INVESTIGATION OF ICE ENCASEMENT SURVIVAL MECHANISMS AND WINTER PREPARATORY MANAGEMENT STRATEGIES IN ANNUAL BLUEGRASS

By

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ABSTRACT

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Annual bluegrass (*Poa annua* L) is a golf course putting green turfgrass species, often in a mixed stand with creeping bentgrass (*Agrostis stolonifera*). Turfgrasses in the transition zone and north often must endure ice encasement or freezing injury during winter months. Annual bluegrass is more susceptible to ice encasement damage than creeping bentgrass. In this thesis, we describe experiments conducted to evaluate applied methods to alleviate ice encasement stress of annual bluegrass through the use of plant growth regulating compounds or protective chemicals. To understand plant physiological responses to chemical treatment and abiotic stress treatments we evaluated parameters such as turf quality, normalized difference vegetative index, fatty acid profiles, and other stress indicators for each experiment.

In the first experiment, chemical applications of various plant protectants and plant growth regulators positively influenced annual bluegrass survival of ice encasement and low temperature. Civitas, mefluidide and propiconazole treatments increased annual bluegrass regrowth following prolonged ice encasement and may have been related to beneficial shifts in fatty acid ratios to favor unsaturated fatty acid content. It was not clear whether the most commonly used plant growth regulator, trinexapac-ethyl, altered winter survival. Some decreases in survival were found due to trinexapac-ethyl treatment but not in a consistent manner.

In the second and third experiment, the phytohormone ethylene was explored in its role in survival of annual bluegrass after ice encasement and low temperature treatment. Ethephon treated annual bluegrass plants were found to have decreased regrowth under ice and non-ice-

covered conditions. Treatment of annual bluegrass with the ethylene inhibitory product ReTain, increased annual bluegrass regrowth after ice encasement and low temperature treatment. Effective ethylene applications of ethephon to annual bluegrass decreased regrowth after ice encasement and low temperature treatment. Our research further shows that ethephon treated annual bluegrass increased lipid peroxidation, saturated fatty acid content, and decreased antioxidant activity when compared to untreated annual bluegrass after winter conditions. Ethylene inhibition treatment of annual bluegrass resulted in increases in apoplastic proteins, antioxidant activity, and plant cell membrane unsaturated fatty acid contents when compared to untreated annual bluegrass. In conclusion, annual bluegrass treated with chemical plant protectants or ethylene inhibitory treatments such as propiconazole, mefluidide, Civitas or ReTain can be beneficial to aid in recovery after winter conditions. Annual bluegrass treated with trinexapac-ethyl or ethephon could potentially be detrimental to turfgrass recovery following ice encasement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ABG: annual bluegrass;

FA: fatty acid;

TE: trinexapac-ethyl;

ACC: 1-aminocyclopropane-1-carboxylic acid;

AVG: aminoethoxyvinyl glycine;

NDVI: normalized difference vegetation index;

TQ: turf quality

PGR: plant growth regulator

CHAPTER 1

WINTER ACCLIMATION AND ICE STRESS RESPONSES

Introduction

Turfgrass species commonly used on golf course putting greens are susceptible to various winter related injuries such as low temperature stress, freezing stress, ice cover, and spring freeze-thaw related injuries. Warm-season grasses, or those primarily grown in southern regions of the U.S. (C4 photosynthetic pathways), can be particularly sensitive to winter stresses if atypical winter conditions occur. Despite their evolution in more temperate climates, cool-season grasses (C3 photosynthetic pathway) also experience winter related injury as these turfgrasses are under strict management, are subjected to intensive cultural practices, and are thought to be metabolically stressed even under optimal environmental conditions (Bell, 2011). The cool-season putting green species, primarily creeping bentgrass (*Agrostis stolonifera*) and annual bluegrass (*Poa annua*), differ significantly in their inherent ability to tolerate different winter related stresses, particularly during prolonged ice cover. Annual bluegrass is more susceptible to ice cover damage than creeping bentgrass (Beard, 1964). In addition to genetically regulated tolerance, the environment of the turfgrass or how each turfgrass species is managed may play a major role in determining winter survival of each species. Therefore, this chapter outlines winter survival mechanisms plants possess naturally and current man-made strategies than can be used to promote winter survival. As annual bluegrass is particularly sensitive to ice cover, this will be the primary winter stress discussed.

Effects of ice cover on turfgrasses

Turfgrass putting greens can be subjected to prolonged periods of ice cover in many northern or temperate regions of the world. Ice cover can be impermeable or permeable to gases. Prolonged periods of impermeable or non-porous ice can readily cause anoxic conditions and buildup of toxic gases. Turfgrasses growing underneath of this ice will experience crown necrosis or crown hydration injury (McKersie and Leshem, 1995; Andrews, 1977). As crowns are the essential overwintering structure for perennial cool-season turfgrass putting green species, loss of crowns will negate any regrowth in the spring. The primary cause of death to turfgrass under impermeable ice sheets is most likely from oxygen depletion, buildup of carbon dioxide, and toxic gas accumulation such as ethyl butyrate (Aamlid et al., 2009). Anoxic conditions have been found to be exacerbated by soils with high respiration rates related to higher organic matter content in experiments with impermeable covers (Rochette et al., 2000). Chemicals such as ethyl butyrate can cause electrolyte leakage and membrane dysfunction (Andrews and Pomeroy, 1989). Experiments done with high concentrations of butyrate and ice encasement on orchard grass show decreased establishment after injury occurred from winter (Brandsaeter et al., 2005). Impermeable sheets of ice are the most damaging to ice cover sensitive turf species such as annual bluegrass. Research is needed to elucidate methods that may promote crown protection and annual bluegrass survival of prolonged impermeable ice conditions.

Permeable or porous ice is typically not as detrimental to turf health when compared to impermeable sheets of ice. Porous ice usually occurs when top layers of snow melt and freeze into snow underneath. Porous ice allows for air movement between the environment and the turfgrass canopy, reducing the incidence of gas accumulation that could cause necrosis of crown

tissue (Olien and Smith, 1981). Uneven turfgrass surfaces can allow for differing depths of ice accumulation resulting in thicker ice formations, which can cause more intense injury (Andrews and Pomeroy 1975). However, ice encasement does not mean intense injury to putting green will occur. Unsaturated soils have been shown to allow for gas exchange from leaf tissue under ice encasement, making it less lethal to putting greens (Andrews, 1996).

Cold acclimation

A major way that plants prepare for any winter related stress, including ice cover, is through acclimation. Acclimation to winter is induced by the reduction in photoperiod and reduction in the day/nighttime temperatures (Atkin and Tjoelker, 2003; Strand et al., 1997). Temperatures ranging from 0 – 12 °C are common for cool-season turfgrasses to acclimate prior to winter (Limin and Fowler, 1985; Guy, 2003; Levitt, 1980). However, there is no set temperature or length of time under cold conditions that will guarantee that acclimation has fully occurred. It is also known that conditions to acclimate can change year to year within a single plant depending on environmental conditions (Gay and Eagles, 1991). Despite yearly variation, a significant amount of research has been performed to better understand specific processes associated with plant acclimation, important processes or mechanisms include photosynthesis and respiration changes, carbohydrate allocation, plant water relations, shifts in membrane composition (fatty acids), hormone responses, and other metabolite induced responses.

Photosynthesis and respiration

Cold acclimation involves changes in a plant's photosynthesis and respiration rates, which may occur differentially in different plant tissues. Both photosynthesis and respiration are temperature sensitive (Berry & Bjbrkman, 1980; Loveys et al., 2003; Atkin et al., 2006). In plant leaves subjected to lower temperatures for 24 to 48 hours, respirations rates increased during

cold acclimation mainly through the relatively low concentrations of reduced ubiquinone that can saturate respiratory flux (Atkin and Tjoelker, 2003). The increase in soluble sugars in leaves possibly could cause a feedback inhibition mechanism in photosynthesis (Hurry et al., 1993; Strand et al., 1997). This in turn allows for the down regulation of photosynthetic gene expression (Strand et al., 1997; Hurry et al., 2000). If cold stress occurs for too long of a period, photosynthetic damage can occur. At low temperatures, consumption of ATP and NADPH by the Calvin cycle declines, which results in reduction of the electron transport chain causing a buildup of free radicals and oxidative damage (Somersalo and Krause, 1988; Hurry and Huner, 1992). Thus, photosynthesis rates decrease faster during cold acclimation when compared to respiration rates. This slower decrease of respiration allows for greater respiration than photosynthesis during acclimation. This increase in respiration may cause a decrease in stored energy in the plant otherwise known as carbohydrate storage. Carbohydrates storages are one of the most important aspects in plants acclimated to cold stress. The crown of the plant is essential to winter survival. The crown of the plant contains the meristematic region in which contains the apical meristem which is also where most carbohydrates are stored. The mechanism under excessive variations in carbohydrate storages may be associated with changes in environmental temperature which can affect respiration. It has been well researched that plant respiration is highly affected by changes in temperature. During deacclimation in the winter, mild winter temperatures increased respiration rates and reduced carbohydrates that were stored. This in turn decreased cold hardiness (Ögren, 1996; Ögren et al., 1997). Decreasing respiration of winter wheat and some cool season grasses has been shown to increase survival to winter conditions (Sagisaka et al., 1991 and Bertrand et al., 2003). If this region is not damaged past a threshold. new roots and shoots will develop originating there. Survival of this region can be largely

dependent on where carbohydrates accumulate in relation to it. Accumulating carbohydrates in the crown region may act as a barrier to ice propagation. In grass species, cold acclimation resulted in the accumulation of fructans (Pollock and Cairns, 1991; Livingston, 1991). Fructans have been found to be essential at subfreezing temperatures as a source of cryoprotective sugars (Olien and Clark, 1993; Livingston, 1996).

Water relations

An important way for plants to acclimate to cold temperatures and prepare for many winter stressors is by regulating water content in important survival structures, such as in crowns. During acclimation, water potentials can play a vital role of the survival of the plant. Ice can form inside plant tissue intracellularly and/or extracellularly. In quick, extreme drops in temperature to below freezing, ice may form inside of plant cells. This is denoted as intracellular freezing. This form of freezing can be lethal since the ice crystals can rupture cell membranes. As temperatures drop more slowly to freezing temperatures, extracellular freezing may occur. Typically, extracellular freezing is not lethal to the plant but can be if freezing persists for too long of a time. During extracellular freezing a water potential gradient is formed. Water flows from intracellular spaces reducing the water potential outside of the cell. Less water inside of plant cells and membrane flexibility can attribute to resistance to lethal intracellular ice formation. If temperatures stay below freezing for too long, cell within the plant may become dehydrated and become susceptible to desiccation injury. Therefore, grasses that can avoid intracellular ice have to tolerate extracellular freezing and possible cellular dehydration. In order to properly acclimate to cold temperatures, cellular water concentrations need to decrease. This can be achieved through osmotic adjustment. During osmotic adjustment of acclimated plants, increases of soluble sugars, amino acids, and inorganic solutes has been observed (Dionne et al.,

2001; Fry et al., 1993; Bredemeijer and Esselink, 1995). The accumulation of solutes inside plant cells lowers the freezing point of water within a cell which can allow for greater tolerance to ice formation.

Hormone responses

As in any physiological process, cold acclimation requires hormone signals to become activated and metabolic shifts during acclimation result in altered hormone levels during and post-acclimation. Gibberellic acid is a complex group of plant growth regulators that serve multiple functions in plants such as germination, growth, and reproduction (Hedden et al. 1978). During acclimation, above ground shoot and leaf growth is slowed due to environmental conditions including shorter photoperiod and decreased air temperature. These changes in plant growth can be associated with gibberellic acid which is involved with germination, growth, and reproductive development. Previous studies have found that foliar applications of GA_3 reduce the development of cold hardiness of wheat (Roberts, 1971). Applications of gibberellin synthesis inhibitors have been reported to enhance cold hardening (Carter and Brenner, 1985). The most known cold tolerance regulatory pathway includes the C-repeat binding factor pathway (Jaglo-Ottosen et al. 1998; Liu et al. 1998). This pathway has shown to downregulate active forms of gibberellins by stimulating gibberellin oxidase enzymes. These enzymes inactivate gibberellins. The C-repeat binding factor pathway is also known for stabilizing DELLA proteins which have been shown to repress gibberellin signaling pathway (Achard et al. 2008). The C-repeat binding factor has also been found to be a negative regulator of cytokinin's which are involved in cell division and growth (Jeon et al., 2010). During the alarm phase of plant response, cytokinin's have been shown to be downregulated in wheat (Kosová et al. 2012). A decrease of active cytokinin in leaves is associated with stomatal regulation while cytokinin is known to encourage

stomata opening (Acharya and Assmann, 2009). Exogeneous application of cytokinin was shown to improve cold tolerance in sugar beets (Dix et al., 1994). Cytokinin biosynthetic gene has also been reported to maintain chlorophyll content and increase cold tolerance in callus of *Festuca arundinacea* (Hu et al., 2005). Indole-3-acetic acid is involved in cell elongation, expansion, and division. Research on auxin role in cold stress revealed that auxin efflux carriers inhibited auxin transport (Shibasaki et al., 2009). However, there was no change in signaling when compared to plants not subjected to cold stress.

During cold hardening of wheat (Triticum aestivum L.) plants, GA levels declined, and ABA levels increased (Reid et al., 1974). Increased levels of abscisic acid were found to coincide with decreased levels of other stress hormones, salicylic acid and jasmonic acid early during response to cold stress in wheat (Kosová et al. 2012). Salicylic acid is a stress hormone mainly related to parasite infection, while jasmonic acid is associated with the response to wounding. Salicylic and jasmonic acid have been reported to induce the expression of several dehydrins in a wide variety of plant species, including a relative of chickpea *Cicer pinnatifidum*, and barley (Bhattarai and Fettig 2005; Sun et al. 2009). In some cases, the concentration of jasmonic and salicylic acid strongly influence the response. Treatment with low concentrations of exogenous salicylic acid (0.25 mM or less) stimulates barley Dhn5 mRNA expression as well as protein levels, while higher concentrations (more than 0.25 mM) that mimic severe biotic stress lead to decrease in both Dhn5 mRNA and protein levels (Sun et al., 2009). Thus, hormone levels of abscisic, jasmonic, and salicylic acid play a role in tolerance to cold/ice stress as well as regulate gene expression during acclimation and may play a role in regulating other plant hormones such as ethylene.

