	Harmonizer		13.57cd
	Civitas		13.3cd
	Two pack		12.98f
	Civitas One		13.09ef
2	Control		14.18a
	Harmonizer		14.08ab
	Civitas		13.78bc
	Two pack		14.12a
	Civitas One		13.95ab
	ANCOVA		
Source of variation			
Treatment (T)		4	***
Photon Flux Density (PPFD)		1	***
Run (R)		1	***
T x PPFD		4	ns
T x R		4	*
PPFD x R		1	ns
T x PPFD x R		4	ns

* Significant at the 0.05 probability level.

*** Significant at the 0.001 probability level.

† Means followed by the same letter are not significantly different according to LSD (0.05).

Table 3.8. Carbon exchange rate ANOVA as affected by treatment, photon flux density, and run.

Source	df	F-ratio
Treatment (T)	4	28.9***
Photon Flux Density (PPFD)	6	1184.1***
Run (R)	1	8.6**
T x PPFD	24	2.4***
T x R	4	2.13
PPFD x R	6	0.02
T x PPFD x R	24	0.25

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

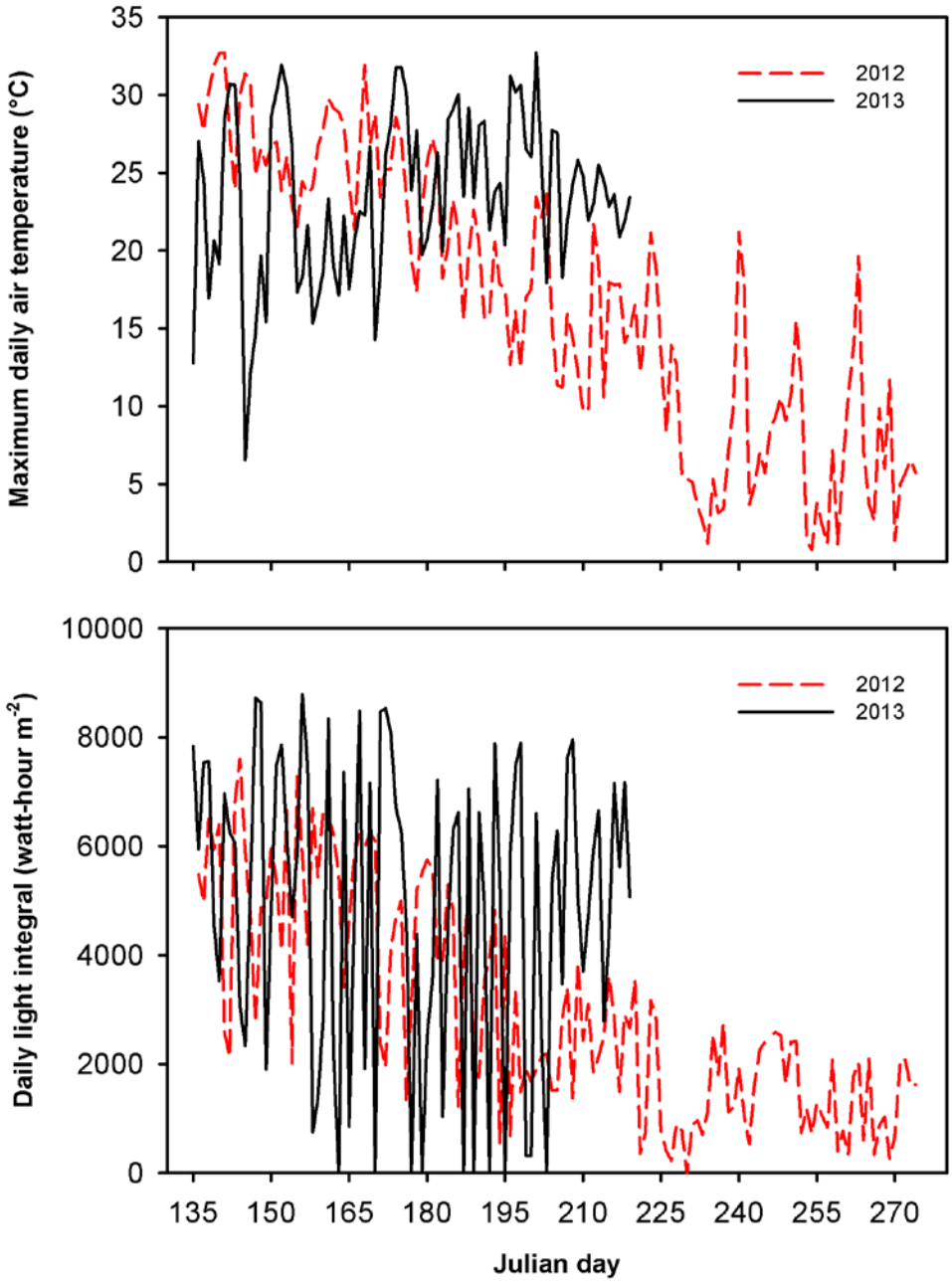

Figure 3.1. Daily maximum and minimum temperatures and daily light integral during 2012 and 2013 at the Bluegrass Land Turf and Landscape Research Facility in Ithaca, NY. There were no measurements after 8 Aug. 2013 because the weather station was struck by lightning.

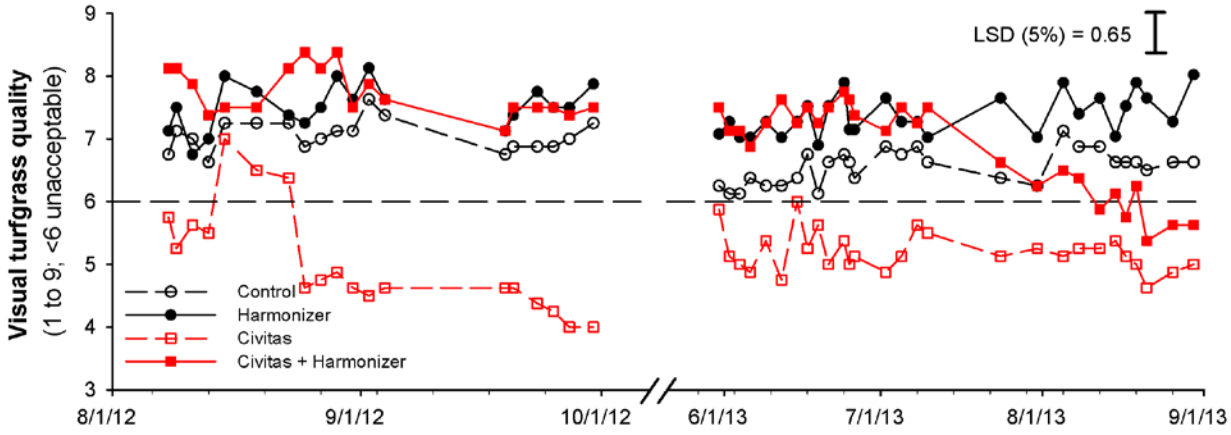

Figure 3.2. Turfgrass visual quality as affected by Civitas and Harmonizer.

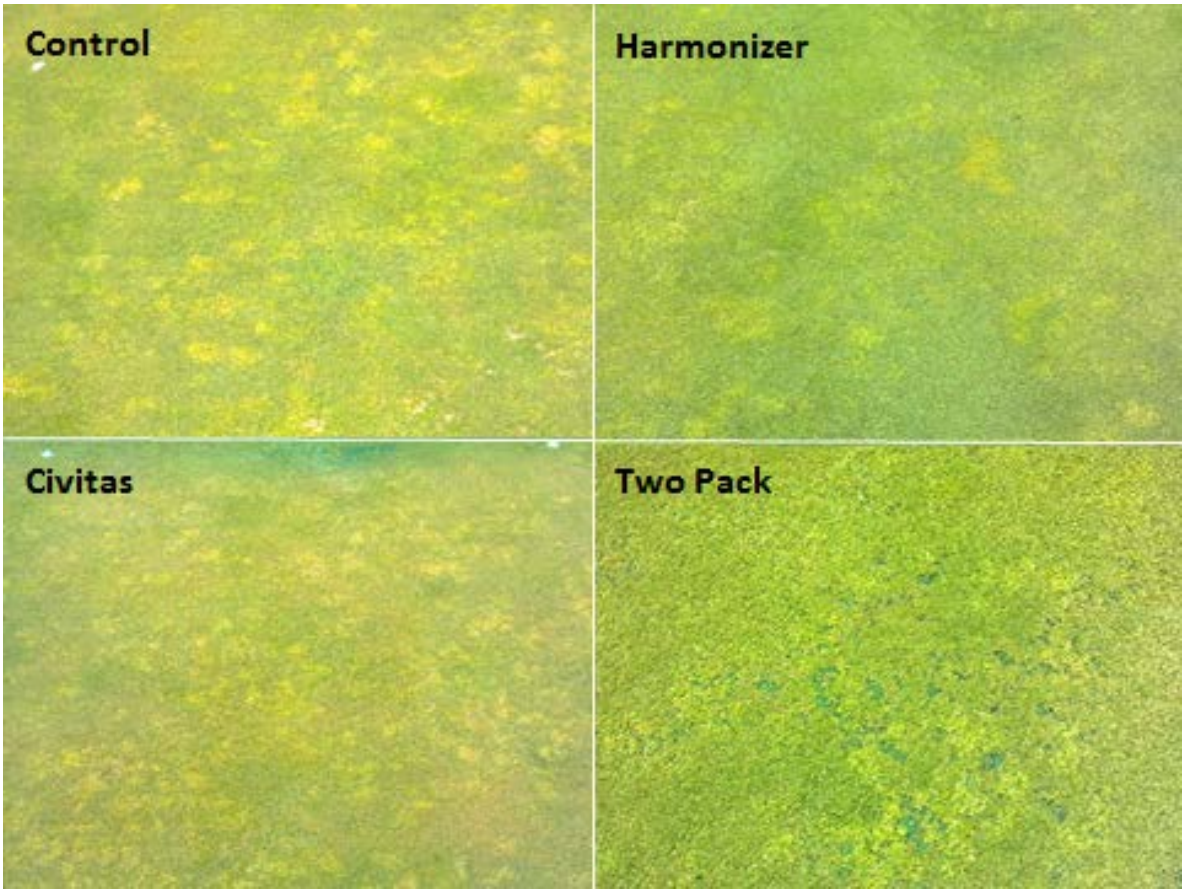

Figure 3.3. An illustration of phytotoxicity at the end of the study in 2013.

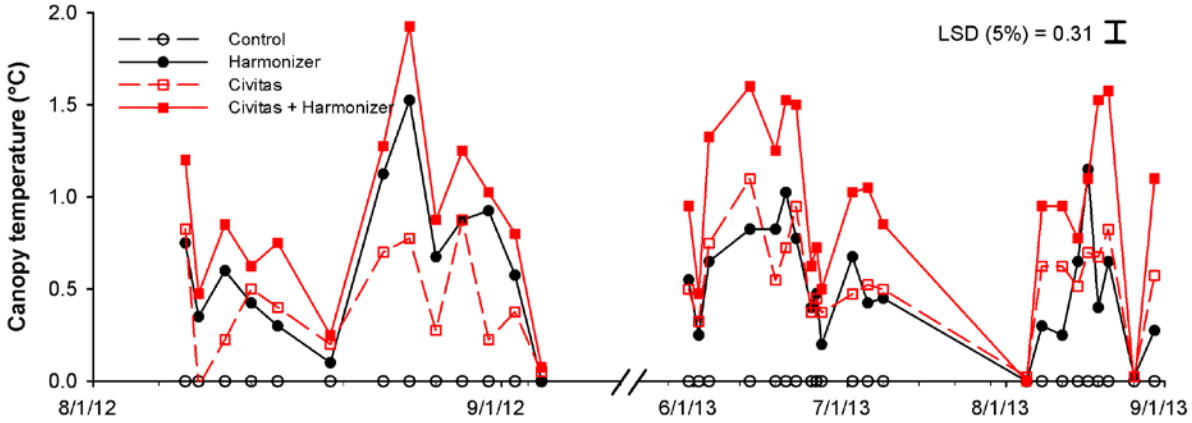

Figure 3.4. Canopy temperature as affected by Civitas and Harmonizer.