Ethylene level, usually estimated via the content of its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), was found to increase rapidly during the alarm phase in the wheat leaves and crowns (Kosová et al. 2012). Ethylene has been associated with abscission of leaves, flowers, buds, gravitropic responses, fruit ripening, and senescence as well as most plant stresses (Taiz and Zeiger, 2006). The gaseous nature of this hormone allows it to effectively signal communities or multiple plant organs via rapid diffusion from plant tissues. Ethylene is biosynthesized via the Yang Cycle with methionine as a precursor (Adams and Yang, 1979; Kende, 1993). Methionine is converted into S-adenosylmethionine (AdoMet) through the enzyme AdoMet synthase. AdoMet is then converted by ACC synthase into 1-aminocyclopropane-1-carboxylic acid (ACC) before being converted into ethylene by ACCoxidase (Adams and Yang, 1979; Taiz and Zeiger, 2002). Environmental and physiological factors regulate ACC synthase activity, but in general, is stimulated by external stress such as wounding, temperature, and water (Kende, 1993; Taiz and Zeiger, 2002). Significant differences in hormonal responses and sensitivity of various species under non-stress and stress conditions within the Poa genus exist. Fiorani et al. (2002) have shown that faster growing *Poa spp* are less sensitive to the accumulation of the growth inhibiting, senescence stimulating hormone, ethylene. Simultaneously, it was found that the fast growing *Poa spp* (*Poa annua* and *Poa trivialis)* produce greater levels of ethylene when compared to slower growing ones (*Poa alpina* and *Poa compressa*). These influences were evaluated only within the ABG genera and only on plant leaves. CBG has greater sensitivity to this hormone, which has been demonstrated by the greater sensitivity of CBG to Ethephon (Bayer Crop Science) treatment (i.e. effective ethylene) compared to ABG (McCullough et al., 2006). However, very little information is available on the role of ethylene applications effects on turfgrass plants under cold/ice stress. It is unknow

whether effective ethylene application to annual bluegrass or creeping bentgrass will increase or decrease tolerance to cold/ice stress or other effects it may have on plant metabolic activity.

Fatty acid composition

Under cold temperatures, fatty acids in cell membranes that normally move and slide against each other at normal temperatures, start to aggregate and pack among themselves This process decreases the overall fluidity of the cell membrane and a state of gel is initiated. Under these conditions a lateral phase separation of membrane lipids could occur (Xin and Browse, 2000). The plasma membrane thus becomes permeable to water and solutes. These released ions cause disruption in processes like water transport, osmotic adjustment and enzyme activities which promotes further membrane destruction and increased cellular damage. In grasses, the extent of modification of the membrane to reduce risk of ruptures is not clear. Some studies in warm season grasses have found an increase in the unsaturation of fatty acids when exposed to cold temperatures (Baird et al., 1998; Shang et al., 2006). Other types of membrane modifications also relay the ability to survive winter conditions. Cells dehydrated by extracellular ice for an extended period, reduce their total surface area. When extracellular ice thaws, the low water potential promotes water influx in the cell. The required expansion in volume must be accommodated or the cell bursts. Freeze tolerant plants avoid expansion-induced lysis by the formation of exocytotic membrane extrusions that are incorporated into the plasma membrane of the expanding cell during thawing (Guy, 2003). No evidence of such tolerant mechanisms is available for annual bluegrass or creeping bentgrass. In wheat, passive efflux of amino acids following mild freezing stress supports the view that changes in membrane properties are an early display of injury (Gao et al., 1983). Protection of cell membranes against freeze-dehydration-induced damage is an important factor in freezing tolerance (Pearce, 2001).

Other metabolites

Aside from fatty acids changes within the cell membrane, the accumulation of antifreeze proteins, compatible solutes, and production of antioxidants assist with cold acclimation (Thomashow, 1999). The main effects of antifreeze proteins on plants to freezing temperatures are to lower the freezing point of water and allow the plant to supercool and inhibit ice recrystallizing that would allow for small ice crystals to form into larger ones which could lead to more injury (Griffin et al. 1992; Kumble et al. 2008; Sandve et al. 2008). 8). Many plant antifreeze proteins are homologous to pathogenesis-related proteins such as ß-l, 3-glucanases, chitinases, and thaumatin-like proteins. Whereas the pathogenesis- related proteins that accumulate in rye during cold acclimation exhibited anti-freeze activity, those that accumulate in non-cold acclimated rye plants lacked such activity (Griffith and Yaish 2004), implying that depressing freezing point is not the main function of plant antifreeze protein. Instead, the main function of plant antifreeze protein has been postulated to be in ice recrystallization inhibition (IRI). Indeed, the IRI activity of plant AFPs has been reported for nearly all the known plant AFPs (Griffith et al. 1992; Worrall et al. 1998; Sidebottom et al. 2000; Kumble et al. 2008; John et al. 2009). Many of these antifreeze proteins are associated with drought stress related genes (Ingram and Bartels, 1996; Dure, 1993; Close, 1997). Many of these proteins are hydrophilic, they have relatively simple amino acid structures with relatively few amino acids, many are composed largely of repeated amino acid sequences, and many are predicted to contain regions capable of forming amphipathic alpha-helices. Antioxidants gene regulations can be associated with cold acclimations have been observed. Enzymes such as cysteine synthase, glutathione synthase, glutathione reductase, and dehydroascorbate reductase have been shown in be upregulated during cold acclimation and are associated with the antioxidant glutathione and

ascorbate. This introduces the idea that antifreeze properties associated with ice stress may be measured through examining antioxidant activities and quantities.

Current winter management strategies

Golf course superintendents do have methods for alleviating ice stress to their putting green surfaces. Some superintendents will dig water channels to move water away from a putting green during the wintertime as snow melt occurs (Quinn, 1990). This can be labor intensive and destructive to the putting green surface as well. Less invasive methods include physical removal of snow and ice from the surface of a putting green if ice accumulation has been prolonged (Frank, 2016). However, these methods can also be damaging if precautions are not taken. Snow and ice removal can cause scuffing and crushing of crown tissue of plants if not performed carefully. Other methods include applying colored sand on top of snow and ice accumulation to assist in melting during the daytime when the sun is out. Colored sands will absorb more heat during the daytime allowing for snow and ice melt. Superintendents also will use covers prior to snow fall to protect putting green canopies (Frank, 2016). These covers typically come in permeable and impermeable types. Permeable covers will allow for gas exchange however, they can also be susceptible to ice accumulation decreasing their effectiveness. Impermeable covers allow for no ice or water to reach tur putting green canopy; however, they do not allow for gas exchange to occur resulting in venting of the putting green to be required so that gas exchange can occur periodically (Rochette et al., 2006). Applying covers to putting green surfaces can be labor intensive and costly for a turf management program, especially since employment during the winter is decreased and cover costs can be great. Thus, the research methodology was aimed at providing golf course superintendents with new tools that could help alleviate ice stress symptoms on putting greens without adding excessive additional costs and not changing

management strategies greatly. Observing commonly used chemicals on golf course putting greens and their effects on ice stress tolerance will give golf course superintendents valuable resources in helping their putting greens survive through winter with minimal damage.

Potential management strategies

Chemical priming can help induce plant response to stress before the stress occurs. Jakab et al. (2005), has observed chemical priming of Arabidopsis with beta-aminobutyric acid induced higher expression of salicylic acid dependent genes prior to drought and salt stress. Cold stress has also been investigated with chemical priming. Exogenous application of hydrogen peroxide demonstrated the same levels of lipid peroxidation in unstressed cold acclimated tomato plants (Iseri et al. 2013). Melatonin has been shown to be associated with abiotic and biotic stress tolerance in plants and more recently has been shown to upregulate drought response genes, cold responsive genes and freeze tolerance transcription factors in *Arabidopsis thaliana* (Bajwa et al. 2014). However, in turfgrass chemical priming for cold/ice stress tolerance has yet to be examined under field or environmentally controlled conditions in creeping bentgrass or annual bluegrass. This raises the question as to what common chemicals used on golf course putting greens affect the ability of turfgrass to survive winter.

Commonly used chemicals on golf course putting greens include plant growth regulators and fungicides. Demethylation inhibitor fungicides have been found to have plant growth regulatory properties like that of paclobutrazol, an early gibberellic acid biosynthesis inhibitor (Köller, 1988). Trinexapac-ethyl is a commonly used plant growth regulator on golf course putting greens to inhibit late gibberellic acid biosynthesis which limits vertical leaf growth (Adams et al., 1992). However, the effects of applying this plant growth regulator to annual

bluegrass late in the fall on cold/ice stress tolerance are relatively unknown and not well studied. Ethephon (effective ethylene) treatments of annual bluegrass has been widely used to control seed head production of the plant (Eggens et al., 1989). It is unknown what effects that an effective ethylene application may have on cold/ice stress of annual bluegrass or creeping bentgrass. The idea that applying common chemicals used on golf course putting greens during acclimation to cold tolerance could potentially prime plants and activate cold/ice tolerance genes allowing for less susceptibility to damage from ice stress.

Conclusions

In conclusion, there are many aspects of turfgrass growth and management that can affect turfgrasses survival to cold/ice conditions. The plants ability to cold acclimate properly through changes in growth habit, hormone concentrations, accumulation of metabolites, carbohydrate storage, and changes to cell membrane fatty acid composition can all effect how the plants survive winter. Putting green surfaces which are the most susceptible to winter related injury can experience ice cover which can lead to anoxic conditions, crown hydration injury, and the buildup of toxic gases can be detrimental to plant cell membranes. Current cultural practices can be labor intensive, costly, and damaging to putting greens to alleviate stress of winter. The current research was aimed at exploring commonly used chemicals and plant hormones to determine if small changes in already established chemical application programs could be utilized in avoiding winter damage through analyzing plant cell membrane fatty acid ratios and plant regrowth. Exploration into commonly used chemical applications on golf course putting greens may give valuable information as to what superintendents can do or change to alleviate the potential to winter injury and minimize costs. Further research into turfgrass cold acclimation is required to better understand why turfgrass respond the way they do to winter conditions.

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CHAPTER 2

CHEMICAL PLANT PROTECTANTS AND PLANT GROWTH REGULATOR EFFECTS ON ANNUAL BLUEGRASS SURVIVAL OF ICE COVER

Abstract

Annual bluegrass (*Poa annua* L; ABG) is susceptible to damage from ice cover. Effects of chemical treatments on ice survival under low temperature growth chamber conditions were studied. Field putting green plots of ABG were treated in the fall of 2014 and 2015 with Civitas, mefluidide, propiconazole, or trinexapac-ethyl (TE) at label rates. After acclimation, turfgrass cores from each plot were transferred to a low temperature growth chamber, received ice or no ice treatment, and were sampled at 0, 20, 40, and 60 d. Ice was applied by misting plants at low temperature. In 2014 Civitas, mefluidide, and propiconazole treated plugs had the greatest amount of regrowth after ice and no ice treatments on most dates; comparable results were found in 2015 but less statistical differences were detected. TE treated plants were no different from the untreated controls on most dates in either year. Plants that were treated with mefluidide, propiconazole, and Civitas had more polyunsaturated fatty acids (FA) in crown tissue, with linoleic acid approximately 50% greater compared to TE and untreated samples. Civitas, mefluidide, or propiconazole treatments improved ABG survival of simulated ice conditions, which could be associated with changes in unsaturated FA content in crown tissue.

Introduction

Turfgrasses or other perennial crops can be subjected to prolonged periods of ice cover in many northern or temperate regions of the world. Freezing rains, ice storms, poor soil drainage, and refreezing of snow melt can cause ice accumulation on top of plant canopies (Mckersie and Leshem, 1994). Ice cover can cause anoxic conditions, crown hydration, and reduced freeze tolerance in ABG (Olien and Smith, 1981; Rochette et al., 2000; Tompkins et al., 2004). The primary cause of death to turfgrass under ice sheets is most likely from oxygen depletion and toxic gas accumulation (Pessarakli, 2008). Annual bluegrass (*Poa annua*; ABG) is a turfgrass putting green species that is susceptible to ice damage, which has been reported to survive between 75 to 90 d of ice cover (Vargas and Turgeon, 2004; Beard, 1964; Tompkins et al., 2004). Past research performed at Michigan State University in a freezing chamber has shown that ABG necrosis can occur in 45 d at -4°C under a 1.27 cm ice layer (Beard, 1964). For comparison, creeping bentgrass (*Agrostis stolonifera*), another common putting green species, can survive under ice cover for up to 120 d without significant injury. Ice damage is a significant issue for golf courses since ABG areas frequently endure prolonged ice cover in northern regions. This damage is very costly as it causes turfgrass managers to repair or completely renovate ice damaged putting greens.

There are pros and cons of current methods of ice management or ice damage prevention used on golf courses. Ice removal can be an effective way to prevent ice damage; however, it is costly, laborious, and can cause mechanical damage to putting greens. Another difficult factor of this method is that ice removal is also highly time sensitive. Tompkins et al. (2004) showed there was little to no benefit of removing ice from ABG after 45 d of ice cover. Another winter turf management strategy is to cover greens with an impermeable or permeable cover or other mulch

material. These materials can raise crown level soil temperatures to increase overwintering survival; however, inconsistent results in winter protection often occur due to unpredictable winter temperature fluctuations and conditions (Dionne et al., 1999). Better, more reliable, and less risky strategies are needed to protect turfgrasses from winter damage such as ice cover. Therefore, this research aims to evaluate simple chemical management strategies that could promote ABG survival of ice cover. The study also aimed to reveal if any of the chemicals used in the study may reduce winter tolerance. Little to no research has been conducted on chemical treatments that could improve ABG recovery from winter-related stresses such as ice.

In addition to new management practices, a better understanding of the physiological mechanisms of ABG that result in ice sensitivity and those that could promote tolerance are needed. Therefore, another goal of the research is to evaluate physiological changes that occur during treatments and during recovery. Winter preparatory management practices are performed in the fall, when turfgrasses undergo the process of acclimation. Acclimation is induced by a reduction in photoperiod and reduced day/nighttime temperatures (Limin and Fowler, 1985; Guy, 2003). This change in environmental conditions can change physiological characteristics in a plant such as increasing antioxidant scavenging, accumulation of antifreeze proteins, and alteration of cell membrane composition (Munshaw et al., 2006). During acclimation, cell membrane fatty acid (FA) profiles can be altered from a saturated FA profile to a more unsaturated FA profile (Baird et al., 1998; Shang et al., 2006). Shifts in FA profiles have been observed in multiple plant species in response to cold acclimation or cold temperature treatment (Hoffman et al., 2010; Samala et al., 1998; Cyril et al., 2002) but have yet to be investigated in ABG. A shift in fatty acid composition towards more unsaturated FAs than saturated FAs is associated with ice encasement tolerance (Hetherington et al., 1987; Dalmannsdottir, et al.,

2001). Thus, whether chemical treatment of ABG can alter FA profile shifts during acclimation and following ice stress deserves investigation.

The chemical treatments used in this study include plant growth regulators (PGRs; mefluidide, trinexapac-ethyl, TE), a fungicide (propiconazole), and a plant protective treatment registered as a fungicide containing mineral oil (a mixture of food grade isoparaffins) as the active ingredient. Many turfgrass managers already use these compounds during the growing seasons to alter the growth habits of ABG or to prevent disease. It is not yet known whether any of these treatments could be play a negative or positive role in winter survival and regrowth following ice stress of ABG. Therefore, we hypothesize that altering plant growth and other physiological changes associated with applications of PGRs or fungicides during acclimation could alter turfgrass regrowth following winter conditions. Thus, our objectives were to evaluate how percent regrowth and physiological attributes associated with acclimation, such as changes in FA profiles, may be affected by chemical treatments and duration of ice cover of ABG under both ice and no ice in a low temperature growth chamber.