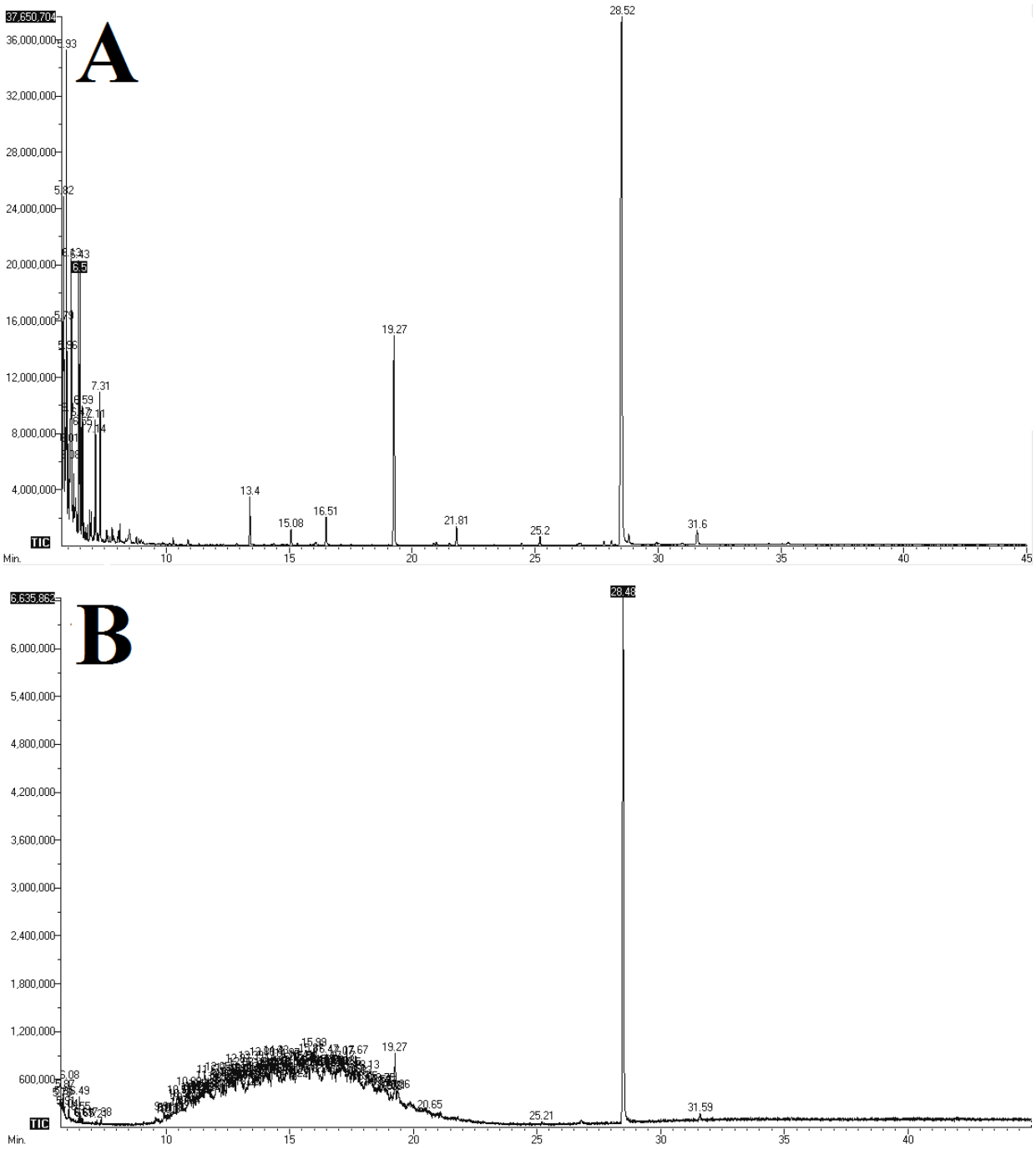

Figure 3.5. A screenshot of two 150 split total ion current chromatographs from (A) epicuticular waxes extracted from a non-treated control and (B) a Civitas One treated sample. Major peaks were a C_{16} and C_{18} fatty acid, a C_{24} alkane internal standard, and C_{18}, C_{22}, C_{24}, C_{26}, and C_{28} primary alcohol in that order.

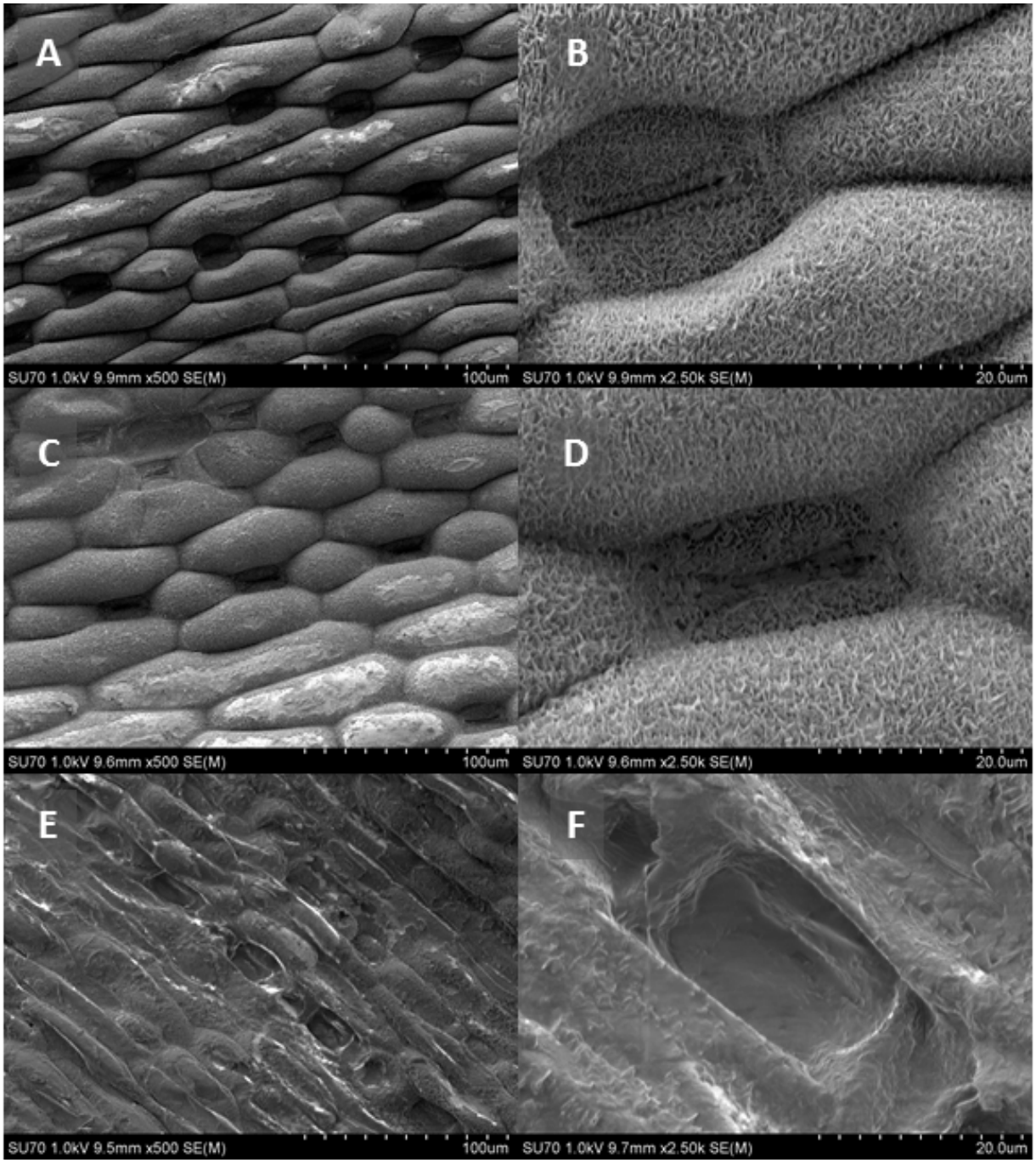

Figure 3.6. Scanning electron micrograph of leaf epicuticular wax morphology and turfgrass stomata. Images A, C, and E are of turfgrass leaves treated with water, 48 h after Civitas Two-Pack application, and 48 h after the second Civitas Two-Pack application, respectively at 500x magnification. Images B, D, and Fare of turfgrass leaves treated with water, 48 h after Civitas Two-Pack application, and 48 h after the second Civitas Two-Pack application, respectively at 2500x magnification. Images were representative of the entire leaf surface.

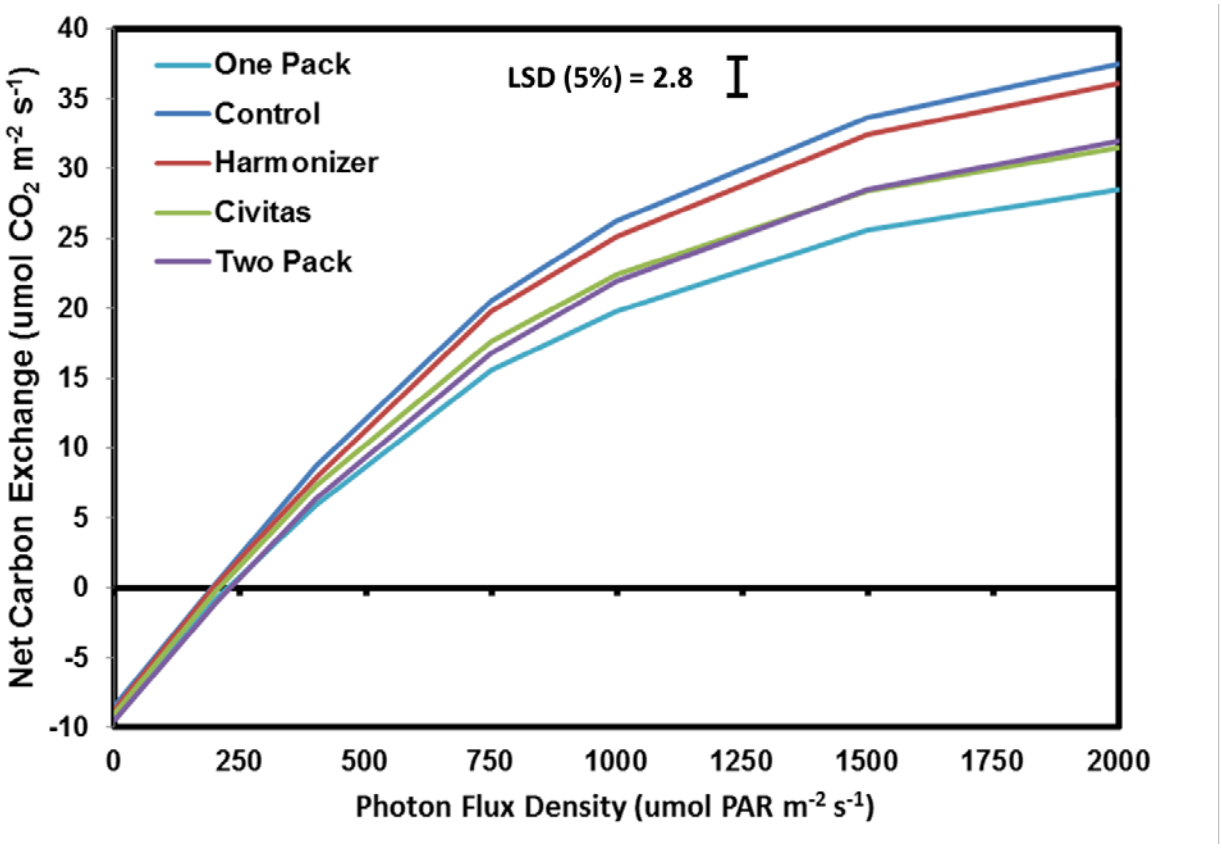

Figure 3.7. The effect of photosynthetic photo flux density and Civitas component or formulation on carbon exchange rate.

Chapter 4: Civitas Formulation and Application Rate have Lasting Effects on Carbon Assimilation and Transpiration in Turfgrass

ABSTRACT

The petroleum derived spray oil (PDSO), Civitas, is used in the turfgrass industry to reduce reliance on traditional pesticides. Civitas was found to reduce carbon assimilation (*A*) and transpiration (*E*) 24 h after it was applied. The Civitas One formulation was more detrimental than the PDSO Civitas formulation. The objectives were to i) determine the persistence of the PDSO Civitas effects on plant *A* and *E*, ii) elucidate the mechanism of *A* inhibition and iii) ascertain if a dose-response exists for the PDSO Civitas effect on *A* and *E*. A Li-Cor 6400 with Arabidopsis chamber was used to construct light response and ambient CO_2 response curves. Cone-tainers of annual bluegrass (*Poa annua* L.) were treated with PDSO Civitas alone, the new Civitas One formulation, or a non-treated control. Light and CO_2 response curves were constructed 3 hours, and 1, 2, 5, and 10 days after treatment (DAT). The effect of Civitas dose rate was also investigated with light response curves 24 h after application of 0, 1.3, 2.5, 5.1, or 10.2 mL m^{-2} with PDSO Civitas. Both Civitas formulations reduced *A* and *E* 3 hours after application. Transpiration recovered more quickly than *A* which was reduce up to 10 DAT. Correlation of *A* to *E* indicated limitation of leaf conductance reduced carboxylation efficacy and is supported by scanning electron micrographs of turfgrass leaves which show debris around plant stomata. The lasting reduction in *A* may have implications on turfgrass carbon partitioning and may reduce total nonstructural carbohydrate concentrations.

INTRODUCTION

The petroleum derived spray oil Civitas™ (Petro-Canada, Mississauga, ON, CA) is used as a fungicide in turfgrass. It has been shown to increase plant disease resistance by inducing plant systemic resistance (Cortes et al., 2010a, b). Induced systemic resistance has been shown to increase plant growth through increased photosynthesis (Ryu et al., 2004; Vallad and Goodman, 2004; Shoresh et al., 2010; Kwon et al., 2010). However, previous research with the PDSO Civitas found it can persist on the turfgrass leaves and reduce carbon assimilation rate (*A*) and transpiration rate (*E*) 48 h after application. Scanning electron micrographs suggest stomatal impedance by oil or wax debris causes the decline in *A* and *E*.

Plant growth is directly related to *A* (Monteith, 1977). Processes that reduce photosynthesis, such as photoinhibition or drought stress which limits gas exchange, reduce plant growth rate and in extreme cases can cause plant death (Taiz and Leigher, 2006). Any reduction in *A* and *E* with Civitas may have serious consequences on plant growth carbon partitioning, accumulation of storage carbohydrates, and may lead to oxidative stress. A more detailed study of turfgrass *A* and *E* are essential to understand how these fundamental plant processes are affected by different Civitas formulations and rates and determine the duration of the effect.

Gas exchange measurements have been used to study plant ecology and physiology in response to environmental stimuli (Long et al., 1996; Long and Bernacchi, 2003). The development of portable open air gas exchange systems has given researchers the ability to easily and accurately measure photosynthesis (*A*), transpiration (*E*), leaf conductance (g_l), and intercellular CO_2 mole fraction (C_i) (Long and Bernacchi, 2003). These values are calculated by measurement of the differential of CO_2 and H_2O concentrations of air moving through a leaf

cuvette and corrected with air pressure, leaf temperature, and diffusion constants (Farquhar et al., 1980).

Traditionally *A* is modeled as a function of photosynthetic photon flux density (*Q*) or C_i. While these *A/Q* and A/C_i curves directly relate *A* to *Q* and C_i, they also provide insight on leaf conductance and efficiency, the sub-processes of photosynthesis (Hay and Porter, 2006). *A/Q* curves can be used to measure maximum dark respiration (R_d), the light compensation point where gross photosynthesis equals total respiration, apparent quantum efficiency (A_{qe}), and maximum photosynthetic capacity (A_{max}). Apparent quantum efficiency represents the moles CO_2 assimilated $mole^{-1}$ Q (Hay and Porter, 2006). *A* decays at higher *Q* intensity due to limitation of CO_2 diffusion or ribulose-1,5-bisphosphate (RuBP) regeneration in the Calvin cycle (Hay and Porter, 2006). A/C_i curves are typically used to determine Rubisco activity ($V_{c,max}$) and regeneration of RuBP (J_{max}) (Hay and Porter, 2006). At low C_i, *A* is limited by Rubisco activity with an inflection as C_i increases where RuBP regeneration inhibits *A*. Analysis of *A/Q* and A/C_i can be used in conjunction to understand the state of a plant's photosynthetic apparatus.

The objective of this project was to determine the temporal effects of Civitas formulation and dose response of the PDSO Civitas on turfgrass carbon and water dynamics with a Li-Cor 6400 Portable Photosynthesis System. Specifically experiments were designed to i) determine the persistence of the PDSO Civitas effects on plant *A* and *E*, ii) elucidate the mechanism of *A* inhibition and iii) ascertain if a dose-response exists for the PDSO Civitas effect on *A* and *E*.

MATERIALS AND METHODS

Plant Establishment

Annual bluegrass (*Poa annua* L.) was established in a Percival WE-1012 growth chamber fitted with a mixed incandescent & fluorescent light source (Percival Scientific, Perry, IA) at Cornell University in Ithaca, NY. Conditions were designed to simulate conditions similar to natural weather conditions in Ithaca, NY during early summer. Specifically, plants received approximately 450 umol PAR m^{-2} s^{-1} for 16 h at 30°C and 50% relative humidity and 8 h of darkness at 20°C and 70% relative humidity.

Ray Leach SC10 3.8 x 210 cm cone-tainers (Stuewe & Sons, Tangent, OR) were filled and packed with non-amended medium textured sand meeting USGA specifications for putting green construction (USGA, 2004). Approximately 10 seeds of annual bluegrass were placed in cones that were filled with sand to 15mm below top of cone to allow for uniform clipping. Cones were fertilized weekly with 10 mL of a fertilizer solution containing 200, 50, 200, and 1 ppm N, P, K, and chelated Fe, respectively for the first ten weeks of establishment. After establishment, cones received 8 mL of the fertilizer mix every two weeks. Cones were clipped several time wk^{-1} and were watered daily to prevent drought stress with tap water. Cones were fully established and deemed ready for experiments twelve weeks after seeding.

Gas Exchange Measurements

Carbon assimilation rate and transpiration were measured with a Li-Cor 6400XT photosynthesis meter with 6400-17L Lighted Whole Plant *Arabidopsis* Chamber (Li-Cor, Lincoln, NE). The chamber was configured to provide constant 60% relative humidity at 30°C and 400ppm CO_2 concentration. The Li-Cor 6400 automatically adjusts flow rate to maintain constant relative humidity; approximately 500 µmol air s^{-1}. Light response curves were created

with an AutoProgram that adjusted photosynthetic photon flux density (*Q*) from 2000 to 0 µmol photosynthetically active radiation (PAR) m^{-2} s^{-1}. Measurements were taken at *Q* values of 2000, 1500, 1000, 750, 500, 250, 125, and 0 µmol PAR m^{-2} s^{-1}. Immediately following light response measurements, CO_2 response was measured at a *Q* of 500 µmol PAR m^{-2} s^{-1}. Measurements were taken at 600, 200, and 50 ppm reference air CO_2 concentrations. Carbon exchange rate and transpiration where measured automatically after CO_2 and H_2O concentrations of the sample had CV values less than 5%.

Experiment 1 – Civitas Formulation

The formulation study was a completely randomized design with four replicates which was repeated twice. Treatments included PDSO Civitas alone, and Civitas One Pack applied at the rates of 5.0 and 5.5 ml m^{-2}, respectively to supply same rate of PDSO. Applications were made with a CO_2 powered backpack sprayer equipped with a TeeJet AI 8004 nozzle (TeeJet Technologies, Wheaton, IL) calibrated to deliver 81 mL m^{-2} at 275kPa. All cones were sorted into a single line to ensure each cone was beneath the nozzle tip. Applications were staggered by one hour across replications to allow for time to make gas exchange measurements. *A/Q and* A/C_a curves were created 3 hours, 1, 2, 5, and 10 days after application of treatments (DAT).

Experiment 2 - Dose Response

The dose response study was a randomized complete block design with four replicates and was repeated twice. The PDSO Civitas was applied at rates of 0, 1.3, 2.5, 5, and 10 L m^{-2}. Applications were applied with the same described in Civitas formulation experiment. Light response curves were created 24 h after application with the technique described in previously.

Statistical Analysis

The statistical method described by Peek et al., (2002) was used to create and compare light response curves in both experiments. Light response curves were constructed with *A* as a function of *Q* where values greater than zero indicate net C fixation/photosynthesis. Light response models were created with the nonlinear mixed models procedure in SAS Version 9 (SAS, 2000). Nonlinear regression curves were fit to each cone-tainer with the model:

$$A = R_d + A_{max} * (1\text{-}e^{(-k*Q)})$$

where R_d represents the maximum respiration rate, A_{max} represents the asymptote where the system becomes light saturated, and *k* represents the initial slope. The output parameter estimates and associated error values were then subjected to repeated measures ANOVA. Means were separated with Fisher's LSD (0.05) when appropriate. Transpiration response to *Q* and *A* response to external CO_2 content was modelled with least squares analysis in JMP 10 (SAS Institute, Cary, NC). The A/C_a curves were created with quadratic regression in JMP 10 (SAS, Cary, NC). Models were analyzed separately when higher order interactions were significant. Means were separated with Fisher's LSD (0.05) when appropriate.

RESULTS AND DISCUSSION

Civitas Formulation – A/Q Curves

Carbon assimilation rate increased as *Q* increased independent of treatment (Fig. 4.1). However, the assimilation rate declined as *A* approached A_{max} at high *Q*. Under optimal conditions for photosynthesis there is a linear increase in *A* with increasing *Q* (Hay and Porter, 2006). During this phase, photosynthesis is light limited and not substrate limited by CO_2

diffusion into the leaf. As *Q* continues to increase, the canopy is no longer light limited due to light saturation, and becomes substrate limited through reduced CO_2 diffusion limits *A*.

On an *A/Q* curve, the change from light limitation to CO_2 limitation is rapid under ideal conditions for photosynthesis. This is particularly true of single leaves of C_3 grasses which typically light saturate near 500 µmol PAR m^{-2} s^{-1} (Beard, 1973; Hay and Porter, 2006). While light saturation is slower on canopy scale because of leaf shading (Sinclair and Muchow, 1999), the gradual transition in *A* to A_{max} during this study reflect the supraoptimal environmental conditions. The day air temperature in the growth chamber was 30°C. Photorespiration is known to hinder net *A* as air temperature exceeds 25°C in C_3 plants (Edwards and Walker, 1983). The supraoptimal air temperature likely contributed to the slow decline in *A* as *Q* increased and the relatively high light compensation point in the control plants.

The inhibition of *A* with PDSO Civitas was substantial, especially at higher values of *Q* (Fig. 4.1). Civitas One had more of a detrimental effect on A_{max} than the PDSO Civitas applied alone (Tables 4.1 & 4.2). The initial slope of the equation, *k*, increased with PDSO Civitas and Civitas One, however A_{qe} is a function of *k* times A_{max}. Although *k* increased with both Civitas formulations, the scalar A_{max} term increased A_{qe} to a larger value in the non-treated control. The reduced A_{qe} with both Civitas formulations is obvious in Fig. 4.1. Civitas formulation did not have a dramatic effect on R_d (Fig. 4.1). Civitas formulation had small and inconsistent effects on R_d over the course of 10 days but there was not an obvious treatment difference (Table 4.4).

The inhibition of *A* was greatest immediately after application and over time approached the level of the non-treated 10 days after application (Tables 4.2 & 4.3). There was a significant run effect which was most pronounced within the first 24 h following application. This was particularly evident 3 h after application during the first experimental run when the Civitas One

treatment never reached the light compensation point. This suppression of A_{max} suggests that there is a strong influence on substrate availability. Hay and Porter (2006) concluded was a suppression of A_{max} can result from decreased CO_2 diffusion or RuBP regeneration.

Research in prior chapters of this dissertation visualized the accumulation of debris around stomata that could result in stomatal impedance. Photosynthesis has been long correlated with stomatal conductance (Wong et al., 1979). The inhibition of stomatal conductance by the PDSO Civitas has the potential to decrease CO_2 diffusion which can reduce A_{max} and A_{qe} with little to no effect on respiration.

Transpiration rate was also reduced with both formulations of Civitas although the decline in *E* was less dramatic than the decline in *A*. Transpiration increased linearly as with *Q* (Table 4.1). As with A, there was DAT x Run interaction. Specifically, during the first experimental run the PDSO Civitas and Civitas One Pack reduced transpiration by 3 h after application but *E* recovered by 24 h after application for the PDSO Civitas (Table 4.5). Comparatively, Civitas One reduced *E* until 10 DAT during the first run. Interestingly, the PDSO Civitas reduced *E* 48 h after application while the Civitas One reduced *E* for the first 48 h after treatment before returning to the level of the control in the second run (Table. 4.5)

Civitas Formulation – A/C_a Curves

A/C_i curves are commonly used in plant physiology to examine Rubisco activity, gas exchange, and RuBP regeneration (Long and Bernacchi, 2003). Due to the limitations of the canopy scale measurements, A/C_a curves were constructed instead of A/C_i curves. A/C_a curves are inherently more difficult to interpret than A/C_i curves because they are affected by the boundary layer and stomatal conductance (Long and Bernacchi, 2003). Assuming the boundary layer component of a dense grass canopy is fairly consistent and soil evaporation is minimal;

A/C$_a$ curves can provide indirect information about Rubisco activity and gas conductance when other factors such as relative humidity, air temperature, and light intensity are controlled.