Materials and methods

Plant materials and growing conditions

This study was conducted from July to November in 2014 and 2015 on the same ABG field each year at the Michigan State University Hancock Turfgrass Research Center in East Lansing, Michigan. Field plots consisted of mature ABG grown on a top-dressed Colwood brookston loam soil. A light sand topdressing was brushed into the canopy weekly from May through September at a depth of 2.0 mm. The site was maintained at a 3.3 mm mowing height and was mown three times weekly. The plots were irrigated nightly to replace approximately 100% potential evapotranspiration to avoid drought stress. Irrigation was withheld when rainfall

events exceeded 100% potential evapotranspiration. Potential evapotranspiration was retrieved from the weather station located at the research facility based on the FAO Penman-Monteith Equation (Allen et al., 1998). The study area was fertilized with 127.2 kg N ha^{-1} each year including foliar feeding 4.9 kg N ha^{-1} weekly June through September of 2014 and 2015. Plots were fertilized with 24.4 kg N ha^{-1} on 2 June and 1 September, using a 18-9-18 (N-P-K) granular fertilizer (Andersons Golf Products, Maumee, OH). Fungicides and insecticides were applied preventatively and curatively to avoid turfgrass loss due to disease or insect damage respectively to all plots equally and should not have had an influence on our results.

Chemical treatments

All chemical treatments began on 31 July 2014 and on 4 August 2015 and were applied at label recommended rates every two weeks (Figure 1:). All treatments were applied with a pressure-calibrated backpack sprayer (591 L ha^{-1} at 275 kPa) equipped with four flat fan nozzles (DG8002 DS, Teejet Technologies, Wheaton, IL.). The foliar chemical treatments included a fungicide product containing a mineral oil [CIVITAS TURF DEFENSE^{TM} (Civitas), Petro-Canada, Mississauga, Ontario] at a rate of 40.6 L ha^{-1}, mefluidide (Embark T&O, PBI-Gordon Corp., Kansas City, MO) at a rate of 1.6 L ha^{-1}, 3), propiconazole (Banner Maxx, Syngenta Crop Protection, Greensboro, NC) at a rate of 6.4 L ha^{-1}, and 4), TE (Primo Maxx, Syngenta Crop Protection, Greensboro, NC) at a rate of 0.4 L ha^{-1}. Untreated plots were sprayed with water and were utilized as the control plots.

Turfgrass plugs (10 cm diameter) were taken by hand with a turf corer from each plot on 11 November 2014 and 25 November 2015 to a depth of 10 cm, after adequate natural plant acclimation. Temperatures optimum for cold hardening were based on Dionne et al. (2001) where plants experienced approximately two weeks of daily average temperatures at or below

2°C. Eight turfgrass plugs were taken from each plot (five chemical treatments, four sampling time points, two ice cover treatments, with four replications) with a total of 160 turfgrass plugs being used for the experiment. Once all plugs were removed from plots, they were immediately planted in 10.2 cm diameter plastic pots with a depth of 15.2 cm in sandy loam soil and transferred to a low temperature growth chamber and allowed to acclimate at -2°C for two weeks prior to low temperature treatment. Growth chamber conditions included a light level of 200 µmol m^{-2} s^{-1} with a 10-h photoperiod and an ambient temperature of -4°C. Treatments inside the growth chamber consisted of 1) no ice or 2) ice cover treatments. Ice cover treatment involved misting deionized water over pots to form an ice layer (1.3 cm thick). Ice layers were monitored for depth throughout the duration of the study and water was added by misting if ice layers showed any loss of depth. Four turfgrass plugs were taken out from the low temperature growth chamber from each chemical treatment, at 0, 20, 40, and 60 d from both ice and no-ice covered plants.

Fatty acid analysis

Extraction of FAs was performed with approximately 200 mg crown tissue (fresh weight) from ice covered and non-ice-covered samples at 0, 20, 40, and 60 d according to the method of Welti et al. (2002) with modifications. Frozen crown material was transferred into test tubes containing 3 mL of preheated isopropanol (75°C) with 0.01% butylated hydroxytoluene (BHT). Samples were placed in a 75°C water bath for 15 min. After the samples had cooled, 1.5 mL chloroform and 0.6 mL distilled water was added, and the samples were capped and shaken at room temperature for 5 h. After 5 h, the lower layer containing chloroform and the lipids were transferred into new test tubes. An additional 4 mL of chloroform/methanol (2:1) with 0.01% BHT was added to the tubes containing the crown material. The tubes were recapped and placed

on a shaker for 15 h. After extraction, 1 mL KCL was added to the tubes containing the extracted lipids and chloroform and centrifuged at 5000 × g for 10 min. After 10 min, the top layer was removed, 2 mL of distilled water was added, and the tubes were centrifuged for an additional 10 min at 5000 × g. The top thin layer was removed, and the remaining sample was evaporated using vacuum centrifugation. Samples were preserved in 1 mL chloroform and stored at –80°C until analysis. Remaining crown material was dried in an oven 70°C for determination of dry weight. Gas chromatography mass spectroscopy was utilized to quantify FAs. As a measure of fatty acid unsaturation (Cyril et al., 2002), the double bond index (DBI) for each chemical treatment was then calculated as DBI = 0 × ([16:0] + [18:0]) + 1 × 1 x ([16:1] + [18:1]) + 2 x ([16:2] + [18:2]) + 3 x [18:3].

Percent regrowth

After either 0, 20, 40 or 60 d in the low temperature growth chamber, a set of turfgrass plugs were removed from the low temperature chamber and transferred to a 4°C chamber for 7 d, which served as a de-acclimation period. Plants were then transferred to a greenhouse. Greenhouse conditions were maintained at average day/night temperatures of 23/16 °C and an average 12-h photoperiod at 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. Visual percent regrowth was observed of surviving plants after a 20 d regrowth period in the greenhouse. Regrowth was determined by inspecting turfgrass plugs for growing ABG plants and plants that were not growing. The number of living plants divided by the number of deceased plants multiplied by 100 achieved the percent regrowth. These numbers were determined by manual counting of crowns and tillers for each plant.

Experimental design and statistical analysis

Field plots were arranged as a completely randomized design with four replications. The growth chamber experiment was conducted as a completely randomized block design with ice treatment as the main block. Chemical treatment and sampling time were completely randomized within the main block. The experiment was repeated using the same growth chamber. All data were subjected to analysis of variance (ANOVA) using SAS 9.4 (SAS institute Inc., Cary, NC) mixed model procedure. For analyzing the data, time (year) was a fixed factor in the model. When ANOVA indicated a significant year effect, results are presented separately by year. Mean separations were performed by using Fischer's Protected Least Significant Differences (LSD) at the $P \leq 0.05$ level.

Results

Main effects and the interactions of chemical treatment and date were significant for percent regrowth, fatty acid analysis, and DBI in 2014 and 2015 (Table 1:). Ice cover main effects were only significant in 2014 and 2015 for percent regrowth of ABG, therefore fatty acid analysis and DBI were pooled over the ice treatment data (ice or no-ice). Significant years effects were observed within percent regrowth, fatty acid analysis and DBI data collections resulting in data being presented separately by year.

Average fall weather conditions were recorded during the chemical treatments in the field, which indicates plants acclimated naturally during treatments (Figure 1:). Chemical treatments had a significant effect on non-ice-covered regrowth after 20 d at -4°C in 2014 with Civitas treatment having greater regrowth than all other treatments while mefluidide and propiconazole treatments had greater regrowth than TE and untreated controls (Figure 2A:).

After 40 d of incubation at -4°C, Civitas treated plants again had the greatest regrowth when compared to other treatments while propiconazole treatment had greater regrowth than mefluidide, TE, and untreated plants (Figure 2A:). After 60 d in the low temperature growth chamber, TE and the untreated had the least amount of regrowth when compared to other treatments while the mefluidide treatment had the greatest regrowth during both years of the experiment (Figure 2A:).

Percent regrowth

Chemical treatments had a significant effect on regrowth of ice covered ABG at 20, 40, and 60 d in the low temperature growth chamber. In general, during both years of the experiment under ice cover, TE and untreated control plants had the lowest regrowth on most days compared to the other PGR treatments (Figure 2B:). Specifically, after 20 d at -4^{o}C under ice cover, Civitas, mefluidide, and propiconazole treated plants had the greatest regrowth in 2014 while TE and the untreated plants had the least amount of regrowth. In 2015, there was no significant treatment effect after 20 d in the low temperature growth chamber; however, after 40 d under ice cover, Civitas had the greatest regrowth while TE had the lowest percent regrowth compared to mefluidide, propiconazole, and the untreated, which were not significantly different from each other. Comparable results were observed after 60 d under ice cover in 2015 with Civitas having the greatest percent regrowth and TE having the least amount of regrowth compared to the other treatments.

Fatty acids

Six FAs, either unsaturated or saturated, were detected in crown tissue of ABG plants. The saturated FAs included palmitic acid (16:0) and stearic acid (18:0). The monounsaturated

FAs were palmitoleic acid (16:1) and oleic acid (18:1) and the polyunsaturated FAs were linoleic acid (18:2) and α-linolenic acid (18:3). The fatty acid hexdecadienoic acid (16:2) was not observed in measurable quantities. Significant effects from PGR treatments were observed on FA composition of ABG crown membrane profiles and were consistent for each time point of sampling (0, 20, 40, or 60 d of low temperature or ice treatment). Ice treatment duration and ice treatment did not change FA composition within a given treatment.

Concentrations of the saturated FA palmitic acid (16:0) were significantly lower in the Civitas, propiconazole and mefluidide treatments when compared to the untreated control and TE treated plants after 20 and 40 d of ice or no ice treated plants in 2014 (Table 2:). For instance, in 2014 after 40 d, Civitas, mefluidide, and propiconazole treated ABG had 16.1, 30.5, and 21.5% lower palmitic acid content when compared to the untreated control, respectively (Table 2). Civitas, propiconazole, and mefluidide had lower concentrations of the saturated FA stearic acid (18:0) when compared to the untreated controls except after 40 d in 2015; TE treated ABG had the same stearic acid content as the untreated control except 0 d in 2014 (Table 2:). Civitas had increased concentrations of the monounsaturated FA palmitoleic (16:1) at 20, 40 and 60 d in 2014 when compared to the untreated control (Table 3:). Oleic acid (18:1) concentration was only increased by Civitas treatments after 0 d in 2014 and 20 and 40 d in 2015 when compared to the untreated control (Table 3:). Civitas, propiconazole, and mefluidide increased concentrations of the polyunsaturated FA linoleic acid (18:2) in both 2014 and 2015 and at 0, 20, 40 and 60 d compared to the untreated control, except for Civitas after 40 d while TE treated ABG was never different from the untreated control (Table 3:). For instance, Civitas, propiconazole, and mefluidide had 39.7, 60.3, and 63.6% greater linoleic acid concentrations, respectively, when compared to the untreated control after 0 d in 2014. The polyunsaturated FA linolenic acid (18:3)

was increased by Civitas treatment after 60 d in 2014 and 20, 40 and 60 d of ice cover in 2015. For example, Civitas had 47.1 and 37.5% higher amounts of linolenic acid when compared to the untreated control after 60 d of ice cover in 2014 and 2015, respectively (Table 3:).

Double bond index

Double bond index indicates a degree to which chemical treatments altered unsaturated fatty acids within ABG. In both 2014 and 2015, Civitas, propiconazole, and mefluidide treated ABG had a greater DBI than the untreated control on all sampling days except 0 d in 2014 (Figure 3:). For example, in 2015 after 40 days in the growth chamber, Civitas, propiconazole and mefluidide each had 19.7, 19.6 and 19.6 % greater DBI than the untreated control, respectively. The DBIs of TE treated plants were generally not significantly different than the DBI of untreated control plants.

Discussion

A reduction in ABG regrowth occurred in 20 d at -4°C under either ice cover or non-ice-covered conditions in 2014. While a reduction in ABG regrowth occurred in 40 d under ice cover in 2015. Previous research has found a survival range of 45 to 90 d for ABG (Vargas and Turgeon, 2004; Beard, 1964; Tompkins et al., 2004). The differences between studies in the number of days under ice that causes damage are likely due to differences in experimental factors, different turf management strategies prior to turf plugs being put into low temperature conditions (management practices have changed significantly in the past 50 years), or since ABG can exhibit a large degree of ecotype variability (Beard et al., 2014; Dionne, 2010). Compared to the past, mowing heights are lower, new chemistries are applied, and enhanced cultural practices that can add traffic stress of turf are commonly employed (Beard et al., 2014). It is important to

note that the cold and ice treatments in the low temperature growth chamber may not be representative of actual field conditions. This growth chamber condition causes freezing of the entire canopy and root zone (-4°C) and would be a more extreme form of stress than in the field. Under field conditions, soil temperatures at crown level (0.5 cm depth) typically remain just below 0ºC while soil temperatures at 10 and 20 cm depths are typically warmer than at the crown level during the winter months (Dionne et al., 1999).

Shifts in membrane FA compositions can play a significant role in plant acclimation to low temperatures, particularly in perennial turfgrass species. Un-acclimated cell membranes can become rigid under low temperatures and increases in unsaturated FAs can help plant membranes retain some degree of fluidity (Iba, 2002). Cell membrane FA composition that is common in most plants includes saturated FAs [palmitic acid (16:0) and stearic acid (18:0)], monounsaturated FAs [palmitoleic acid (16:1) and oleic acid (18:1)], and the polyunsaturated FAs [linoleic acid (18:2) and α-linolenic acid (18:3)] (Millar et al., 2000). In perennial ryegrass (*Lolium perenne*), saturated FAs (stearic and palmitic acid) were found to decrease in concentration while polyunsaturated (linoleic and linolenic) were found to increase in concentration due to cold acclimation in crown tissue after 21 d of acclimation (Hoffman et al., 2010). ABG crown FAs did not change significantly between each sampling date or between ice cover treatments. This suggests that ABG crown FAs were not altered during ice cover treatment and that any FA changes that occurred happened during chemical treatments during the acclimation period. Thus, future work could more specifically evaluate ABG FA changes during the acclimation or chemical treatment period. Some chemical treatments evaluated in this study did significantly alter FA composition in ABG crown tissue, which could have promoted ice survival and regrowth during recover from ice conditions. Whether any FA changes were a direct

or indirect effect of chemical treatment cannot be determined by the results of the study and could be investigated in the future.

PGRs are used primarily in turfgrass management to reduce above ground growth, thereby reducing mowing requirements, or to inhibit seed head production. Mefluidide inhibits cell elongation and division and is applied to limit ABG flowering and seed head production in golf course putting greens in the spring (Tautvydas, 1984; Haguewood et al., 2013). The mode of action of mefluidide was only recently determined to be associated with inhibition of 3-ketoacyl-CoA synthase, which is involved in very long chain fatty acid synthesis (Tresch et al., 2012). Inhibition of long chain FAs could be related to our results regarding mefluidide effects on saturated and unsaturated FA found here; however, more detailed biochemical analysis would be needed to determine this. Mefluidide, a mitosis inhibitor, had similar FA contents to Civitas and propiconazole treatments. Previous research with mefluidide shows that mefluidide treated maize had increased abscisic acid content when compared to untreated maize prior and during cold (26°C) treatment (Zhang et al., 1986). Increases of abscisic acid contents have been shown to increase cold hardiness in plants (Li et al., 1989). Mefluidide treatment of corn seedling leaves increased linoleic acid concentrations and decreased palmitic acid concentrations when compared to untreated corn leaves (Zhang and Chen, 1991).