Assimilation rate increased with increased *C*$_a$ (Fig. 4.2). The quadratic term, CO_2 x CO_2, was significant (Table 4.6). Civitas One and PDSO Civitas reduced the y-intercept (*R*$_d$) following product application (Table 4.7). The *R*$_d$ value in an *A/C*$_a$ represents the amount of respiration occurring under irradiation of 500 µmol PAR m^{-2} s^{-1} which is different than the *R*$_d$ from an *A/Q* curve which represents dark respiration. Both Civitas formulations reduced R_d on all days in both runs. The lone exception occurred 10 DAT in the second run where PDSO Civitas had similar R_d as the control. The Civitas One was more detrimental than PDSO Civitas. The increase in R_d likely occurred due to photorespiration because there were no meaningful differences between treatments in the absence of light.

The initial slope of the *A/C*$_a$ reflects Rubisco activity and gas exchange (Long and Bernacchi, 2003). There was a significant Civitas x DAT x CO_2 interaction regarding the linear term (Table 4.6). They closely followed the trends observed in the *A/Q* curves (Figs. 4.1 & 4.2). Both Civitas formulations reduced the initial slopes 3 h after application compared to non-treated turf (Table 4.8). Similar to other measured parameters, the effect declined over time independent of run and there was a significant formulation effect. There were also significant Run x DAT and Run x Civitas interactions (Tables 4.9 & 4.10). The Civitas x DAT x CO_2 x CO_2 interaction was also significant (Table 4.11). These interactive effects, while significant, do not contradict the hypothesis that Civitas formulation inhibits *A* and *E* due to reduced gas exchange.

Unfortunately, *A* and *E* were measured on a canopy scale. This complicates accurate determination of leaf water conductance (*g*$_l$) and stomatal conductance. However, transpiration is intimately linked to *g*$_l$ by vapor pressure deficit (VPD) which serves as the driving force (von

Caemmerer and Farquhar, 1981). The Li-Cor 6400 was configured to maintain constant relative humidity and air temperature. Under constant *Q*, air temperature, and air relative humidity VPD should be fairly constant. Therefore *E* is directly proportional to g_l and can be used to estimate the effect of Civitas formulation in gas exchange.

Reductions in E from PDSO Civitas led to a dramatic decline in *A* and these reductions were slower to recover than *E*. The large VPD may overpower the reduction in stomatal conductance while exerting little influence on CO_2 diffusion. Additionally, CO_2 diffusion into leaves via stomata can be hindered by convection processes which result from transpiration (Farquhar and Sharkey, 1982). Alternatively, the PDSO Civitas may have the potential to alter photosynthesis at a more fundamental level than simply inhibiting CO_2 diffusion.

Carbon assimilation rate data from the A/C_a curves at 600 ppm CO_2 were plotted as a function of transpiration to isolate the effect of leaf conductance on carbon fixation (Fig. 4.3). The *A* in the non-treated control was the least sensitive to transpiration rate/leaf conductance. This indicates that *A* in the control plants was limited by other factors than CO_2 availability, most likely *Q*. Both Civitas formulations were more sensitive to transpiration/leaf conductance than the control. This provides more evidence that gas exchange is inhibited with both Civitas formulations. There is also evidence that the carboxylation capacity is reduced with both Civitas formulations. At higher levels of transpiration/conductance, both PDSO Civitas and Civitas One values were typically less than the non-treated control.

Inhibition of gas exchange can have profound effects on plant physiology. Both PDSO Civitas and Civitas One reduced net photosynthesis and transpiration rate while photorespiration and Rubisco inactivation increased. The Civitas One had a more dramatic and lasting effect than

the PDSO Civitas. Although both products have equivalent concentrations of oil there could be differences in the formulation components between the two products.

The PDSO Civitas is marketed as a product that enhances plant health. However, it seems reasonable from these results that PDSO Civitas leads to increases in plant oxidative stress through increased photorespiration and the reduction in photosynthetic efficiency (Taiz and Zeigler, 2006). Therefore, optimization of the PDSO Civitas application rate may minimize these deleterious responses as evidenced by the differential effects on A by 1.3 and 2.5 mL m^{-2} application rate compared the 5.1 and 10.2 mL m^{-2} rates.

Alternatively, some level of oxidative stress may be required to prime plant defense. Recent advances in plant molecular biology have found that reactive oxygen species play a role in cell signaling and response to abiotic and biotic stresses (Niyogi, 1999). If PDSO Civitas primes defense by inducing non-lethal oxidative stress, application rate needs to be optimized to both elicit induced systemic resistance yet minimize negative effects on carbon assimilation, carbon dynamics, and transpiration.

Civitas Dose Response – A/Q Curves

The shape of *A/Q* and A/C_i curves differed between the two experimental runs (Fig. 4.1 & 4.2). The control treatments were similar between the two runs, but *A* was inhibited by PDSO Civitas formulation more in the first experimental run, likely the result of experimental error in product application. The CO_2 powered sprayer was calibrated for the nozzle to be 50 cm above the turfgrass canopy. However, the nozzle was approximately 35 cm above the canopy during the first experimental run that resulted in an application increase in rate. This was corrected and the difference between experimental runs suggests PDSO Civitas application rate may have a significant effect on *A.*

A significant negative linear dose response of PDSO Civitas was observed in this study for both *A/Q* and *E/Q* (Tables 4.12 & 4.l3). Increased oil application rate most likely further inhibited leaf gas exchange and reduced *A* and *E*. Despite best efforts to apply equal quantities of PDSO Civitas in both runs of the dose response experiments, there was a significant Run x Rate interaction for *E*. Transpiration was inhibited more in first dose response run than the second run.

CONCLUSIONS

There is significant evidence that PDSO Civitas inhibited gas exchange which reduced *A* and *E*. The Civitas One formulation was more detrimental than the PDSO applied alone. Although stomatal conductance could not be directly measured in this study, the decline in *E* and *A*, and increase in photorespiration and canopy temperature indicate stomatal conductance is being inhibited. These responses were exacerbated as PDSO Civitas application rate increased and persisted several days after application. The decline in *A* likely has an effect on carbon partitioning, carbon storage, and creation of oxidative stress within the turfgrass plant which requires further investigation.

TABLES AND FIGURES

Table 4.1. Civitas formulation, photosynthetic photon flux density (PPFD), and run on maximum dark respiration (R_d), initial slope (k), the maximum photosynthetic rate (A_{max}), and transpiration rate (E) over ten days after application (DAT) in the Civitas formulation experiment.

Source	df	p-value			
		R_d	k	A_{max}	E
Formulation (F)	2	ns	***	***	**
DAT (D)	4	***	***	***	***
Run (R)	1	*	***	***	ns
F x D	8	*	***	***	***
R x F	2	ns	***	***	ns
R x D	4	***	***	***	***
R x F x D	8	ns	***	**	***
PPFD (P)	1	na	na	na	***
F x P	2	na	na	na	ns
P x D	4	na	na	na	ns
F x P x D	8	na	na	na	ns
R x P	1	na	na	na	ns
R x F x P	2	na	na	na	ns
R x P x D	4	na	na	na	ns
R x F x P x D	8	na	na	na	ns

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 4.2. Model parameter estimates for A_{max} as affect by Civitas formulation, time since application, and run in the Civitas formulation experiment.

Run	Civitas Formulation	Time after treatment application				
		3 hours	1 day	2 days	5 days	10 days
		---------- A_{max} (µmol CO_2 m^{-2} s^{-1}) ----------				
1	Control	22b	35b	39b	53b	57b
	Civitas	53a	63a	54a	64a	65a
	Civitas One	14c	32b	33c	48c	55b
2	Control	46b	52b	51b	54b	51ab
	Civitas	58a	64a	61a	63a	55a
	Civitas One	23c	39c	45b	57ab	47b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.3. Model parameter estimates for *k* as affect by Civitas formulation, time after application, and run in the Civitas formulation experiment.

Run	Civitas Formulation	Time after treatment application				
		3 hours	1 day	2 days	5 days	10 days
		---------- *k* x 10^{-4} ----------				
1	Civitas	18.5b	13.7a	13.0a	10.5ab	10.7ab
	Control	11.3c	10.7b	11.1b	9.4b	9.4b
	Civitas One	21.7a	14.0a	14.0a	11.8a	10.9a
2	Civitas	11.6b	12.1a	11.4a	11.8a	12.2a
	Control	9.9c	9.6b	9.8a	9.7b	11.1a
	Civitas One	16.9a	10.1b	10.7a	9.6b	12.4a

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.4. Model parameter estimates for R_d as affect by Civitas formulation, time since application, and run in the Civitas formulation experiment.

Civitas Formulation	Time after treatment application				
	3 hours	1 day	2 days	5 days	10 days
	---------- R_d (µmol CO_2 m^{-2} s^{-1}) ----------				
Control	-17.2a	-17.6a	-16.5a	-18.8b	-18.8a
Civitas	-17.4a	-17.6a	-16.9a	-17.2a	-17.3a
Civitas One	-16.6a	-16.3a	-16.4a	-18.5ab	-18.3a
Run					
1	-16.9a	-18.0b	-17.1a	-18.4a	-19.5b
2	-17.3a	-16.4a	-16.1a	-18.0a	-16.8a

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.5. Intercept parameter estimates for transpiration rate as affect by Civitas formulation and time since application in Civitas formulation experiment.

Run	Civitas Formulation	Time after treatment application				
		3 hours	1 day	2 days	5 days	10 days
		---------- y-intercept (mmol H_2O m^{-2} s^{-1}) ----------				
1	Control	14.1a	16.2a	14.9ab	14.9ab	16.3a
	Civitas	12.0b	15.5ab	15.7a	15.6a	15.9a
	Civitas One	11.8b	14.8b	14.6b	14.6b	15.4a
2	Control	16.1a	16.3a	15.6a	16.9a	15.5ab
	Civitas	14.9ab	15.9a	13.2b	14.8b	15.2b
	Civitas One	11.7b	13.8b	14.0b	16.6a	16.1a

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.6. The effect of Civitas formulation and ambient CO_2 concentration (C_a) on net carbon assimilation (*A*) over the course of 10 days and two runs in the Civitas formulation experiment.

Source	df	p-value
CO_2 (C)	1	***
Civitas Formulation (F)	2	ns
Days after treatment (D)	4	ns
Run (R)	1	***
C x C	1	***
F x C	2	***
D x C	4	***
R x C	1	***
F x D x C	8	***
F x C x C	2	***
D x C x C	4	***
F x R x C	2	**
D x R x C	4	***
F x D	8	ns
F x R	2	*
D x R	4	*
F x D x R	8	***
F x D x C x C	8	**

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 4.7. The influence of Civitas formulation on model y-intercept (R_d) over the course of 10 days and two runs in the Civitas formulation experiment. R_d represents maximum respiration at 500 µmol PAR m^{-2} s^{-1}.

Run	Civitas Formulation	Time after treatment application				
		3 hours	1 day	2 days	5 days	10 days
		---------- y-intercept (µmol CO_2 m^{-2} s^{-1}) ----------				
1	Control	2.3a	1.5a	2.7a	3.1a	3.0a
	Civitas	-5.5b	-2.4b	-0.8b	0.0b	-0.3c
	Civitas One	-8.7c	-4.1c	-1.9c	0.1b	1.1b
2	Control	1.3a	4.7a	4.5a	4.3a	4.2a
	Civitas	-1.0b	1.8b	3.2b	2.5b	3.6a
	Civitas One	-6.6c	-2.4c	-0.6c	0.3c	1.4b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.8. The influence of Civitas formulation linear term of the model over the course of 10 days in the Civitas formulation experiment.

Civitas Formulation	Time after treatment application				
	3 hours	1 day	2 days	5 days	10 days
	---------- Linear term $x\ 10^{-2}$ (µmol CO_2 m^{-2} s^{-1} ppm^{-1} C_a) ----------				
Control	6.16a	5.13a	6.56a	6.66a	6.44a
Civitas	3.50b	3.99ab	5.58ab	5.60a	5.30ab
Civitas One	2.07b	2.60b	4.68b	5.88a	3.91b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.9. The influence of run on model linear term of the model over the course of 10 days in the Civitas formulation experiment.

Run	Time after treatment application				
	3 hours	1 day	2 days	5 days	10 days
	---------- Linear term $x\ 10^{-2}$ (µmol CO_2 m^{-2} s^{-1} ppm^{-1} C_a) ----------				
1	3.29b	4.71a	5.36a	6.20a	6.15a
2	4.87a	5.91a	5.92a	6.45a	6.35a

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.10. The influence Civitas formulation on the linear term of the model over the course during the two experimental runs in the Civitas formulation experiment.

Civitas Formulation	Run	
	1	2
	------- Linear term x 10^{-2} (µmol CO_2 m^{-2} s^{-1} ppm^{-1} C_a) -------	
Control	4.93a	4.77a
Civitas	3.82b	4.16a
Civitas One	2.97b	2.79b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.11. The influence of Civitas formulation quadratic term of the model over the course of 10 days in the Civitas formulation experiment.

Civitas Formulation	Time after treatment application				
	3 hours	1 day	2 days	5 days	10 days
	---------- Quadratic term x 10^{-5} (µmol CO_2 m^{-2} s^{-1} ppm^{-1} C_a^2) ----------				
Control	-4.05a	-2.50a	-4.12a	-3.88a	-3.87a
Civitas	-1.87a	-2.35a	-2.57a	-3.33a	-3.24a
Civitas One	-1.24a	-0.84b	-2.02a	-3.81a	-3.87a

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.12. Multiple regression effect tests for Civitas rate, photosynthetic photon flux density (*Q*), and Run in the Civitas dose rate experiment.

Source	df	p-value			
		R_d	k	A_{max}	E
Rate	1	*ns*	***	***	***
Run	1	***	***	***	***
Rate x Run	1	ns	ns	ns	*
Q	1	na	na	na	***
Rate x *Q*	1	na	na	na	ns
Run x *Q*	1	na	na	na	ns
Rate x Run x *Q*	1	na	na	na	ns

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 4.13. Parameter estimates for transpiration rate as affected by photosynthetic photon flux density (*Q*) and Civitas application rate in the Civitas dose rate experiment.

Term	Parameter estimate			
	R_d	k x 10^{-4}	A_{max}	E
Intercept	-18.9***	13.7***	45.1***	11.800***
Civitas rate†	0.014	0.1***	-0.506***	-0.114***
Run [1]	-4.4***	2.8***	11.2***	0.750***
Civitas rate x Run [1]	ns	ns	ns	0.057***
PPFD‡	na	na	na	0.003***

*** Significantly different than 0 at the 0.001 probability level.

† mL Civitas m^{-2}

‡µmol photosynthetically active radiation m^{-2} s^{-1}

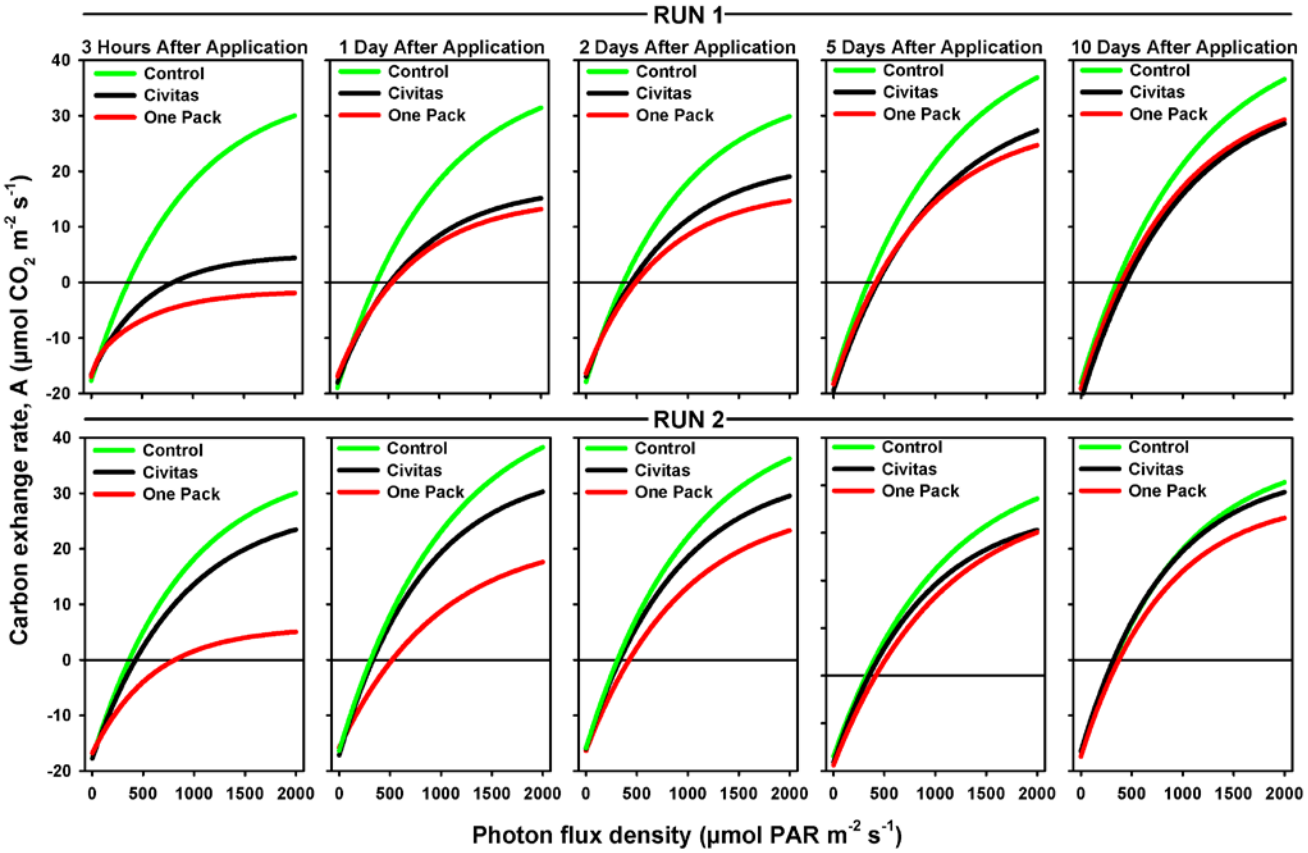

Figure 4.1. *A/Q* models output of Civitas formulation, days after treatment and run in the Civitas formulation experiment. These output curves represent the average response of four experimental units. R^2 values for individual curves were always greater than 0.9.

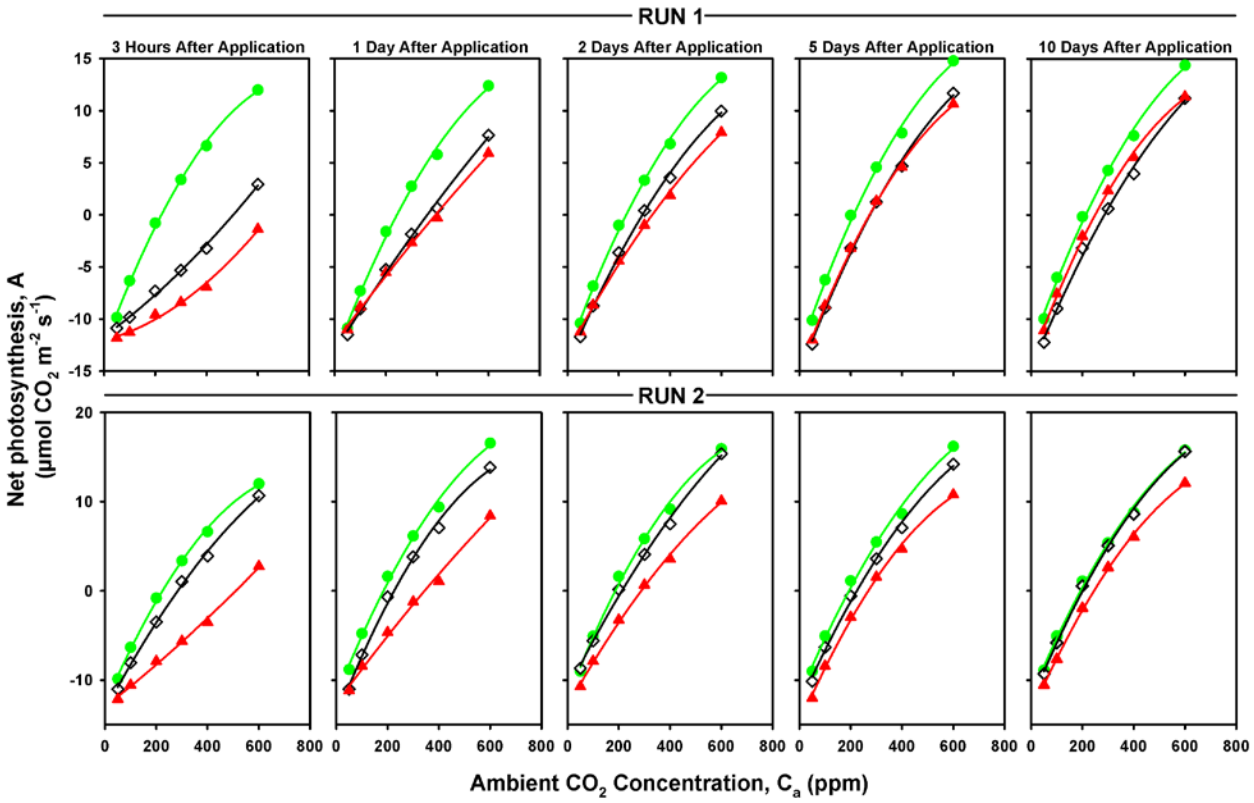

Figure 4.2. *A/C*$_a$ models output of Civitas formulation, days after treatment and run in the Civitas formulation experiment. These output curves represent the average response of four experimental units. R^2 values for individual curves were always greater than 0.9.

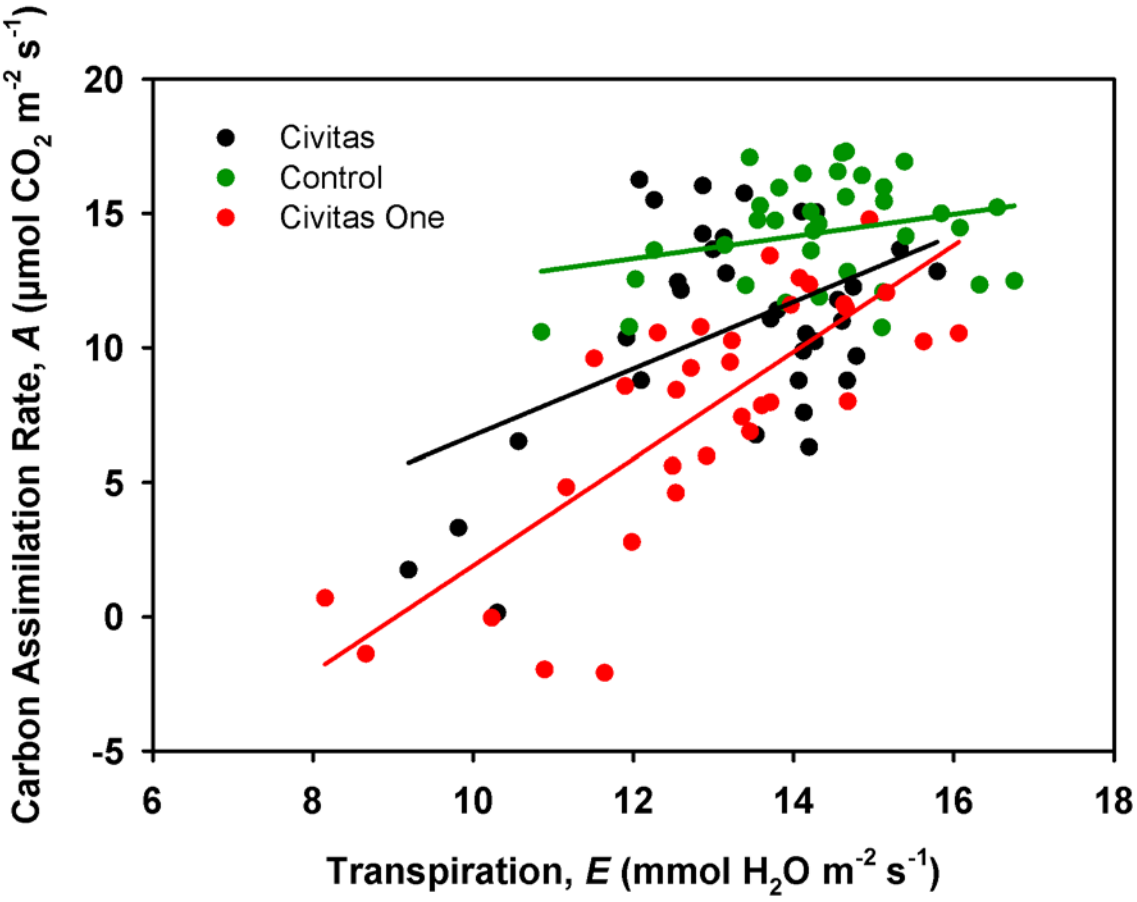

Figure 4.3. The effect of transpiration rate *(E)* on carbon assimilation rate (*A*). The Li-Cor was configured to sustain 30°C air temperature, 60% relative humidity, 600 ppm CO_2 air concentration, and photosynthetic photon flux density of 500 µmol PAR m^{-2} s^{-1} to provide a similar vapor pressure deficit for all measurements.