Gibberellic acid (GA) biosynthesis is inhibited by TE, which reduces cell elongation. It has been shown to reduce grass clipping yield in multiple grass species including Kentucky bluegrass (*Poa pratensis*), perennial ryegrass, creeping bentgrass, and ABG (Ervin and Koski, 1998; Ervin and Koski, 2001; Kreuser and Soldat, 2012; Landry and Murphy, 2000). The chemical TE has been shown to decrease tolerance to heat stress in Kentucky bluegrass (Heckman et al., 2001). However, TE has also been shown to play a role in improving heat

tolerance when combined with drought stress in creeping bentgrass (McCann and Huang, 2006). Heckman et al. (2001) concluded that it could have been due to TE being in the same class as cyclohexanedione, which interrupts lipid synthesis. Effects on lipid synthesis is relevant to the FA results found here. In TE treated plants, palmitoleic acid, an unsaturated fatty acid, was higher on some dates in 2014 in crown tissue than in untreated control plants. We did not see any effects of TE treatment of plants on FA profiles compared to untreated plants. Therefore, it is not yet fully clear how TE may alter lipid or FA synthesis and how it may impact abiotic stress tolerance.

TE treated ABG had the same regrowth as the untreated controls on most dates measured, except for after ice cover at 40 and 60 d in 2015 it had significantly less regrowth than the controls. Murata et al. (1982) found that there was no relationship between freeze sensitive plants and freeze tolerant plants and their palmitoleic acid content. Thus, it is not yet clear why TE treatment may have reduced regrowth following prolonged low temperature or ice cover conditions. It is possible that TE repression of GA lasted into the spring or that other factors such as carbohydrate or hormonal responses to TE treatment could play a role in this reduction of regrowth. In Kentucky bluegrass, TE application inhibited growth by reducing gibberellic acid (GA) and reducing the amount of labeled carbon in the form of photosynthates in the crown of the plant when compared to plants not treated with TE (Hanson and Branham, 1987). Rossi and Buelow (1997) showed that applying recommended label rates of TE in the fall on fairway height ABG caused increased necrosis when compared to untreated areas the following spring when ratings occurred. However, GA accumulation in plants during acclimation is thought to be negatively associated with cold tolerance (Shan et al., 2007). Future research is needed to determine if carbohydrates or GA may play a role in ABG sensitivity or tolerance to winter

conditions. Based on our results, applying TE to ABG in the late summer into fall during acclimation could cause no change or could decrease ABG survival.

The fungicide products used in this study, propiconazole and Civitas, are both thought to have PGR-like effects on turfgrasses (Elliott, 1999). Propiconazole is a demethylation inhibitor fungicide with a triazole chemistry. Literature regarding the effects of fungicide treatments on abiotic stresses is lacking, particularly for those associated with winter conditions. Paclobutrazol, a PGR with a similar chemistry to propiconazole, increased protection to chilling injury of bell pepper fruits (*Capsicum annuum*; Lurie et al., 1994) The researchers were not able to determine the mechanism. For instance, paclobutrazol treatment did not alter phospholipid content of bell pepper fruits when compared to untreated plants (Lurie et al., 1994). Civitas can cause PGR-like effects such as altering carbon assimilation and carbon partitioning in the plant (Kreuser, 2014). Civitas is also known to be an induced systemic resistance (ISR) activator. The ISR pathway is a plant defense response pathway that can be induced by chemicals, pathogens, or other abiotic stresses (Cortes et al., 2010; Hsiang et al., 2011). Despite their differences in chemistry or mechanism of action, Civitas, propiconazole, or mefluidide each may be effective treatments to increase ABG survival of winter or ice conditions. However, the mechanism associated with these treatments are not yet clear.

The major FA that was up-regulated by Civitas, mefluidide, and propiconazole during acclimation was linolenic acid, which is an 18-carbon omega-3 trienoic polyunsaturated FA found in membrane lipids of plants (Upchurch, 2008). For Civitas, effects on linonenic acid could be through the promotion of ISR, for which the primary up-regulated signaling hormone is jasmonic acid. Linolenic acid is a precursor to jasmonic acid (Farmer and Ryan, 1992); however, whether ISR and jasmonic acid played a role in effecting linolenic during winter stresses cannot

be directly concluded from the results of our study. Linolenic acid is known to play a role in cold tolerance in some species. For instance, a cold tolerant variety of seashore paspalum (*Paspalum vaginatum* Sw) was found to have higher concentrations of linolenic acid when compared to cultivars that were not cold tolerant (Cyril et al., 2002). However, past research on wheat varieties reveals that increases of linolenic acid synthesis does not necessarily indicate that tolerance to freezing will occur (de la Roche, 1979). Our research suggests that applications of Civitas, mefluidide, or propiconazole may cause an increase in linolenic acid of ABG crown membrane tissue, which could be associated with increased tolerance to low temperature and ice stress.

It is possible that regulation of FAs by Civitas, mefluidide, and propiconazole could have improved ice and low temperature tolerance. These three treatments generally caused greater levels of monounsaturated and polyunsaturated accumulation in crown tissues, which may have played a role in maintaining membrane fluidity during acclimation to maintain membrane integrity during low temperature and ice conditions. Future research could investigate these treatments specifically for membrane integrity maintenance, as this was not directly measured in the current study.

It is important to note that ABG is notorious for being a turfgrass species that is highly plastic and variable in response to spring freeze-thaw cycles. ABG frequently can undergo premature de-acclimation (Espevig et al., 2014; Hoffman et al., 2014). De-acclimation is a change in physiological attributes within the plant that lead to increased plant growth and a decrease in freeze tolerance. Optimally, de-acclimation happens gradually in response to an increase in environmental temperatures during the spring (Arora et al., 2004; Kalberer et al., 2006). Applying the PGR mefluidide in the field to winter wheat varieties delayed the loss of

cold hardiness during the winter in Saskatchewan (Gusta et al., 1990). It is not yet clear if increases in spring regrowth following winter would be a benefit or detriment to ABG if unseasonable winter or spring conditions existed. Evaluating the treatments used in our study under ice in the field and under experimental de-acclimation cycles would help to better understand potential ABG responses to PGR treatment. Additionally, Civitas and demethylation inhibitor fungicides can cause phytotoxicity to some turfgrasses at certain rates and times of year (Elliott, 1995; Kreuser and Rossi, 2014). Therefore, it is important to note that the treatments proposed here could slightly reduce fall turf quality.

Conclusions

Civitas, mefluidide and propiconazole treatments increased unsaturated FA ratios and percent regrowth when compared to untreated controls on most dates measured. Enhanced crown survival due to the treatments could have played a role in increased regrowth after ice or no ice treatments. A shift in crown tissue FAs from saturated to unsaturated FAs in response to chemical treatments could have played a role in enhanced survival of annual bluegrass plants following simulated winter conditions. It is not clear whether TE treatment may decrease or cause no change in low temperature or ice survival of ABG. Future work should evaluate these treatments under ice in field conditions, determine potential effects on de-acclimation, and tested on other turfgrass species that are sensitive to cold or winterkill stresses. Additionally, investigating the direct mechanism of these treatments on enhanced regrowth may be warranted such as whether other physiological factors, like shifts in carbohydrate storage, could have played a role in ABG acclimation. Our results indicate that some of the chemical treatments described here could be feasible treatment methods to enhance ABG survival of winter conditions.

APPENDIX

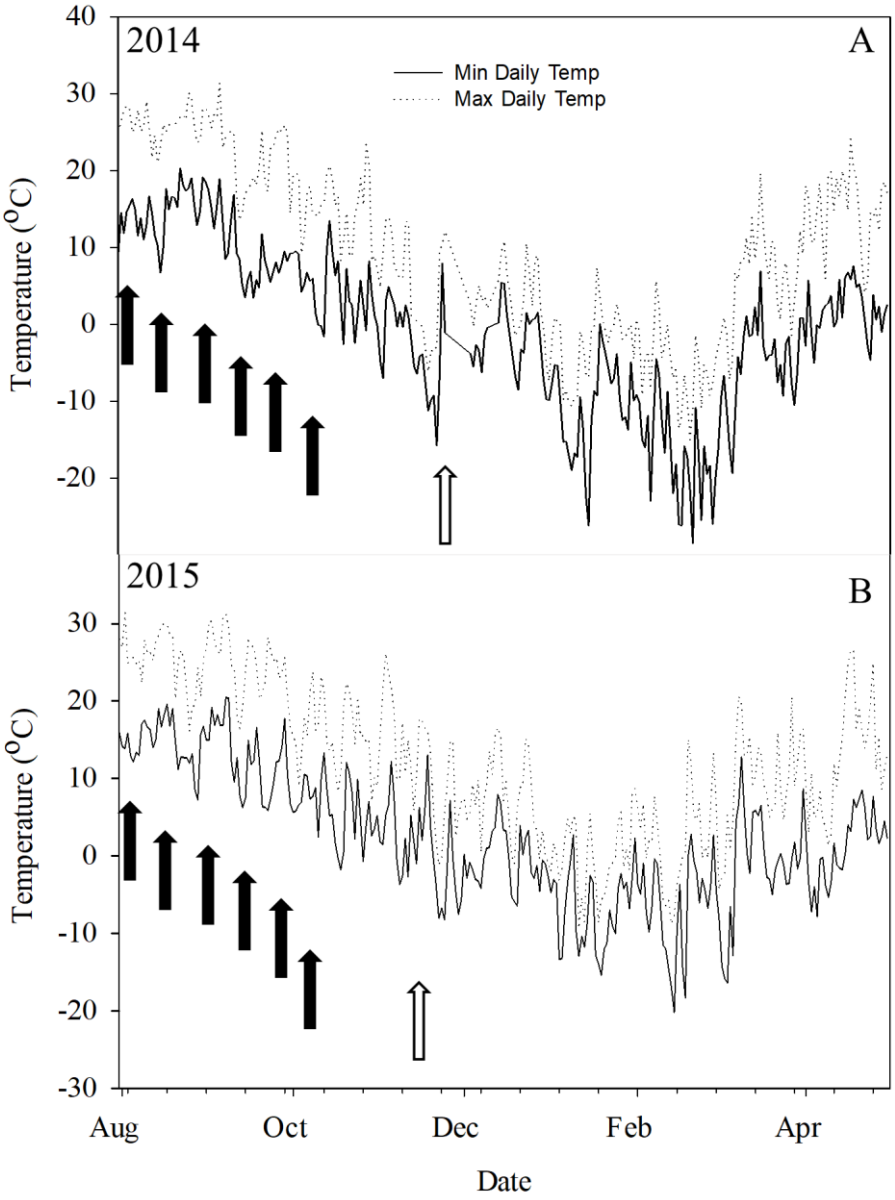

Figure 1: *Daily minimum and maximum air temperature for the Hancock Turfgrass Research Center in East Lansing, MI, from A) 31 July 2014 to 3 April 2015 and 31 July 2015 to 30 April 2016.* Black filled in arrows indicate when field treatment applications were applied in each year. Open arrows indicate when annual bluegrass plugs were removed from the field and transferred to the low temperature growth chamber.

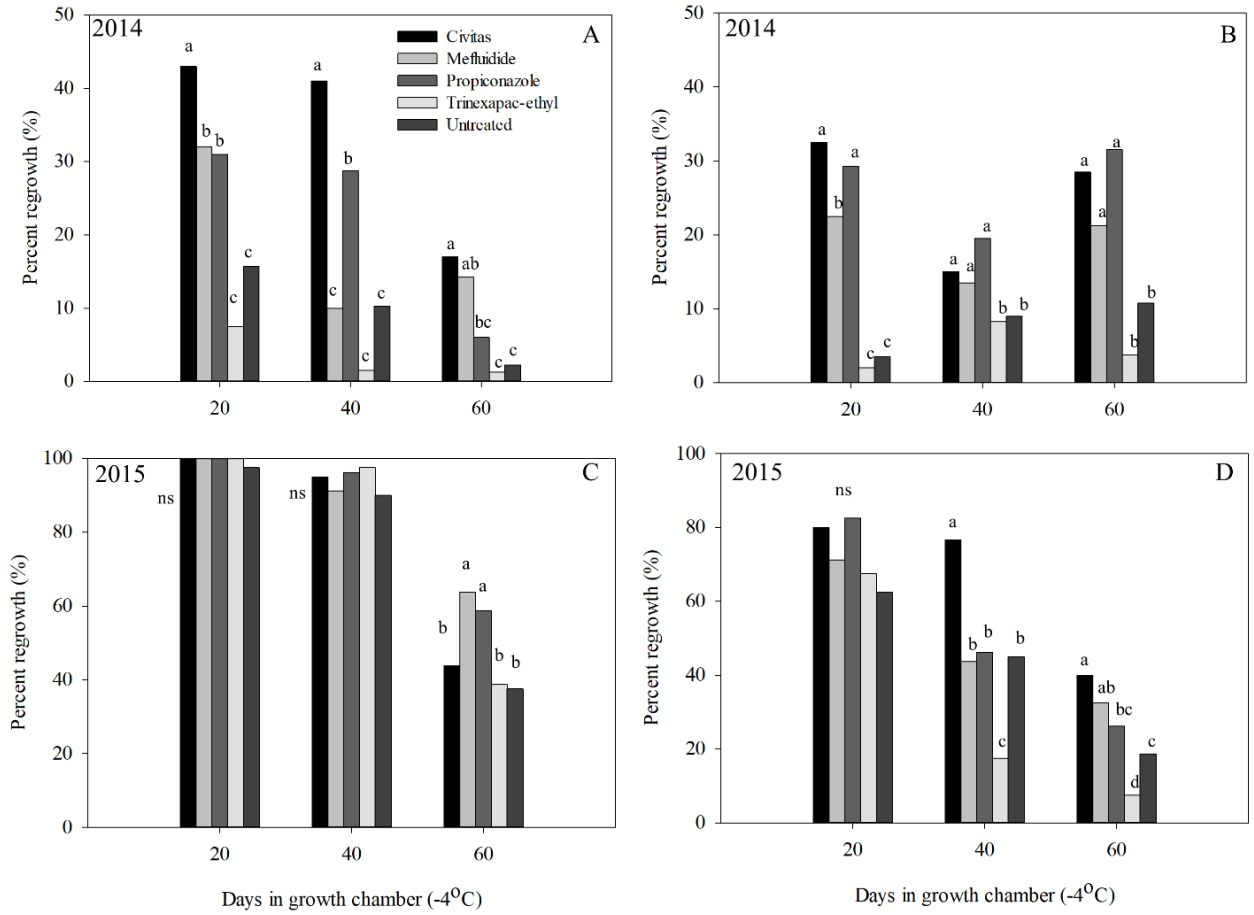

Figure 2: *Percent regrowth of annual bluegrass treated with a given chemical treatment after 20, 40, and 60 days in the low temperature growth chamber (-4°C) under A) non-ice cover conditions in 2014, B) ice covered conditions in 2014, C) non-ice cover conditions in 2015 and D) ice covered conditions in 2015.* Means with the same letter on a given rating date are not significantly different based on least significant difference values ($P \leq 0.05$).