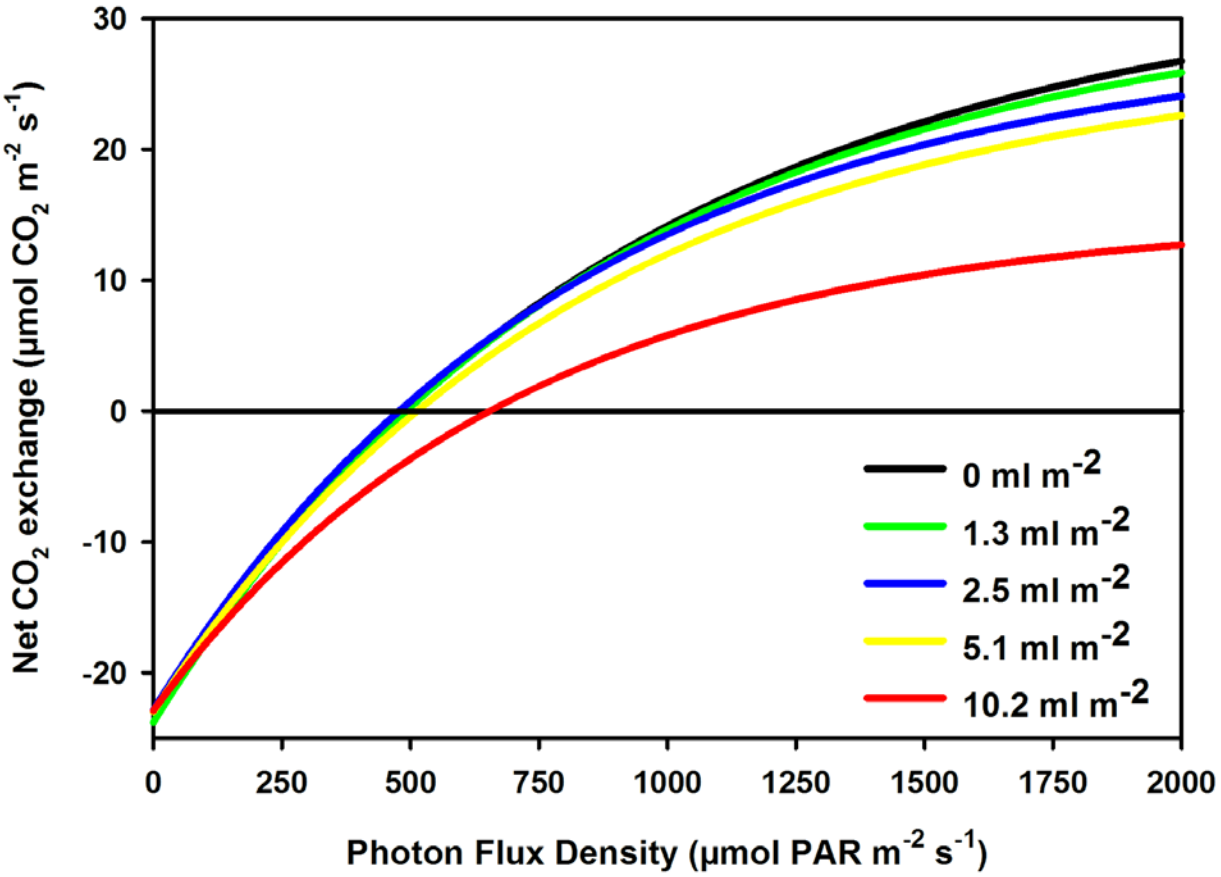

Figure 4.4. *A/Q* models output of response to Civitas dose rate. These output curves represent the average response of four experimental units. R^2 values for individual curves were always greater than 0.9.

Chapter 5: Civitas Alters Turfgrass Carbon Partitioning

ABSTRACT

Total nonstructural carbohydrates are considered a plant's energy currency. They provide the energy required for plants to survive and recover from environmental stress. Civitas™ is a petroleum-derived spray oil (PDSO) that is used in turfgrass management to induce plant systemic resistance. Prior research found that Civitas reduced carbon exchange rate (CER) for a period of 10 days. The objective of this research was to determine how Civitas affected carbon partitioning and turfgrass nonstructural carbohydrate status. Annual bluegrass (*Poa annua* L.) was treated with Civitas or an antitranspirant resin (Wilt Stop), which served as a positive control, every two weeks in a growth chamber. Environmental conditions were designed to provide high light intensity and daily water stress. Visual turfgrass quality rating, CER, and transpiration were measured 1, 2, 3, 4, 6, and 8 weeks after initial application (WAIT). Plants were harvested 2, 4, and 8 WAIT to quantify carbon partitioning and storage and soluble carbohydrates. Civitas and Wilt Stop reduced CER on all rating days. Civitas also reduced turfgrass quality rating shortly after the initial application while Wilt Stop had similar quality as the non-treated control plants until 8 WAIT. Civitas increased turfgrass dry clipping yield. Above ground verdure and root dry mass were not affected by either treatment. Chronic light and temperature stress increased the root to shoot ratio and reduced verdure water content regardless of treatment. Water soluble fructan content increased and starch content declined as the study progressed. Civitas consistently reduced fructan and starch content. Civitas also altered the composition of soluble sugars 8 WAIT compared to the control. Civitas treated plants had higher levels of glucose and fructose and lower levels of sucrose compared to the non-treated control. Civitas did not cause an accumulation of sorbitol or trehalose, carbohydrates

known to accumulate during water stress. These results indicate Civitas had a negative effect on storage carbohydrates and altered the way annual bluegrass responds to environmental stress.

INTRODUCTION

The PDSO Civitas is used in the turfgrass industry as a fungicide because it has been shown to induce systemic resistance (Cortes et al., 2010a, 2010b). Previous studies found PDSO Civitas increased turfgrass clipping yield and reduced photosynthesis and transpiration rate for greater than 10 days as a result of stomatal impedance; however respiration rate was not affected. These processes are known to reduced turfgrass carbohydrate status.

Most plants are photoautotrophic organisms that reduce CO_2 to complex carbohydrates in the presence of light through the process of photosynthesis. Carbohydrates produced by the plant are involved with plant structure, energy transfer and storage, osmoregulation, and ultimately affect plant growth and development (Taiz and Zeiger, 2006). Total nonstructural carbohydrates (TNC) are the total quantity of carbohydrate moieties that are not directly involved in the structural elements of plant tissue (Hull, 1992). They are composed of both storage carbohydrate polymers and water soluble mono- and disaccharides. Fructans and, to a lesser extent in C_3 grasses, starch are the prominent energy storage carbohydrates (Pollock and Cairns, 1991). Water soluble carbohydrates such as glucose, fructose, and sucrose provide energy for metabolic processes and are important for osmoregulation (Lee et al., 2008).

Total nonstructural carbohydrates have been described as the energy currency of plants (Hull, 1992). Although TNC quantity and composition can be dynamic, quantification of plant TNC content has historically been used to assess plant physiological status (Sheffer et al., 1979; Volenec, 1986; Hull, 1992; DaCosta and Huang, 2006). Higher levels of TNC content are associated with better environmental stress tolerance and increased recuperative potential. Ultimately, TNC content depends on the rate of photosynthesis, growth, and respiration. Environmental conditions or management practices that hinder photosynthesis or stimulate

growth and respiration have the potential to reduce TNC (Volenec, 1986; Xu and Huang; Narra et al., 2004).

The previous research reported in the prior chapters of this dissertation has demonstrated reduction in carbon assimilation which could have a dramatic effect on the vital need for carbohydrates. Therefore the objective of this project was to elucidate the effect of PDSO Civitas on turfgrass carbon partitioning as a result of stomatal impedance.

MATERIALS AND METHODS

Plant Establishment

Annual bluegrass (*Poa annua* L.) was established in a Percival WE-1012 growth chamber fitted with a mixed incandescent & fluorescent light source (Percival Scientific, Perry, IA) at Cornell University in Ithaca, NY. Conditions were designed to simulate conditions similar to natural weather conditions in Ithaca, NY during early summer. Specifically, plants received approximately 450 umol PAR m^{-2} s^{-1} for 16 h at 30°C and 50% relative humidity and 8 h of darkness at 20°C and 70% relative humidity.

Ray Leach SC10 3.8 x 210 cm cone-tainers (Stuewe & Sons, Tangent, OR) were filled and packed with non-amended medium textured sand meeting USGA specifications for putting green construction (USGA, 2004). Approximately 10 seeds of annual bluegrass were placed in cones that were filled to 15mm with sand below top of cone to allow for uniform clipping. Cones were fertilized weekly with 10 mL of a fertilizer solution containing 200, 50, 200, and 1 ppm N, P, K, and chelated Fe, respectively for the first ten weeks of establishment. After establishment, cones received 8 mL of the fertilizer mix every two weeks. Cones were clipped several time wk^{-}

[1] and were watered daily to prevent drought stress with tap water. Cones were fully established and deemed ready for experiments twelve weeks after seeding.

Supraoptimal environmental conditions for cool-season turfgrass were used to assess the effect of PDSO Civitas on carbon partitioning during stressful environmental conditions. Once plants were established they were transferred to a Percival Scientific growth chamber outfitted with a mixed high pressure sodium and metal halide light source that increased PPFD to approximately 950 µmol PAR m^{-2} s^{-1} at bench top level. All other environmental controls were similar to those described during establishment.

Experimental Design

The experiment was a completely randomized design with four replicates and was repeated. Treatments included Civitas applied at 5 mL m^{-2}, an anti-transpirant positive control plant derived resin (PDR) (Wilt-Stop) applied at 8 mL m^{-2}, and a water negative control. The PDR application rate was experimentally determined to provide similar CER rate as the Civitas treatment (data not shown). Treatments were applied every two weeks with a CO_2 powered backpack sprayer equipped with a TeeJet AI 8004 nozzle (TeeJet Technologies, Wheaton, IL) calibrated to deliver 81 mL m^{-2} at 275kPa. All cones were sorted into a single line to ensure each cone was 50cm beneath the nozzle tip. To allow for multiple harvests during each eight week run, 36 cone-tainers of turf were used in each run. Sixteen cones were treated with PDSO Civitas, PDR, or water. Four cones were then harvested from each treatment 2, 4, and 8 weeks after initial application of treatments (WAIT).

Data Collection

Visual turfgrass quality rating was assessed 1, 2, 3, 4, 6, and 8 WAIT. Visual turfgrass quality rating is a visual composite rating accounting for factors such as color, canopy density,

and surface uniformity (Skogley and Sawyer, 1992). It was rated on a 1 to 9 scale where 1 represents completely dead turf and 9 represents perfect turfgrass quality. Values greater than 6 were deemed minimally acceptable turfgrass quality. Ratings were only taken from the cones designated for harvest 8 WAIT.

Prior to harvest, CER and transpiration were measured with a Li-Cor 6400XT photosynthesis meter with 6400-17L Lighted Whole Plant *Arabidopsis* Chamber (Li-Cor, Lincoln, NE). The chamber was configured to provide constant 65% relative humidity at 30°C, 400ppm CO_2, and 950 µmol PAR m^{-2} s^{-1} PPFD. The Li-Cor 6400 adjusted flow rate to maintain constant relative humidity; approximately 500 µmol air s^{-1}. The PPFD and CO_2 concentration roughly approximated conditions at bench height in the growth chamber. Immediately following gas exchange measurement, clippings were collected by clipping the turf flush with the top of the cone-tainer. The remaining turf tissue and sand media was extracted from the cone-tainer, the verdure was cut away from the sand and weighed wet. 500 mg of wet verdure tissue was frozen in liquid N_2 for nonstructural carbohydrate analysis while the remaining tissue was dried at 65°C for 48 h. Relative water content and total dry mass were calculated by as:

$$Water\ content = 1 - \left(\frac{\text{Dry mass of sample}}{\text{Verdure wet mass} - \text{mass of subsample}}\right)$$

$$Total\ dry\ verdure\ mass = Verdure\ wet\ mass\ x\ (1 - water\ content)$$

The sand root zone media was gently shaken from the roots. Root tissue was then carefully washed under a stream of tap water and dried at 65°C for 48 h. Dry roots were then

placed into a crucible, weighed with an analytical balance, and ashed at 450°C for four hours in a muffle furnace. Root mass was calculated as:

$$Root\ mass = Preashed\ mass - Post\ ashed\ mass$$

Nonstructural Carbohydrate Analysis

The frozen subsamples of tissue were freeze dried for 48 h at -40°C and 0.013 kPa or less. Samples were allowed to return to room temperature in the presence of the vacuum to avoid condensation which could rehydrate the tissue. Dried tissue was then passed through a Wiley mill (Thomas Scientific, Swendesboro, NJ) in preparation for carbohydrate extraction.