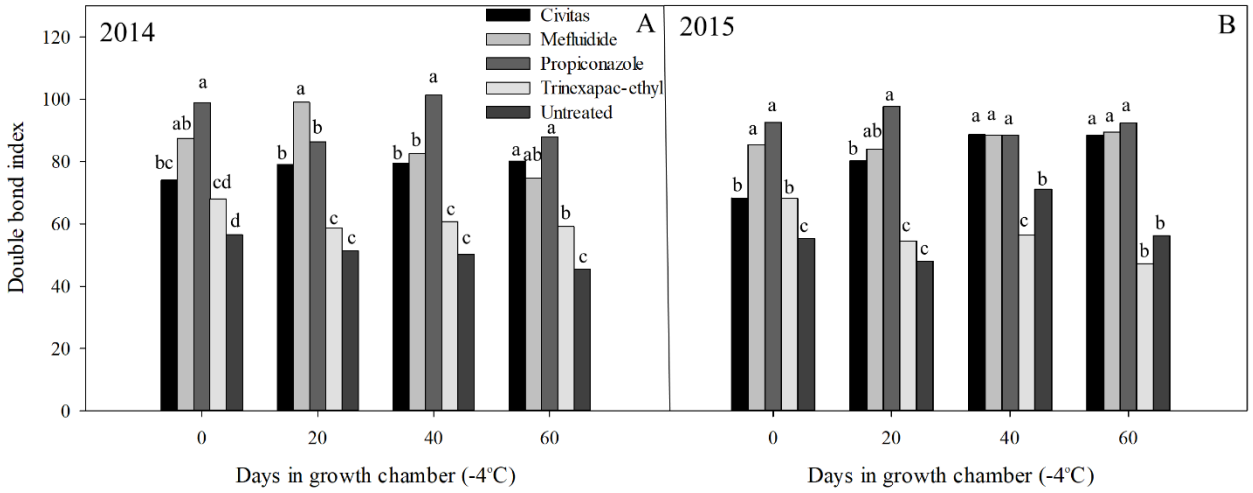

Figure 3: *Double bond index for fatty acids of annual bluegrass treated with foliar applications of a given chemical treatment after 0, 20, 40, and 60 days in the low temperature growth chamber (-4°C) in A) 2014 and B) 2015.* Means with the same letter on a given rating date are not significantly different based on least significant difference values ($P \leq 0.05$).

Table 1: *Analysis of variance for main treatment factors and interactions of percent regrowth, fatty acid analysis, double bond index, and ratio of 18:3 /18:2 fatty acids under chemical and ice cover treatments of annual bluegrass in East Lansing, MI during 2014 and 2015.*

Effect	Percent regrowth		Fatty acids		Double bond index	
	2014	2015	2014	2015	2014	2015
Rep						
Chemical treatment (T)	***	***	***	***	***	***
Ice treatment (I)	*	**	ns	ns	ns	ns
T x I	nsł	ns	ns	ns	ns	ns
Date (D)	***	***	***	***	**	***
T x D	***	**	***	***	***	**
I x D	ns	ns	ns	ns	ns	ns
T x I x D	ns	ns	ns	ns	ns	ns

* p values ≤ 0.05
** p value ≤ 0.01
*** p value ≤ 0.001
ł ns = not significant with p value > 0.05.

T indicates effect of chemical treatment; I indicates effect of ice treatment; D indicates effect of date; T x I indicates interaction of chemical treatment by ice treatment; T x D indicates interaction of chemical treatment by date; I x D indicates the interaction of Ice treatment by date; and T x I x D indicates the three way interaction of chemical treatment by ice treatment by date. . NS indicates a non-significant difference was detected; *, **, *** represents significant effect at the level of 0.05, 0.01, and 0.001, respectively.

Table 2: *Changes in the saturated fatty acid contents of crown tissue of annual bluegrass treated in the field with foliar chemical applications and then exposed to 0, 20, 40 and 60 days in a low temperature growth chamber (-4 °C) in 2014 and 2015.* Within each column, means followed by the same letter are not significantly different ($P \leq 0.05$).

	2014				2015			
	Stress treatment period (d)				Stress treatment period (d)			
	0	20	40	60	0	20	40	60
16:0[ϟ]	Molar percentage (mol %)							
Civitas	29.50 a[ł]	30.50 bc	29.25 bc	26.95 c	29.2 b	29.27 bc	27.63 b	26.63 b
Propiconazole	29.20 a	26.90 c	27.38 bc	26.95 c	30.07 b	28.87 bc	25.68 b	28.50 b
Mefluidide	23.75 b	29.30 c	24.25 c	29.58 bc	26.37 b	25.32 c	26.05 b	27.75 b
Trinexapac-ethyl	33.75 a	33.40 ab	31.98 ab	34.18 a	34.37 a	33.25 ab	33.35 a	35.03 a
Untreated	31.75 a	35.00 a	34.88 a	32.63 ab	34.77 a	34.52 a	33.15 a	34.30 a
18:0								
Civitas	28.27 bc	29.10 b	28.83 b	29.03 b	30.17 b	28.40 b	26.70 b	27.55 b
Propiconazole	25.60 c	26.20 b	25.90 b	26.25 b	25.22 c	26.00 b	26.48 b	25.18 b
Mefluidide	27.57 c	26.20 b	26.18 b	25.88 b	28.77 b	26.20 b	26.80 b	26.48 b
Trinexapac-ethyl	32.22 b	35.40 a	36.43 a	35.43 a	30.57 ab	35.50 a	36.98 a	36.80 a
Untreated	38.07 a	37.00 a	36.90 a	36.38 a	32.97 a	37.47 a	29.05 b	35.38 a

ϟ The fatty acid ratios are (C, number of carbon atoms)/ (D, number of double bonds) and are composed of palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.

ł Means followed by the same letter within each column for each fatty acid are not significantly different based on Fisher's protected LSD ($\alpha = 0.05$).

Table 3: *Changes in the unsaturated fatty acid contents of crown tissue of annual bluegrass treated in the field with foliar chemical applications and then exposed to 0, 20, 40 and 60 days of ice cover in a low temperature growth chamber (-4 °C) in 2014 and 2015.* Within each column for each fatty acid, means followed by the same letter are not significantly different ($P \leq 0.05$). Columns with no letters indicate no significant differences among chemical treatment.

	2014				2015			
	Stress treatment period (d)				Stress treatment period (d)			
	0	20	40	60	0	20	40	60
16:1[l]	Molar percentage (mol %)							
Civitas	7.80	6.60 ab	6.28 a	6.08 a	8.08	6.65 a	6.60	6.10
Propiconazole	6.05	4.70 c	6.65 a	4.00 b	5.90	6.30 a	4.98	6.50
Mefluidide	4.78	6.30 ab	4.15 b	6.60 a	4.50	4.70 c	4.78	5.20
Trinexapac-ethyl	5.85	7.20 a	7.05 a	6.33 a	7.10	6.20 ab	5.83	4.93
Untreated	5.93	5.50 bc	4.78 b	4.33 b	5.28	4.97 bc	5.28	4.95
18:1								
Civitas	9.37 a[1]	6.70	7.08	6.93 a	8.80 a	8.45 a	7.85 a	6.75
Propiconazole	7.55 ab	5.30	6.33	4.40 b	7.52 ab	6.77 ab	5.13 b	6.20
Mefluidide	5.67 b	7.10	5.35	6.60 ab	4.85 c	6.57 bc	5.85 b	4.78
Trinexapac-ethyl	5.72 b	5.80	6.08	5.90 ab	5.72 bc	4.85 c	5.43 b	5.65
Untreated	6.42 b	5.60	5.73	5.73 ab	7.32 ab	6.50 bc	5.90 b	7.08
18:2								
Civitas	13.55 b	13.50 b	12.93 bc	13.53 bc	12.47 b	14.05 b	12.90 b	17.00 b
Propiconazole	20.57 a	21.60 a	19.30 a	17.25 ab	20.07 a	18.60 a	23.53 a	24.03 ab
Mefluidide	22.42 a	19.00 a	22.03 a	22.00 a	19.92 a	20.42 a	24.28 a	21.20 a
Trinexapac-ethyl	8.15 c	9.00 bc	9.33 bc	9.73 cd	8.37 c	8.65 c	8.40 c	8.80 c
Untreated	8.17 c	8.00 c	7.63 c	7.13 d	8.90 c	7.95 c	15.50 b	9.13 c
18:3								
Civitas	9.95	12.90 ab	13.43 ab	13.38 a	8.78	12.35 ab	16.10 a	13.85 a
Propiconazole	10.93	15.30 a	10.33 ab	10.60 ab	10.60	11.25 abc	10.45 b	10.53 ab
Mefluidide	14.58	11.70 ab	15.93 a	10.25 ab	14.43	15.17 a	9.78 b	12.35 bc
Trinexapac-ethyl	13.40	9.30 b	9.65 b	9.18 b	12.83	8.70 bc	9.43 b	6.30 d
Untreated	9.30	8.10 b	8.15 b	7.08 b	8.28	6.87 c	9.63 b	8.65 cd

ł The fatty acid ratios are (C, number of carbon atoms)/ (D, number of double bonds) and are composed of palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.
ł Means followed by the same letter within each column for each fatty acid are not significantly different based on Fisher's protected LSD ($\alpha = 0.05$).

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CHAPTER 3

ETHYLENE REGULATORY TREATMENT EFFECTS ON ANNUAL BLUEGRASS SURVIVAL OF FREEZING TEMPERATURE AND ICE COVER

Abstract

Annual bluegrass (*Poa annua* L) is a golf course turfgrass species, often in a mixed stand with creeping bentgrass (*Agrostis stolonifera*). Annual bluegrass is more susceptible to ice cover damage than creeping bentgrass. The objective was to determine how fall applications of substances promotive [1-aminocyclopropane-1-carboxylic acid (ACC) or ethephon] or inhibitory [Retain or aminoethoxyvinylglycine (AVG)] to ethylene production influence turfgrass quality, normalized difference vegetation index (NDVI), ethylene production, canopy respiration rates, and recovery from ice encasement under field and/or growth chamber conditions. The field was monitored for fall quality and acclimation parameters and the growth chamber study was used for ice treatments and recovery of annual bluegrass only, the winter-sensitive species. Under field conditions, ethephon application increased ethylene production and whole plant respiration of both annual bluegrass and creeping bentgrass and decreased turf quality and NDVI when compared to controls. Ethylene inhibition treatments did not affect turf quality, respiration or NDVI when compared to the untreated controls but AVG treated annual bluegrass had greater ethylene production than the untreated controls after 35 and 42 d of treatment. In the growth chamber experiment, ethephon decreased annual bluegrass regrowth while Retain improved annual bluegrass (ABG) regrowth after 40 or 80 days of exposure to -4°C with or without ice cover. Treatments that inhibit turfgrass canopy ethylene production may be viable methods to improve annual bluegrass winter survival of ice encasement conditions and may have no negative effects on creeping bentgrass. Testing these treatments for ice survival under field conditions is still needed.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; NDVI, normalized difference vegetation index; TQ, turf quality

Introduction

Perennial crops such as turfgrasses, and others can be exposed to prolonged periods of ice cover in many temperate regions of the world. Ice accumulation has been cited as a primary cause of stress during the winter in these regions (Mckersie and Leshem, 1994; Gudleifsson, 2013). Many factors affect how ice accumulation may cause stress in perennials, such as duration of ice cover and porousness of the ice cover. If the ice is porous, gas exchange from the plant canopy to the atmosphere may be limited and can cause hypoxia. Prolonged, non-porous ice typically leads to anoxic conditions and the presence of toxic gases in the plant canopy (Olien and Smith, 1981; Rochette et al., 2000; Tompkins et al., 2004). Toxic gases such as ethyl-butyrate have been found to accumulate under ice cover and can cause cell membrane disruption in turfgrass plants (Aamlid et al., 2009). Ice cover can be significantly damaging to plants and reduce spring regrowth or yields by damaging crown tissues.

The perennial form of annual bluegrass (*Poa annua* var *reptans*) is a turfgrass putting green and fairway species that is susceptible to damage from ice cover. In various studies, annual bluegrass has been reported to survive an average of 20-75 d of ice cover (Beard, 1964; Tompkins et al., 2004; Aamlid et al., 2009; Waalen et al., 2017). In a freezer-based experiment, annual bluegrass necrosis occurred in 45 d at -4°C under a 1.27 cm, non-porous ice layer (Beard, 1964). For comparison, creeping bentgrass (*Agrostis stolonifera*), another common putting green and fairway species, can survive under ice cover for up to 120 d without significant injury (Beard, 1964). Annual bluegrass is also susceptible to various other winter damages such as

freezing stress and premature de-acclimation in the spring (Hoffman et al., 2013). In Hoffman et al. (2013) annual bluegrass freezing tolerance was significantly lower than in creeping bentgrass. Putting greens and fairways are often a mixed stand of annual bluegrass and creeping bentgrass; thus, it is important to examine the effects of winter protective treatments on both species to evaluate potential negative and positive effects of each treatment. This can also help provide a mechanistic understanding of treatments based on comparisons of how a more ice tolerant compared to a more sensitive species responds to treatments.

Ice cover can severely reduce annual bluegrass survival of crown tissue, the primary overwintering organ, and lead to high renovation costs for golf courses (Kvalbein et al., 2017). Research to evaluate best winter preparation practices and determine new strategies for enhance ice encasement tolerance is needed to reduce these costs. Current strategies of ice cover management for golf courses mainly include the prevention of ice buildup or removal of existing ice. This may include crafting trenches through a putting green for water drainage, covering entire putting greens with protective covers, or physically removing snow or ice from putting green surfaces. These strategies can be costly and not always reliable due to unpredictable winter temperature fluctuations and precipitation events (Tompkins et al., 2004; Dionne et al., 1999). Some of these practices, which may be practical for putting greens, are not feasible on larger areas of annual bluegrass, such as on fairways. Sensitive turf areas may not be readily accessible by machinery required to perform some of these practices. Thus, new and more broadly applicable and cost-effective strategies that can be implemented prior to winter are needed to protect annual bluegrass and creeping bentgrass under ice cover.