Carbohydrates were determined with the methods described by Ranwala and Miller (2008). Soluble carbohydrates were extracted from 50 mg of dried tissue using three sequential extractions with 3 mL of 80% ethanol for 30 minutes at 70°C. The tissue suspensions were then centrifuged at 3,000g for 10 minutes and the supernatants were poured into clean test tubes (20 x150 disposable culture tubes, VWR International, Radnor, PA). 100 µg of lactose was added to the initial extractions to serve as the internal standard. The combined supernatants were passed through ion exchange columns consisting of 1 ml of Amberlite IRA-67 (acetate form) and Dowex 50W (hydrogen form, Sigma-Aldrich, St. Louis, MO) to remove charged material. Samples were then evaporated to dryness with a Rotary Evapo-Mix (Buchler Instruments, Fort Lee, NJ) at 60°C and re-dissolved with 10 mL Milli-Q ultrapure water (EMD Millipore, Billerica, MA).

Water soluble fructans were then extracted from the dried pellets using three sequential extractions with 3 mL of Milli-Q ultrapure water at 80°C for one h. 100 µg of xylitol was added

to the initial extractions to serve as the internal standard for the water extractions. The tissue suspensions were centrifuged, combined, and filtered through the ion exchange columns described above. The resulting filtrates were diluted to 15 mL with Milli-Q water, and hydrolyzed with equal parts 0.5 M HCl at 100°C for 5 min. The solutions were then neutralized with 0.5 M NaOH.

Samples were analyzed with high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) using a Dionex ICS-5000 series chromatograph with Carbopac PA-1 guard column (4 x 50 mm) and analytical column (4 x 250 mm), pulsed amperometric detector, and gold electrode (Dionex, Sunnyvale, CA). The HPAE-PAD was configured for a 25 µL injection volume. Carbohydrates were eluted in 200 mM NaOH at 1.0 ml min^{-1} for 20 min. Standard curves were created for glucose, fructose, sucrose, myo-inositol, sorbitol, trehalose, and the internal standards (Sigma Aldrich, St. Louis, MO) to quantify carbohydrate content.

Starch was extracted from the pellets following the water extraction of fructans based on the methods of Ranwala and Miller (2008). Pellets were re-suspended in 100 mM Na-acetate buffer (ph 4.5) and incubated at 100°C for 1 h. After the suspension cooled to approximately 25°C, 50 units of amyloglucosidase (Sigma Aldrich) were added to the samples. Samples were incubated for 2 d at 55°C. The resulting starch digests were then centrifuged at 3,000g for 10 minutes and 100 µL of the supernantants were added to a clean 13x100 glass culture tube (VWR International, Radnor, PA). A solution containing 5 units of glucose oxidase, 1 unit of peroxidase, and 0.04 mg of *o*-dianisidine mL^{-1} was prepared on ice. 5 mL the solution was added to each starch digest and glucose standards. Tubes were mixed and incubated for 30 min at 30°C. Following incubation, color development was stabilized with 1 mL 2.2 HCl and

absorbance was read in a spectrophotometer at 450 nm. To calculate mg starch g^{-1} dry tissue, µmol of glucose $digest^{-1}$ was calculated from the standard curve. After accounting for the dilution factor and beginning tissue dry mass, µmol of glucose was converted to mg glucose g^{-1} tissue. The resulting value was then multiplied by 0.9 to account for the mass of the water added during starch hydrolysis.

Statistical Analysis

Visual turfgrass quality ratings, water content, plant biomass, CER, transpiration rate, and carbohydrates were subjected to ANOVA. Run was treated as a random variable in the model and was typically 5% or less of total variance. All biomass data was square root transformed and sucrose content data were log transformed to satisfy the assumption of equal variance. Means were separated with Fisher's Protected LSD (α=0.05) when appropriate.

RESULTS

At the end of plant establishment the cone-tainers had a uniform visual quality rating of 8 (1 to 9 scale). Visual turfgrass quality rating declined as the experiment progressed (Table 5.1, 5.2) independent of treatment suggesting plants were under supraoptimal growing conditions and experiencing stress. Leaf chlorosis was the predominant cause of the decline in quality and several cone-tainers of turf died 8 WAIT.

There was a significant Treatment x WAIT interaction for visual turfgrass quality rating (Table 5.1). Visual turfgrass quality rating was highest 2 WAIT for all treatments. The control treatments remained near the limits of minimal acceptable quality all eight weeks with a mean average visual turfgrass quality of 6.1 (Table 5.2) suggesting plants were under stress. The positive control, PDR Wilt Stop, had similar visual turfgrass quality to the water control until the

8 WAIT when quality dramatically declined. The PDSO Civitas treatment reduced turfgrass quality below the level of the control on three of the last four rating dates. Unlike PDR Wilt Stop that had acceptable turfgrass quality until replicates began to die, replicates treated with PDSO Civitas had uniform chlorosis across all replicates but only one PDSO Civitas replicate died 8 WAIT during the second run.

The PDSO Civitas and PDR Wilt Stop reduced CER while only PDR Wilt Stop reduced transpiration compared to the control (Table 5.3). The PDR Wilt Stop and PDSO Civitas had statistically similar CER and transpiration rate during the two eight week runs. Carbon exchange and transpiration rates increased during the first four weeks before a sharp decline 6 WAIT. There was not a significant Treatment x WAIT interaction indicating transpiration was inhibited with PDR Wilt Stop and PDSO Civitas during the entire study.

Clipping biomass was affected by treatment (Table 5.4). The PDSO Civitas increased clipping yield while PDR Wilt Stop did not. Treatments did not affect verdure, verdure water content or root biomass. However, changes in total biomass during the study mirrored CER, transpiration rate, and visual turfgrass quality rating, suggesting an intimate link between carbon fixation and utilization. Clipping biomass was greatest 4 WAIT when CER was greatest. Total biomass declined from 76.63 to 53.09 g dry tissue m^{-2} between the four and eight week harvest (Table 5.4).

Verdure biomass declined as the study progressed while root dry biomass increased and then declined (Table 5.4). Carbon partitioning between clippings, verdure, and root biomass changed from the 2, 4, and 8 week harvests. The root to shoot ratio dramatically increased as the study progressed; 0.5, 1.0, and 1.7 at 2, 4, and 8 WAIT, respectively. Verdure water content also

declined from 71% 2 WAIT to 50% 8 WAIT which is common in the drought literature (Table 5.4).

Leaf starch and water soluble fructan content were inversely related as the experiment progressed (Table 5.5). Starch content was greatest 2 WAIT and declined over the course of each experimental run. Conversely, water soluble fructans increased over the course of 8 weeks. The PDSO Civitas treatments had less leaf starch and water soluble fructan content than the control while the PDR Wilt Stop did not reduce starch or fructan content relative to the control (Table 5.5).

There was a significant Treatment x WAIT interaction for all soluble sugars except myo-inositol which declined rapidly from 4 to 8 WAIT (Table 5.6). Treatment differences did not occur until 8 WAIT (Fig. 5.1). Leaf fructose content rapidly declined from 4 to 8 WAIT. The control plants had the most dramatic decline in leaf fructose content on week 8. Civitas treatments resulted in greater fructose than the control 8 WAIT. Leaf glucose content declined in the control 8 WAIT but not for the Civitas and Wilt Stop treatments. Sucrose and trehalose content increased as the experiment progressed for the control and Wilt Stop treatments but not the Civitas treatment. Finally, leaf sorbitol content increased from 4 to 8 WAIT in all treatments. Wilt Stop sorbitol content 8 WAIT was significantly higher than the control and Civitas treatment which were the same.

DISCUSSION

Annual Bluegrass Response to Multiple Stresses

The 16 h photoperiod, high PPFD, and supraoptimal air temperature resulted in significant reductions in all measured parameters independent of treatment suggesting the

imposition of stress. Furthermore, visual quality ratings declined dramatically when plants moved from the establishment chamber to the high light chamber. Supraoptimal levels of temperature and light are known to cause photorespiration and chronic photooxidative stress (Taiz and Zeiger, 2006). Both processes reduce photosynthetic efficiency and lead to the production of damaging reactive oxygen species (ROS) which cause damage to plant lipids, proteins, pigments, and DNA (Polle, 1997).

The dramatic decline in visual quality ratings from time 0 to 1 WAIT likely resulted from the abrupt change in light intensity. Visual quality ratings of the control plants were largely unchanged after the decline during the first week. Plants adapt to changes in PPFD over the course of seconds to days via photoprotection (Demming and Adams, 1992; Niyogi, 1999). Excess light reduces thylakoid pH and cell redox state, increases production of ROS, and leads to the accumulation of pigment metabolites. These responses activate signal cascades which results in nonphotochemical quenching (qE), chloroplast avoidance, and changes in gene expression (Demming and Adams, 1992; Niyogi, 1999). These photoprotective responses likely prevented a further decline in visual turfgrass quality rating during the remainder of the experiment.

The increased CER during the first four weeks support plant acclimation to the supraoptimal temperature and higher PPFD, i.e., as the plant acclimated, radiation use efficiency increased. Clipping yield was also greatest 4 WAIT which corresponded with the high CER. Similar relationships observed in other crops. For example, maize biomass is directly related the radiation use efficiency (Lindquist et al., 2005).

Despite daily irrigation in excess of field capacity, there was significant evidence of water stress during the study. Each cone-tainer had an average of 20 mm of plant available soil

water. Although evapotranspiration was not measured directly in this study, it is possible that plant available soil water declined enough to elicit drought responses at the end of the 16 h photoperiod from high evaporative demand. Furthermore, increased root-to-shoot ratio, reduced relative water content and transpiration rate, and accumulation of sucrose have been associated with drought avoidance and tolerance (Kameli and Lösel, 1993; DaCosta and Huang, 2006; Zang et al., 2013).

Accumulation of soluble carbohydrates is known to confer increased drought tolerance (Julander, 1945). Kameli and Lösel (1993) found that glucose and fructose content increased more dramatically than sucrose in wheat when exposed to drought. Additionally, Kentucky bluegrass (*Poa pratensis*) cultivars with high levels of glucose, fructose, and sucrose accumulation during drought were found to have better drought tolerance (Huang and Gao, 1999; Huang and Fu, 2000, 2001; Fu and Dernoeden, 2008; DaCosta and Huang, 2006; Yang et al., 2013). In fact, Yang et al. (2013) suggested sucrose accumulation is more important in Kentucky bluegrass.

In this study, glucose, fructose, and myo-inositol levels declined dramatically 8 WAIT while sucrose, sorbitol, and trehalose increased. Sorbitol and trehalose are known to be involved with cell osmoregulation and stress signal transduction (Loescher, 1987; Garg et al., 2002). Accumulation of sorbitol and trehalose is common when plants are under water stress. The decline in glucose and fructose is interesting. Research in forage grasses has found that glucose and fructose content increase rapidly during the onset of water stress (Kameli and Lösel, 1993). Yang et al. (2013) found that Kentucky bluegrass responded to drought with a rapid increase in sucrose. Synthesis of sucrose for soluble glucose and fructose moieties like explain changes in sugar content. The observed increase in fructan content and reduced starch content is consistent

with the literature which suggests plants accumulate fructans during stress to provide energy for regrowth after the stress subsides (Busso et al. 1990; Da Silva and Arrabaca, 2004; Harb et al., 2010).