A general response of plants to stress is an increased production of the gaseous hormone ethylene (Nilsen and Orcutt, 1996). The production of ethylene is known to be differential in

both creeping bentgrass and annual bluegrass. During the summer, annual bluegrass produced more ethylene than creeping bentgrass (Laskowski, 2017). It is not yet known if the two species differ in ethylene production during acclimation and it is not yet clear if or how ethylene regulation may be used by plants to prepare for winter. Investigating ethylene regulation during acclimation could result in valuable management strategies to enhance winter survival of various plant species. Two commonly used ethylene promotive treatments are ethephon (2-chloroethyl phosphonic acid) and aminocyclopropane-1-carboxylic acid (ACC). Ethephon breakdown releases ethylene (Cho et al., 1988) and ACC is a precursor to ethylene in the ethylene biosynthesis pathway in plants (Amrhein, 1981). Ethephon treatment was deleterious to warm-season (C4 photosynthetic pathway) turfgrasses for winter survival (Munshaw et al., 2010) and reduced the freeze tolerance of *Arabidopsis thaliana* (Shi et al., 2012). Conversely, ethylene has been shown to improve plant tolerance of freezing stress by increasing antifreeze protein expression in winter rye plants (*Secale cereal*) treated with ethephon and ACC (Yu et al., 2001). In maize (*Zea mays*) leaf tissue, ACC content was observed to increase under chilling stress and during acclimation when compared to non-acclimated maize (Szalai, 2000). This evidence suggests ethylene may play a major role in plant acclimation but a better understanding of how ethylene effects acclimation characteristics and winter survival is needed.

If ethylene may reduce freezing or winter tolerance in some plant species, use of ethylene inhibitors may be warranted. Aminoethoxyvinylglycine (AVG) is an inhibitor of the ethylene biosynthesis pathway and has potential to be used for promoting plant tolerance of certain stresses (Saltveit, 2004). For instance, creeping bentgrass treated with AVG had 25% less ethylene after 21 d of heat stress at 35 °C in a growth chamber and showed improved tolerance to heat stress (Xu and Huang, 2009). Whether regulation of ethylene, via inhibition or promotion of

ethylene during acclimation, plays a role in annual bluegrass or creeping bentgrass survival of winter conditions has yet to be determined and is needed.

Ethylene regulatory treatments would be readily applicable and cost-effective management strategies to promote annual bluegrass tolerance of winter conditions. To our knowledge, ethylene production by turfgrasses during acclimation, effects of ethylene on turfgrass respiration rates during acclimation, and whether ethylene may be associated with the difference in tolerance to various winterkill stresses, particularly ice cover stress, of cool-season turfgrasses have all yet to be investigated. We hypothesize that 1) annual bluegrass may produce higher levels of ethylene than creeping bentgrass during acclimation and high levels of ethylene production by annual bluegrass may have negative effects on regrowth following ice encasement or freezing temperatures and 2) treatments that inhibit ethylene production may improve regrowth and ethylene promotive treatments may reduce regrowth following ice encasement or freezing temperatures. The objectives of the study were to investigate the effects of ethylene regulation on annual bluegrass and creeping bentgrass ethylene production, respiration rates, field performance during fall acclimation and annual bluegrass survival of freezing temperature and ice cover based on plant regrowth abilities.

Materials and methods

Plant materials and growing conditions

This study was conducted from July to November in 2016 and 2017 on two individual annual bluegrass field and creeping bentgrass fields each year at the Michigan State University Hancock Turfgrass Research Center in East Lansing, Michigan. Field plots consisted of mature turfgrass grown on a top-dressed native soil (Colwood brookston loam). A light sand topdressing was

brushed into the canopy weekly from May through September to total a depth of 2.0 mm. The site was maintained at a 3.3 mm mowing height and was mown three times weekly. The plots were irrigated nightly to replace approximately 100% potential evapotranspiration to avoid drought stress. Irrigation was withheld when rainfall events exceeded 100% potential evapotranspiration. Potential evapotranspiration was retrieved from the weather station located at the research facility based on the Penman-Monteith Equation (Allen et al., 1998). Each study area was fertilized with 127.2 kg N ha^{-1} each year including foliar feeding 4.9 kg N ha^{-1} weekly June through September of 2014 and 2015. Plots were fertilized with 24.4 kg N ha^{-1} on 2 June and 1 September using a 18-9-18 (N-P-K) granular fertilizer (Andersons Golf Products, Maumee, OH). Fungicides and insecticides were applied preventatively and curatively to avoid turfgrass loss due to disease or insect damage respectively to all plots equally and to not influence the results of the study.

Chemical treatments

All chemical treatments began on 3 October 2016 and on 6 October 2017 and were applied weekly for six weeks (Figure 1). All treatments were applied with a pressure-calibrated backpack sprayer (591 L ha^{-1} at 275 kPa) equipped with four flat fan nozzles (DG8002 DS, Teejet Technologies, Wheaton, IL.) to each field. The foliar chemical treatments included 1) a plant growth regulator product and effective ethylene application containing ethephon [Proxy; Bayer Environmental Science, Research Triangle Park, NC] at a rate of 1.72 L ha^{-1} of ingredient , 2) an ethylene biosynthesis precursor, ACC (Sigma Aldrich., St. Louis, MO) at a concentration of 100µM, 3) a commercially available ethylene biosynthesis inhibitor containing AVG (ReTain, Valent BioSciences Corporation, Libertyville, IL) at a rate of 226 g ha^{-1}, 4) the pure form of AVG (Sigma Aldrich, St. Louis, MO) at a concentration of 2.4 g ha^{-1} and 5) untreated plots were

sprayed with water and were utilized as the control plots. Retain contains 15% of AVG, the active ingredient. The concentration of AVG applied differed between ReTain and pure AVG, in order to test multiple rates. The treatment rate of pure AVG was based on previous literature (Xu and Huang, 2009).

Field plot measurements

Visual assessments and normalized difference vegetative index (NDVI) were determined weekly to document turfgrass performance under field conditions. Turf quality (TQ) was rated visually based on color, density, and uniformity of the grass using a scale of 1 to 9 with 9 representing a fully turgid, dense green canopy and 1 representing completely brown, necrotic and/or sparse plants (Beard, 1964). Canopy reflectance was determined by measuring normalized difference vegetative index (NDVI) with the use of a turfgrass color meter (Field Scout TCM-500; Spectrum technologies Inc. Aurora, IL.).

Canopy ethylene evolution was measured weekly in October and November in 2016 and 2017. Prior to treatments, a 15.2 cm diameter circle was cut into each plot area at 1.27 cm depth for an airtight seal to place a 463 cm^3 circular plexiglass container with septum. Ethylene evolution measurements were obtained by placing the plexiglass container into the turf canopy and allowed to equilibrate for 1 h. A 1-mL gas sample was then withdrawn from the container and subjected to gas chromatographic (GC) analysis as in the methods of Mir et al. (2001). The GC instrument (Carle Series 400 AGC; Hach Co., Loveland, CO.) was fitted with a stainless-steel column (6-m-long, 2-mm inner diameter) packed with activated alumina and detection was via a flame ionization detector (Mir et al., 2001). The ethylene detection limit was approximately 0.005 $\mu L \cdot L^{-1}$. Ethylene concentrations were calculated relative to a certified standard (Matheson Gas Products, Chicago, IL.) with a concentration of 0.995 $\mu L \cdot L^{-1}$.

Canopy dark respiration rates were measured every week in October and November in 2016 and 2017 at 2 h after sunset by enclosing the turf canopy in a transparent circular plastic chamber (15 cm in diameter × 12 cm deep) attached to an infrared gas analyzer system (LI-COR 6400; LICOR, Lincoln, NE.). The respiration rate was expressed as rate of CO_2 evolution per unit turf area. The dark respiration rate of whole plants with soil and that of bare soil without grass was measured. Respiration rate of plants were determined by subtracting soil respiration rate from that of whole plants with soil (Huang et al., 2000).

Low temperature and ice treatments

Turfgrass plugs (10 cm diameter) were taken by hand with a turf corer from each plot on 11 November 2016 and 25 November 2017 to a depth of 10 cm, after adequate natural plant acclimation. Temperatures optimal for cold hardening were based on Dionne et al. (1999) where plants experienced approximately two weeks of temperatures at or below 2°C. Eight turfgrass plugs were taken from each plot with a total of 160 turfgrass plugs being used for the experiment. Once all plugs were removed from plots, they were immediately planted in plastic pots (10.2 cm diameter and 15.2 cm deep) in sandy loam soil and transferred to a low temperature growth chamber and allowed to acclimate at -2°C for two weeks prior to low temperature treatment. The growth chamber study included 160 individual plugs with five chemical treatments, two ice treatments, and four sampling time points each replicated four times. Growth chamber conditions were set to reproduce a typical overcast winter day. Conditions included a light level of 200 μmol m^{-2} s^{-1} with a 10-h photoperiod and an ambient temperature of -4°C. Treatments inside the growth chamber consisted of no ice or ice cover treatments. Four of the eight plugs taken from each plot were covered by ice by misting deionized water over each pot to form an ice layer (1.3 cm thick). Ice layers were monitored for

depth throughout the duration of the study and water was added by misting if ice layers showed any loss of depth. Eight randomly selected turfgrass plugs from a given chemical treatment were taken out of the low temperature growth chamber at 0, 20, 40, and 80 d from the ice and no-ice covered plant groups.

Percent regrowth

After removal from the freezing chamber, turfgrass plugs were transferred to a chamber where they remained for 7 d to allow for ice melt and de-acclimation. The light and other conditions were the same in the 4°C chamber as they were for low temperature chamber. Plants were then transferred to a greenhouse containing supplemental lighting controlled by a timer. Greenhouse conditions were maintained at average day/night temperatures of 23/16 °C and an average 12-h photoperiod at an average of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation. Visual percent regrowth was observed of surviving plants after a 3, 7, 15, and 20 days of regrowth in the greenhouse. Regrowth was determined by inspecting turfgrass plugs for growing annual bluegrass plants and plants that were not growing. The number of living crowns divided by the number of total crowns (deceased and living) multiplied by 100 achieved the percent regrowth. These numbers were determined by manual counting of crowns and tillers for each plant. This method has been used previously in Laskowski et al. (2019).

Experimental design and statistical analysis

Individual fields were used for creeping bentgrass and annual bluegrass. Individual ANOVA tests were performed on creeping bentgrass and annual bluegrass. Each field was divided into plots based on treatment and were arranged as a completely randomized design with four replications. The growth chamber experiment was conducted as a completely randomized block

design with ice and no ice treatment as the main blocks. Chemical treatment and sampling time were completely randomized within the main block. The experiment was first conducted in 2016/2017 and repeated in 2017/18 using the same growth chambers and greenhouse facilities. All data were subjected to analysis of variance (ANOVA) using SAS 9.4 (SAS institute Inc., Cary, NC) mixed model procedure. Normality of data was tested by the Shapiro-Wilk method while homogeneity was tested by the Levene's test. For analyzing the data, time (year and week) was a fixed factor in the model in the field and growth chamber experiment. All data was normally distributed and indicated that ANOVA assumptions were met. Differences between treatment means were separated by Fisher's protected least significance difference test at the 0.05 *P* level.

Results

Species by chemical treatment interactions were only significant while measuring ethylene production. Chemical treatment by days after initial treatment were significant among all measurements and significant three-way interaction of species by chemical treatment by days after initial treatment were present among all measurements except for regrowth in which only annual bluegrass was analyzed (Table 4 and 5:).

Environmental conditions

Typical fall central Michigan weather conditions were recorded during the chemical treatments in the field, which indicates plants acclimated naturally during treatments (Figure 4:). Environmental conditions between 2016 and 2017 were similar. Daily minimum temperatures were similar between years. However, between 30 October and 13 November the maximum daily temperature was warmer in 2016 than 2017 (Figure 4:).

Turf quality

In the field for creeping bentgrass, ethephon application resulted in lower turf quality than the untreated control on all sampling days 21 through 42. For example, ethephon treated creeping bentgrass had 30% lower turf quality when compared to the untreated control on 35 d after initial treatment (Figure 5B:). In annual bluegrass, ethephon treatment caused 31 to 50% lower turf quality from 21 to 42 d after initial treatment when compared to the untreated controls (Figure 5A:). ACC treated annual bluegrass had 12 and 13% higher turf quality when compared to the untreated control on 28 and 35 d after initial treatment, respectively. AVG and ReTain did not cause consistent significant differences in turf quality in annual bluegrass or creeping bentgrass (Figure 5A and 5B:).

Normalized difference vegetation index

During acclimation, annual bluegrass NDVI decreased over time while creeping bentgrass NDVI increased during the acclimation period (Figure 5C and D:). Ethephon treated creeping bentgrass and annual bluegrass plots had lower NDVI when compared to the untreated control starting approximately 21 d after the first treatment. On day 35 after the initial treatment, ethephon-treated annual bluegrass and creeping bentgrass had 6.0 and 5.6 % lower NDVI than their untreated controls, respectively (Figure 5C, D:). Treatments such as ACC had lower NDVI when compared to the untreated control on day 14, 21, and 28 after the first treatment in annual bluegrass. When compared to the control, annual bluegrass treated with Retain or AVG had greater NDVI on day 42 after the first treatment.

Canopy ethylene evolution

During the acclimation period, ethylene production increased in annual bluegrass to 14 d after the first treatment for all chemical treatments and then decreased over time when compared to 14 d. Annual bluegrass had an average of 74% more ethylene production when compared to creeping bentgrass. Chemical ethylene promotive treatments affected ethylene production to different extents in the two turfgrass species. Ethephon treated annual bluegrass plots had greater ethylene production when compared to the untreated controls on several dates. For instance, 42 d after initial chemical treatment, annual bluegrass treated with ethephon had 145% greater ethylene production compared to untreated control annual bluegrass. ACC treated annual bluegrass had 128, 38 and 144% greater ethylene produced when compared to the untreated control on 28, 35, and 42 d after initial treatment, respectively (Figure 6A:). In creeping bentgrass, ethephon treated plots had 52% greater ethylene production on 35 d and 78% greater ethylene production 42 d after initial treatment.

Ethylene inhibition treatments differentially altered ethylene production in the two species and had differences in effectiveness. Ethylene evolution levels were not significantly different than controls in Retain treated plants throughout the 42 d of evaluation. On 35 and 42 d, ethylene evolution was significantly higher due to AVG treatment compared to controls and Retain treated annual bluegrass. Treatment with AVG had greater ethylene production after 35 and 42 d in annual bluegrass when compared to the untreated control. Creeping bentgrass plots treated with AVG did not significantly differ from untreated control plots. Retain treated annual bluegrass and creeping bentgrass did not significantly differ in ethylene production from the untreated controls.

Respiration

In both annual bluegrass and creeping bentgrass plots, CO_2 production decreased over time. For example, CO_2 concentration decreased by 77% from 0 to 42 d after initial treatment in annual bluegrass (Figure 6C:). Ethephon treated plots overall had greater CO_2 production compared to untreated control plots in both annual bluegrass and creeping bentgrass. For instance, ethephon treated plots had 60% greater CO_2 production than untreated controls on 14 d after initial treatment in creeping bentgrass and 42% greater CO_2 production when compared to the untreated control on 28 d after initial treatment in annual bluegrass (Figure 6D:). No consistent significant differences in canopy respiration rates were detected in either species due to ReTain and AVG treatment compared to controls (Figure 3C, D:).

Regrowth

After 0 and 20 d at -4°C under ice and no ice conditions, ethephon treated plants had less regrowth when compared to the untreated control (Figure 7:). Under no ice cover conditions, ReTain treated plants had greater regrowth when compared to the untreated control after 40 d (Figure 7:). After 80 d at -4°C, ReTain treated plants had greater regrowth when compared to the untreated control while ACC treatment had less regrowth than the untreated control under ice cover. Compared to the untreated control, ReTain treated annual bluegrass had 47% and 110% more regrowth after 10 and 80 d under ice cover, respectively. Under non-ice-covered conditions after 80 d, ReTain and AVG treated plants had greater regrowth when compared to the untreated control while ACC treatment had less regrowth when compared to the untreated control (Figure 7:).

Discussion

In this study, we have found that ethylene may reduce tolerance to ice and low temperature in annual bluegrass plants. We also found that ethylene production is differential for annual bluegrass, a winter sensitive species, and creeping bentgrass, a more winter tolerant species, during acclimation. Our results are in line with a study by Fiorani et al (2002) that compared multiple *Poa* sp and found *Poa annua* to have a high amount of ethylene production, as one of the Poa species with a fast growth rate. Thus, it may be possible that the enhanced production of ethylene during acclimation by annual bluegrass may play a role in its sensitivity to winter conditions. Ethylene, the gaseous plant hormone, has been observed to increase in plants in response to various stresses (Nilsen and Orcutt, 1996), but how ethylene regulates acclimation to cold stress and tolerance of winter conditions is not well understood. Ethylene has been shown to be repressed during acclimation in some plant species such as the legume, *Medicago truncatula*. In that *Medicago* species, inhibition of ethylene by biosynthesis inhibitors was found to enhance freezing tolerance (Zhao et al., 2014). Similarly, ethylene reduced freeze tolerance in other species such as Arabidopsis, as ethylene was shown to negatively regulate plant responses to freezing stress (Shi et al., 2012). Conversely, ethylene may have improved plant tolerance of freezing stress by increasing antifreeze protein expression in winter rye plants (*Secale cereal;* Yu et al., 2001).

Additional evidence for ethylene causing winter sensitivity in annual bluegrass is shown through our results of the ethylene application treatments of ethephon and ACC prior to acclimation. Ethephon and ACC treatments were most effective at promoting ethylene evolution from field plots on 14 and 28 d after treatment. There was a slight decrease in ethylene evolution by 35 and 42 d, indicating that the effects of these compounds may have been wearing off by that time or this decrease was due to acclimation progression towards dormancy. On a few dates,

ethephon or ACC treatment reduced regrowth from ice or low temperature conditions in annual bluegrass plants (Figure 4). Ethephon is a commonly used chemical in turfgrass management for annual bluegrass flowering and seed head control (Askew, 2017). Based on our TQ results comparing ethephon (applied as Proxy) to ACC, the turf quality in the fall was more affected by Proxy than ACC, thus it is possible that other constituents in that product could have caused toxicity unrelated to ethylene effects. Turfgrass managers that may be using ethephon, particularly as a replacement for mefluidide, for control of annual bluegrass flowering may see a decrease in turf quality depending on environmental conditions and concentration of ethephon applied. The study is the first to show that ethephon treatments potentially could reduce spring regrowth following winter conditions either under no ice or ice-covered conditions at the rates used in our study. It is possible that turfgrass managers in northern climates may need to be cautious of recommendations for annual bluegrass seedhead control using ethephon during the fall; however, additional field testing for ice survival and spring regrowth is needed.

Interestingly, annual bluegrass was highly responsive to ethephon and ACC treatments in ethylene production, whereas creeping bentgrass was less sensitive to these treatments. Creeping bentgrass produced more ethylene than controls in response to ethephon treatment, but the increases were less pronounced than those detected for annual bluegrass. Creeping bentgrass ethylene production was not influenced by ACC application. This may mean that ethylene regulatory treatments may be effective for annual bluegrass but not have major effects on creeping bentgrass, which is important to note when trying to identify management strategies for mixed stand putting greens.

A potential strategy to improve annual bluegrass tolerance of ice and low temperature conditions may be to inhibit ethylene prior to fall acclimation. We did not detect significant

differences in canopy ethylene production in the field in response to AVG and ReTain treatment compared to control plants. It is not clear why no differences occurred, but it is possible that more controlled environmental conditions may have been needed to detect reductions in ethylene production. Thus, additional research on the effects of ethylene inhibitors on ethylene production in annual bluegrass is needed. Improvements in annual bluegrass tolerance of ice in response to ethylene inhibition treatment were detected since plants treated with ReTain exhibited enhanced regrowth after ice cover and no ice cover compared to controls following the low temperature growth chamber treatments. It is possible that ethylene inhibitory treatment rates and frequencies could be adjusted in future studies to see more of a field response and amplified tolerance to winter conditions. The difference in response in ReTain compared to AVG treatments is likely due to the rate of AVG used and potential surfactants or other ingredients in Retain to promote absorption of AVG. Our research also shows that ReTain did not decrease turf quality in the field when compared to the untreated controls. ReTain, has been tested previously in the southern U.S. to determine the maximum tolerable concentration that can be applied to creeping bentgrass without phytotoxicity symptoms (Strunk et al., 2010). Consistent with our work, their research showed that applying a concentration of 226 g ha^{-1} ReTain or less did not decrease turf quality.

How ethylene influences plant respiration rates are important for understanding acclimation and overwintering potential, as respiration rates will indicate how slowly or quickly plant overwintering tissues, such as crowns, may deplete carbohydrate stores. Treatment of potato tubers (*Solanum tuberosum* L.) with ethylene gas caused a rapid rise in their respiration rate, reaching 5 to 10 times the rate of untreated tubers over 30 hours of treatment and then falling slowly (Reid and Pratt, 1972). In annual bluegrass and creeping bentgrass, respiration rates decreased overtime in response to turf acclimation, regardless of whether they were treated

for ethylene regulation or left untreated. For both species, treatment with ethephon caused plants to maintain higher rates of respiration than controls throughout the duration of the acclimation period, which could have played a role in the reduction of ice and low temperature tolerance found after plants were subjected to the simulated winter conditions in the growth chamber.

Low temperature without ice cover treatment caused a decrease in annual bluegrass regrowth during the recovery period. The duration at -4°C in the low temperature growth chamber cannot be directly correlated to freezing tolerance or duration of cold temperature survival in the field in previous literature. This is because the conditions in the chamber are more severe than under field conditions since the full soil profile freezes in the chamber whereas soil temperatures in the field would not get much below freezing. Retain (ethylene inhibition) increased annual bluegrass tolerance of low temperature conditions and ACC reduced tolerance at 80 d of -4°C. These treatment results are in line with Munshaw et al. (2010) and Shi et al. (2010) that found ethylene reduced low temperature tolerance or freeze tolerance in a turfgrass species and in *A. thaliana*, respectively. Our results contrast with the work of Yu et al. (2001) that showed ethylene improved freezing stress tolerance in winter rye plants. Additional research on low temperature tolerance and ethylene regulation is needed in more plant species prior to practical application of this method.

In conclusion, natural ethylene evolution and ethephon applications during fall acclimation could be detrimental to annual bluegrass winter survival and spring regrowth, depending on the severity of winter conditions. Ethephon treated annual bluegrass plants were found to have decreased regrowth under ice and non-ice-covered conditions. Treatment of annual bluegrass with ethylene inhibitory products, such as ReTain, may be a viable way of increasing annual bluegrass spring regrowth in low temperatures if ice cover persists in the winter. Pure

AVG at the rate used in this study was not effective in promoting regrowth from ice and freezing temperatures. Additional research on application timing, method, frequency, and optimal rates of AVG are needed. A better understanding of the physiological mechanisms associated with ethylene regulation and turfgrass overwintering is also needed.

APPENDIX

Figure 4: *Daily minimum and maximum temperature for the Hancock Turfgrass Research Center in East Lansing, MI, from A) 1 October 2016 to 1 December 2016 and B) 1 October 2017 to 1 December 2017.* Black filled in arrows indicate when turfgrass plugs were taken from annual bluegrass plot areas. Black outlined small arrows indicate when a chemical application was made.

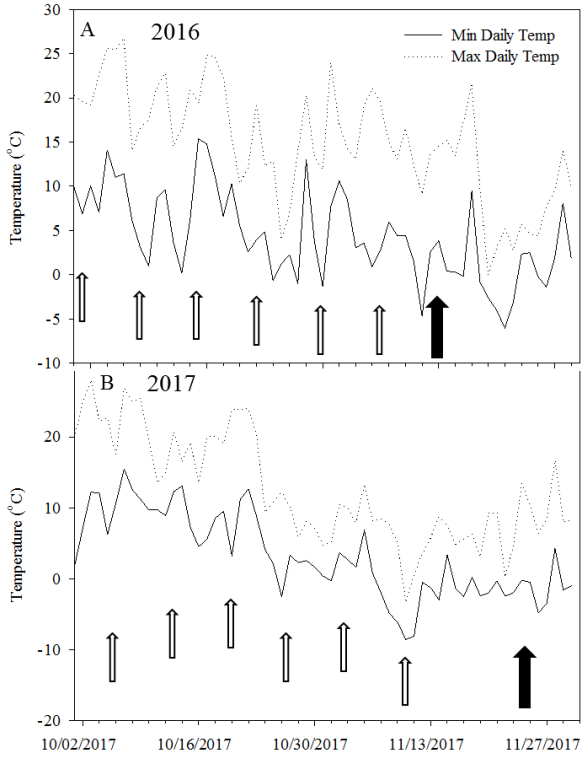

Figure 5: *Visual turfgrass quality (scale of 1-9 with 9 = healthy, 1 = necrotic) of A) annual bluegrass and B) creeping bentgrass and normalized difference vegetation index (NDVI) of C) annual bluegrass and D) creeping bentgrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, ReTain, aminoethoxyvinylglycine (AVG), or untreated.* Means from both 2016 and 2017 are pooled together. Least significant difference (LSD) values are indicated by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment. Asterisks indicate when significant differences occurred in one or more of the treatments over time compared to day 0.

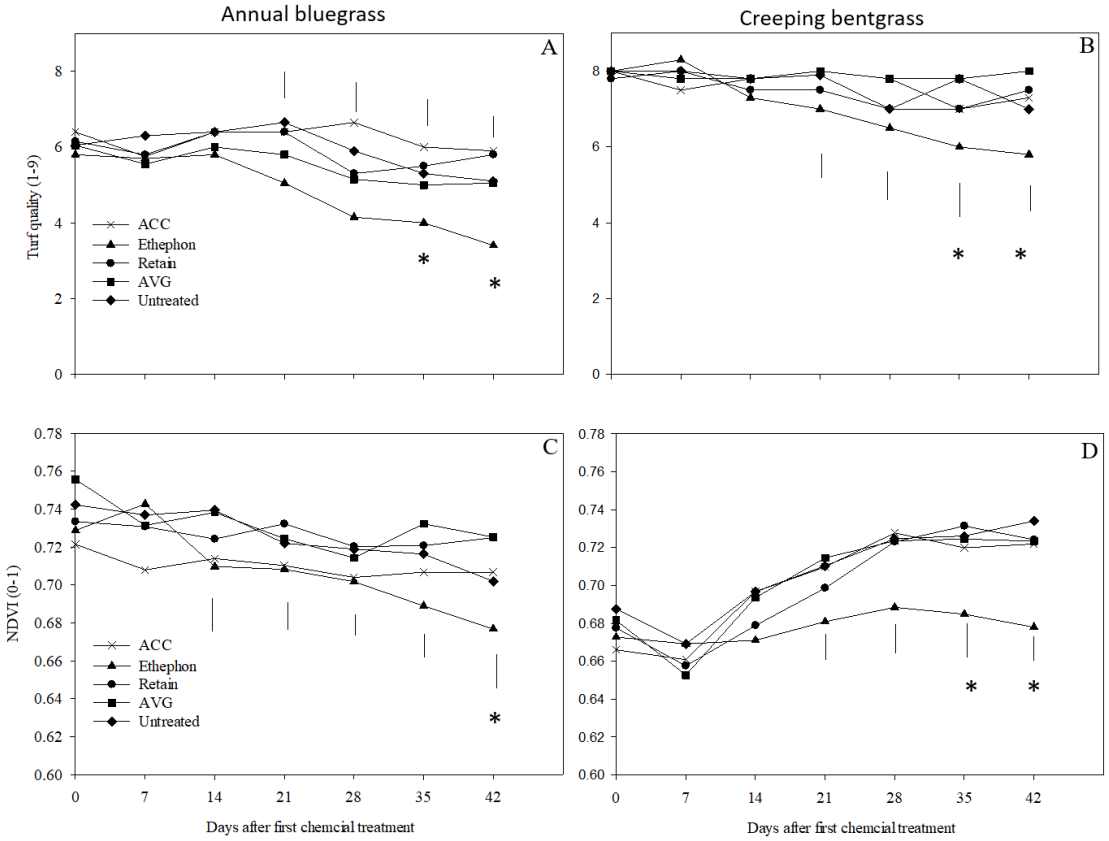

Figure 6: *Ethylene gas production of A) annual bluegrass and B) creeping bentgrass and turfgrass whole plant respiration of C) annual bluegrass and D) creeping bentgrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, ReTain, aminoethoxyvinylglycine (AVG), or untreated.* Means from both 2016 and 2017 are pooled together. Least significant difference (LSD) values are indicated by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment. Asterisks indicate when significant differences occurred in one or more of the treatments over time compared to day 0.

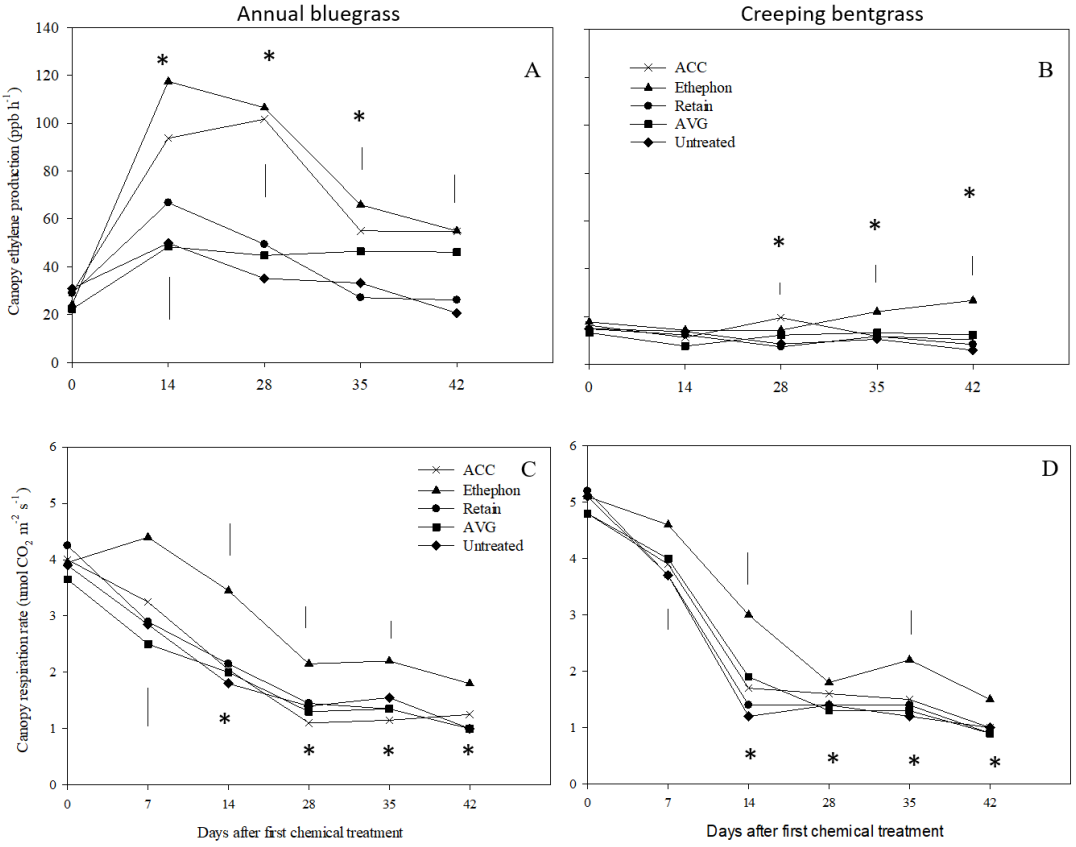

Figure 7: *Regrowth of annual bluegrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, ReTain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in A) ice covered or B) non-ice-covered treatments.* Means from both 2016 and 2017 are pooled together. Means with the same letter on a given rating day are not significantly different based on least significant difference values ($P \leq 0.05$). Capital letters represent least significant difference values ($P \leq 0.05$) between sampling dates.