Impact of Civitas and Wilt Stop on Annual Bluegrass

There were significant differences in the visual quality ratings between PDSO Civitas and PDR Wilt Stop. Specifically, PDSO Civitas caused leaf chlorosis but was rarely fatal while PDR Wilt Stop had similar visual quality to the control until 8 WAIT when quality dramatically declined and plants in some cone-tainers died. It was hypothesized that PDSO Civitas and PDR Wilt Stop would have had the same effect on visual quality rating because both inhibited gas exchange by the same amount. However the more dramatic effect of PDSO Civitas on annual bluegrass physiology suggests the plant response to PDSO Civitas more than simply inhibiting gas exchange.

There was a significant difference in clipping yield between the PDSO Civitas and PDR Wilt Stop. The PDSO Civitas increased clipping yield in this study, specifically PDSO Civitas increased leaf elongation rate. This increase in leaf elongation has not been replicated when turf was treated with other horticultural oils or antitranspirants (unpublished data). It still is unclear why Civitas increases leaf elongation rate. We hypothesized that reduced gas exchange may increase leaf water potential and accelerate leaf elongation rate. This theory is based on the work of Ehlert et al. (2009). However, lack of a clipping response with other products that reduce transpiration disproves that hypothesis.

The decline in storage carbohydrates with PDSO Civitas and PDR Wilt Stop was expected since neither treatment affected verdure or root biomass yet reduced CER. The PDSO Civitas tended to have more of a detrimental effect on storage carbohydrates than PDR Wilt

Stop. The decline is likely required to sustain the high level of clipping yield. The decline in storage carbohydrate suggests Civitas diminished plant's recuperative potential. Grass under environmental stress accumulates carbohydrates to both tolerate and recover from periods of high environmental stress (DaCosta and Huang, 2006). A reduction in this carbohydrate pool directly limits energy available for maintenance respiration and regrowth.

The PDSO Civitas had less of an effect on accumulation of soluble carbohydrates. Accumulation in soluble carbohydrates is an essential mechanism to resist cell dehydration (Lee et al., 2008). Turfgrass species or cultivars with higher carbohydrate accumulation during drought stress typically have better quality during and after water stress (Kameli and Lösel, 1993; DaCosta and Huang, 2006, Lee et al., 2008; Yang et al., 2013). Therefore, Civitas may have either reduced chronic daily drought stress or reduced the plant's ability to respond to drought. The visual quality of the control and Wilt Stop treatments were generally better than the Civitas treatments. A comparison of quality rating with soluble carbohydrates content suggests Civitas may hinder drought acclimation and may have caused the in the decline in turfgrass quality. More experiments are required to understand how Civitas affects plant drought resistance.

CONCLUSIONS

Treatment of annual bluegrass c.v. 'DW-184' with supraoptimal temperatures and high PPFD reduced turfgrass quality until the plants acclimated to the conditions over the course of the first week. Plants increase in root-to-shoot ratio and nonstructural carbohydrates in response to the stressful conditions. It is not known if unimproved ecotypes of annual bluegrass would respond similarly. Application of Civitas reduced storage carbohydrate content while clipping

yield was enhanced. Reduced TNC levels are generally associated with lower plant stress tolerance and recuperative potential. There was also some evidence that Civitas reduced plant drought resistance. Further research is required to determine how Civitas affects stress tolerance.

TABLES AND FIGURES

Table 5.1. Visual Turfgrass Quality ANOVA.

Source	df	F-ratio
Treatment (T)	2	6.89**
Weeks after initial treatment (WAIT)	5	13.48***
T x WAIT	10	2.32*

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 5.2. Visual turfgrass quality as affected by treatment over the course of eight weeks.

Treatment	Weeks after initial treatment						Mean
	1	2	3	4	6	8	
Control	6.0a†	6.4b	6.4a	6.1a	5.8a	6.3a	6.1a
Wilt Stop	6.0a	6.7a	6.4a	6.1a	5.8a	5.5b	5.9ab
Civitas	6.0a	6.3b	6b	5.3b	5.4a	5.8b	5.8b

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 5.3. Turfgrass exchange rate (CER) and transpiration rate as affected by treatment over the course of eight weeks.

Treatment (T)	df	CER	Transpiration
		µmol CO_2 m^{-2} s^{-1}	mmol H_2O m^{-2} s^{-1}
Control		10.3a†	14.8a
Wilt Stop		8.9b	14.1b
Civitas		8.5b	14.4ab
Weeks after initial treatment (WAIT)			
1 weeks		8.1c	13.5c
2 weeks		10.8b	16.9a
3 weeks		9.9bc	15.4b
4 weeks		16.9a	16.0b
6 weeks		5.5d	12.5d
8 weeks		4.2d	12.3d
	ANOVA		
T	2	*	*
WAIT	5	***	***
T x WAIT	10	ns	ns

* Significant at the 0.05 probability level.

*** Significant at the 0.001 probability level.

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 5.4. Turfgrass dry biomass and relative water content as affected by treatment over eight weeks.

Treatment (T)	df	Clippings	Verdure	Roots	Total	Water content
		----------g dry mass m^{-2}----------				$g\ g^{-1}$
Civitas		2.74a†	30.05ns	31.83ns	67.17ns	0.67ns
Wilt Stop		1.63b	33.92	31.74	69.42	0.65
Control		1.55b	31.92	32.45	68.46	0.72
Weeks after initital treatment (WAIT)						
2 weeks		0.92b	49.92a	26.36c	78.38a	0.71b
4 weeks		4.67a	32.10b	37.87a	76.63a	0.82a
8 weeks		1.11b	18.39c	33.03b	53.09b	0.50c
			ANOVA			
T	2	**	ns	ns	ns	ns
WAIT	2	***	***	***	***	***
T x WAIT	4	ns	ns	ns	ns	ns

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 5.5. Turfgrass storage carbohydrates as affected by treatments over eight weeks.

Treatment (T)	df	Starch	Water soluble fructans
		-----mg g^{-1} dry leaf mass-----	
Control		15.8a†	111.8a
Wilt Stop		15.8a	103.4ab
Civitas		14.1b	91.6b
Weeks after initial treatment (WAIT)			
2 weeks		16.4a	94.2b
4 weeks		15.8a	99.1b
8 weeks		13.5b	113.4a
	ANOVA		
T	2	*	*
WAIT	2	**	*
T x WAIT	4	ns	ns

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

Table 5.6. Turfgrass soluble carbohydrate content ANOVA.

Source	df	F-ratio					
		Myo-insolitol	Sorbitol	Trehalose	Glucose	Fructose	Sucrose
Treatment (T)	2	3.1	3.2*	2.1	5.1**	0.9	32.7***
Weeks after initial treatment (WAIT)	2	117.4***	37.5***	13.2***	13.3***	86.7***	99.6***
T x WAIT	4	1.5	3.1*	5.9***	3.2*	3.2*	21.2***

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

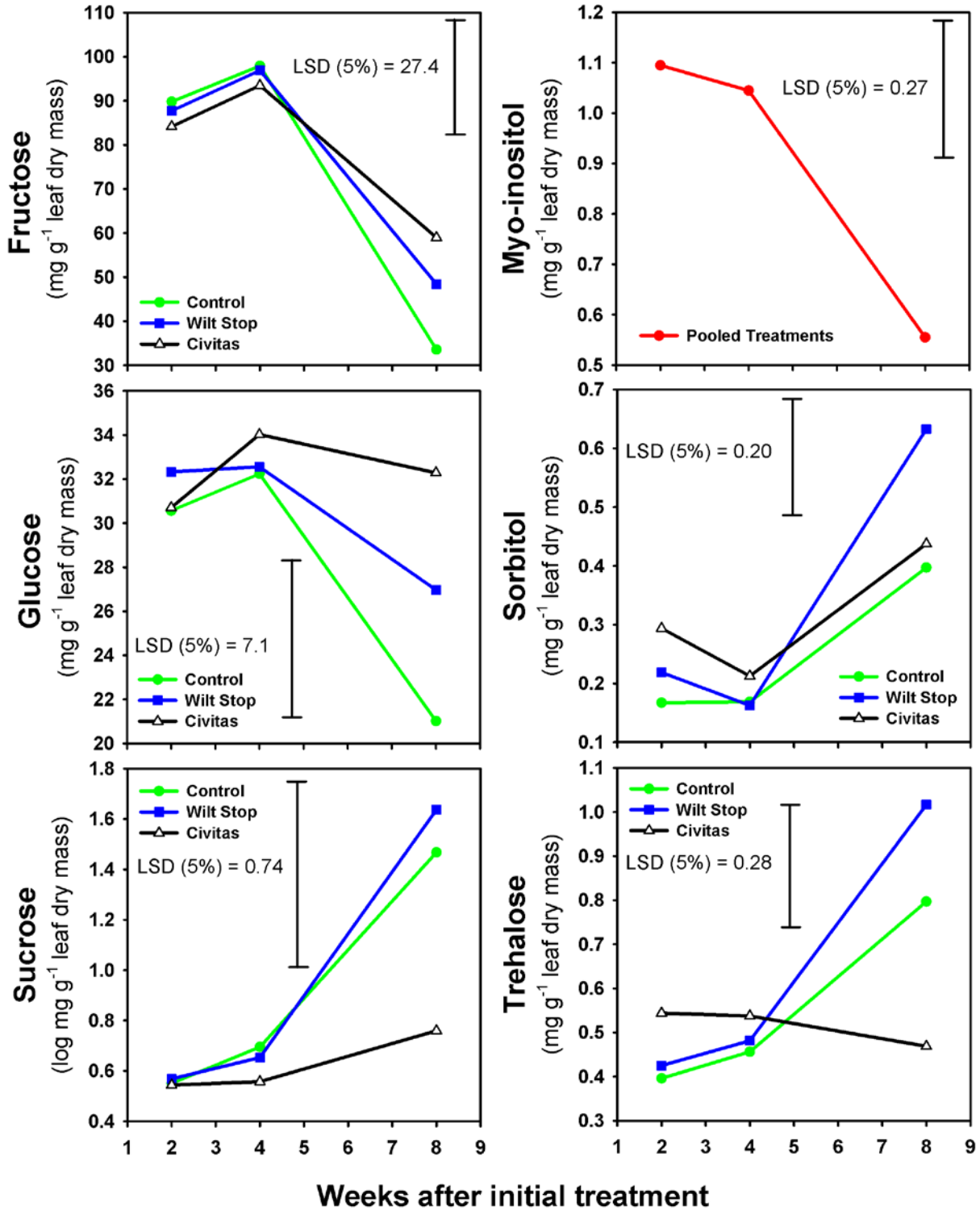

Figure 5.1. Civitas and Wilt Stop affect annual bluegrass leaf carbohydrate content.

Chapter 6: Dissertation Summary and Future Research

The petroleum derived spray oil (PDSO) Civitas has gained moderate acceptance by golf course superintendents. It's ability to reduce turfgrass fungicide and insecticide requirements save time and money and reduce the environmental impact. Initially, widespread adaptation of PDSO Civitas was limited, however, because research regarding the impact of Civitas on turfgrass physiology was lacking. The work of this dissertation studied the impact Civitas on various plant processes. While the results answer many questions, they have highlighted various areas of study that need to be evaluated in the future.

Field evaluations of both the PDSO Civitas and the pigment Harmonizer yielded interesting results. Civitas caused phytotoxicity when applied every two weeks at the high application rate. The phytotoxicity was mitigated and then simply masked by the Harmonizer. The potential for phytotoxicity has cause golf course superintendents to reduce Civitas application rate. Growth chamber studies showed evidence of stomatal impedance caused which was application dose rate dependent and likely caused oxidative stress. Mild levels of environmental stress are known to precondition plants for environmental stress. Mild levels of oxidative stress after Civitas application may be necessary to prime plant defense. Future research is needed to optimize Civitas application rate. The ideal application rate would induce plant systemic resistant yet have minimal impact on leaf gas exchange processes and may vary depending on mowing height and leaf area index.

Turfgrass clipping yield commonly increased immediately following application. Although some of the increase in yield may be attributed to the mass of the oil on the leaf surface, time-lapse imagery illustrated increased leaf elongation. This response is interesting because products that initiate plant defenses typically reduce yield as carbon resources are

partitioned from growth processes to defensive processes. The carbon partitioning study found that carbon was partitioned from green verdure to drive increased clipping production. Quantification of plant hormones, such as gibberellins, may provide some insight on increased growth rate. Transcriptome profiling with technologies such as RNA-seq may provide a more robust.

Finally, there was evidence that Civitas altered plant drought tolerance compared to the control. Future study of environmental stress tolerance is required to understand how changes in leaf carbohydrate content affect turfgrass performance under drought, salinity, and temperature stress. The experiments in this dissertation indicate Civitas has a profound impact on various plant processes. As our understanding of Civitas increases, acceptance of Civitas by the industry should increase which has the potential to reduce pesticide applications on turfgrass systems.

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