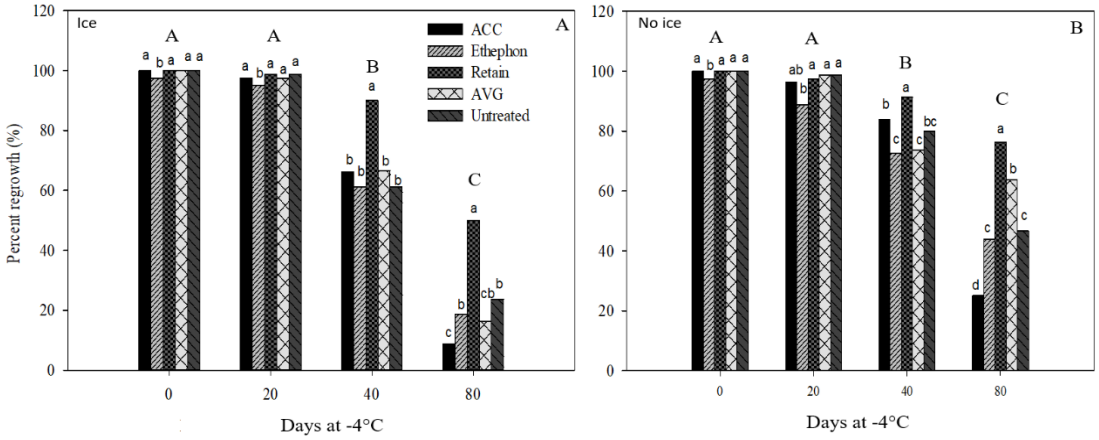

Table 4: *Analysis of variance for main treatment factors and interactions of turf quality, normalized difference vegetative index (NDVI), respiration as measured by CO_2 evolution from field plots, canopy ethylene production and percent regrowth of annual bluegrass in field conditions in East Lansing, MI during 2016 and 2017.*

Effect		Quality	NDVI	Respiration	Ethylene	Percent regrowth
Rep						
Chemical treatment (T)		***	***	***	***	***
Days after initial treatment (D)		***	***	***	***	*
T x D		**	**	*	**	***

* p values ≤ 0.05
** p value ≤ 0.01
*** p value ≤ 0.001
ns = not significant $P > 0.05$.

Table 5: *Analysis of variance for main treatment factors and interactions of turf quality, normalized difference vegetative index (NDVI), respiration as measured by CO_2 evolution from field plots, and canopy ethylene production of creeping bentgrass in field conditions in East Lansing, MI during 2016 and 2017.*

Effect		Quality	NDVI	Respiration	Ethylene
Rep					
Chemical treatment (T)		***	***	***	***
Days after initial treatment (D)		***	***	***	***
T x D		**	**	*	**

* p values ≤ 0.05
** p value ≤ 0.01
*** p value ≤ 0.001
ns = not significant $P > 0.05$.

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CHAPTER 4

INFLUENCE OF ICE AND ETHYLENE REGULATION ON CELLULAR PROTECTION MECHANISMS IN ANNUAL BLUEGRASS

Abstract

Annual bluegrass (*Poa annua* var *reptans*), when grown as a putting green species, is sensitive to winter injury such as ice cover. Regulating annual bluegrass ethylene production alters annual bluegrass tolerance of ice. The goals of this study were to determine how winter conditions and ethylene regulatory treatments affect the antioxidant system, fatty acid composition, and apoplastic proteins of annual bluegrass plant tissues. Ethylene promotive [1-aminocyclopropane-1-carboxylic acid (ACC) or ethephon] and ethylene inhibition treatments [Retain or aminoethoxyvinylglycine (AVG)] were applied to plants in the field during acclimation. Plant plugs were taken and subjected to low temperature and ice treatments in growth chamber conditions. Antioxidant activities of ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were measured along with malondialdehyde content (MDA) and apoplastic protein content in leaf and crown tissue. Saturated and unsaturated fatty acids (FA) contents were measured in leaf, crown, and root tissue. Compared to the untreated controls, ethephon treated annual bluegrass had greater MDA contents, lower POD and SOD activity, greater saturated and decreased unsaturated FAs. Ethylene inhibition treatments caused annual bluegrass to have less saturated FA content and greater unsaturated FA content, a greater content of apoplast proteins, and higher CAT activity when compared to the untreated controls. The activity of APX was greater in AVG treated annual bluegrass than in controls. Ethylene may reduce physiological health overwinter and inhibitory treatments may promote winter tolerance by promoting antioxidant activity, apoplast proteins, and the content unsaturated fatty acids in plant tissues.

Keywords: *Poa annua*, ice encasement, plant growth regulator, acclimation, ice stress, turfgrass, annual bluegrass

Introduction

The process of acclimating to winter conditions and overwintering are both important to the survival of perennial plants in temperate areas. Despite appearances that plants are doing nothing during winter dormancy, limited physiological processes such as respiration are still occurring (Ogren, 2000). Those physiological processes need to be altered during acclimation and protected during winter to maintain functionality under harsh winter conditions for spring regrowth. Plants can protect overwintering tissues via several mechanisms including sugar concentration gradients, changes in fatty acid profiles and antioxidants (Samala et al 1998). Evaluating how cellular protection mechanisms may be affected by winter stresses in species sensitive to winter and determining whether management practices can improve cellular protection mechanisms is important for developing strategies to reduce winter associated damages.

Annual bluegrass (*Poa annua* var *reptans*), when maintained in a perennial growth habit typical in turfgrass management, is an ice encasement sensitive species. It has been reported to survive an average of 60 d of ice cover (Vargas and Turgeon, 2004; Beard, 1964; Tompkins et al., 2004), which contrasts to creeping bentgrass (*Agrostis stolonifera*), which can survive under ice cover for up to 120 d (Beard, 1964). Annual bluegrass and creeping bentgrass are typically found as pure or mixed stands on golf course putting greens and fairways. Previously, we have found that treatments that inhibit ethylene improved annual bluegrass recovery following low temperature and ice conditions and ethylene promotive treatments reduced or had no effect on winter recovery, depending on the duration (Laskowski and

Merewitz, 2019). As inhibiting ethylene may be a viable management practice that could be used to protect annual bluegrass or other turfgrass species during winter dormancy, it is important to understand the mechanism associated with improved tolerance. Additionally, how ethylene regulates acclimation and overwintering is still not fully understood.

Ethylene, the plant hormone commonly associated with stress responses and signaling, is primarily understood for plant stresses such as flooding or submergence (Fukao et al., 2006), thigmomorphogenesis (Biro and Jaffe, 1984) and pathogen responses (Lund et al., 1998). How ethylene plays a role in acclimation and overwintering is less well understood. Ethylene effects on cold or other winter stress tolerances may be based on plant species or environmental conditions since contrasting results have been found in various studies (Munshaw et al., 2010; Shi et al., 2012; Yu et al., 2001; Szalai, 2000; Zhao et al., 2014). It is clear ethylene has a major effect on plant acclimation and tolerance of winter conditions, but a better understanding of the physiological mechanisms associated with ethylene regulation is needed.

One major change that can occur during acclimation to cold is the alteration of cell membrane composition (Shang et al., 2006). Generally, plants more tolerant of winter conditions, including ice encasement, accumulate more unsaturated FAs than saturated fatty acids in their membranes (Shang et al., 2006; Hetherington et al., 1987; Dalmannsdottir, et al., 2001). Shifts in FA profiles occurred in annual bluegrass plants under different fall plant growth regulator treatments during acclimation (Laskowski et al., 2018). Thus, measurement of FA profiles may be a good indicator for the effects of ethylene regulatory treatments on annual bluegrass acclimation and survival of ice stress.

We hypothesized that ethylene inhibition-induced improvements in annual bluegrass recovery from ice stress could be associated with cellular protection mechanisms such as a

promotion or maintenance of antioxidant enzyme activities, increased unsaturated fatty acid contents, and/or accumulations in apoplastic proteins. Therefore, the objectives of this study were to evaluate ethylene promotive treatments [ethephon (2-chloroethyl phosphonic acid) and aminocyclopropane-1-carboxylic acid (ACC)] and ethylene inhibition treatment [aminoethoxyvinylglycine (AVG)] effects on annual bluegrass responses to winter stresses.

Materials and Methods

Plant material and experimental treatments

Plant materials used in this study were generated from the same experiment described in Laskowski and Merewitz (2019). Briefly, turfgrass plants were taken following acclimation in the fall of 2016 and 2017 from a mature field of annual bluegrass growing at the Hancock Turfgrass Research Center, East Lansing MI. The foliar chemical treatments included 1) ethephon (Proxy; Bayer Environmental Science, Research Triangle Park, NC) at a rate of 7.96 L ha^{-1}, 2) an ethylene biosynthesis precursor, ACC (Sigma Aldrich., St. Louis, MO) at a rate of 100 µM, 3), 3) a commercially available ethylene biosynthesis inhibitor containing AVG (ReTain, Valent BioSciences Corporation, Libertyville, IL) at a rate of 226 g ha^{-1} and 4) the pure form of AVG (Sigma Aldrich, St. Louis, MO) at a rate of 25 µM. Untreated plots were sprayed with water and were utilized as the control plots. Eight turfgrass plugs were taken from each plot (five chemical treatments, five sampling time points with four replications) for a total of 160 turfgrass plugs being used for the experiment. The plugs were planted in plastic pots (10.2 x 15.2 cm) in a sandy loam soil and put in a low temperature growth chamber. The plants acclimated to growth chamber conditions at -2 °C for two weeks prior to stress treatment. Growth chamber conditions included a light level of 200 µmol m^{-2} s^{-1} with a 10-h photoperiod and an ambient temperature of

-4°C. Treatments inside the growth chamber consisted of 1) no ice or 2) ice cover treatments. Ice cover treatment involved misting deionized water over pots to form an ice layer (1.3 cm thick), with periodic misting to prevent ice loss. Four randomly selected pots within each treatment were taken out from the low temperature growth chamber on 0, 20, 40, and 80 d of ice or no-ice treated plants. On each sampling day, plants were destructively sampled, and samples were manually split into leaves, crowns, or roots and immediately frozen in liquid nitrogen. All samples were then placed in a freezer (-80 ºC) until further analysis.

Fatty acid analysis

Extraction of FAs was performed with approximately 200 mg (fresh weight) crown, leaf, or root tissue from ice covered and non-ice-covered samples at 0, 20, 40, and 80 d according to the method of Cyril et al. (2002) with modifications. Frozen crown material was transferred into test tubes containing 3 mL of preheated isopropanol (75 °C) with 0.01% butylated hydroxytoluene (BHT). Samples were placed in a 75°C water bath for 15 min. After the samples had cooled, 1.5 mL chloroform and 0.6 mL distilled water was added and the samples were capped and shaken at room temperature for 5 h. After 5 h, the lower layer containing chloroform and the lipids were transferred into new test tubes. An additional 4 mL of chloroform/methanol (2:1) with 0.01% BHT was added to the tubes containing the crown material. The tubes were recapped and placed on a shaker for 15 h. After extraction, 1 mL KCL was added to the tubes containing the extracted lipids and chloroform and centrifuged at 5000 × g for 10 min. After 10 min, the top layer was removed, 2 mL of distilled water was added, and the tubes were centrifuged for an additional 10 min at 5000 × g. The top thin layer was removed and the remaining sample was evaporated using vacuum centrifugation. Samples were preserved in 1 mL chloroform and stored at –80°C until analysis. Remaining crown material was dried in an oven 70°C for determination of dry weight.

Gas chromatography mass spectroscopy was performed at the Mass Spectrometry and Metabolomics Core at Michigan State University utilizing a mass selective detector (5975 inert XL MSD detector; Agilent Technologies, Santa Clara, CA). Separation and quantification of FAs was achieved by injection of 1 μL of extract into a column (VF-5ms; 30 m × 0.25 mm × 0.25 μm; Agilent) using the following temperature profile: 30°C for 4 min; 10°C min^{-1} to 320°C; 320°C for 2 min.

Antioxidant activity and lipid peroxidation

Antioxidant activity was determined by using 250 mg of leaf and crown tissue which was ground to a fine powder using a mortar and pestle and extracted using 4 mL of extraction buffer [50mM potassium phosphate buffer (pH 7.0), and 1% polyvinylpyrrolidone]. The extractions were centrifuged at 15,000 gn for 30 min at 4°C and supernatant was collected for subsequent enzyme assay and quantification. Ascorbate peroxidase (APX) activity was measured based on the oxidation of ascorbate as described by Nakano and Assada (1981) with modifications. The reaction solution (3 mL) contained 100 mM sodium acetate (pH 5.8), 0.003 mM ethylenediaminetetraacetic acid, 10 mM ascorbate, and 100 mL leaf extract. The absorbance changes at 290 nm were measured every 10 s for 60 s using a spectrophotometer (Genesys 10S; Thermo Scientific, Madison, WI). The absorbance change of 0.01/min was taken as one unit of APX activity. The peroxidase (POD) activity was measured by monitoring the increase in absorbency at 460 nm every 10 s for 60 s as guaiacol was oxidized according to the method of Chance and Maehly (1955). The POD reaction solution (3 mL) contained 0.1 M sodium acetate buffer (pH 5.0), 0.25%guaiacol (resolved in 50% ethanol), 0.75% H_2O_2, and 100 mL of leaf extract. CAT activity was measured by the rate of decomposition of H_2O_2 at 240 nm in 3 mL of reaction mixture consisting of 50mM sodium phosphate buffer and 45 mM H_2O_2 (Chance and

Maehly 1955). SOD activity was measured by its ability to inhibit p-nitro-blue tetrazolium chloride reduction at 560 nm (Giannopolitis and Ries 1977).

Lipid peroxidation level was determined based on malondialdehyde (MDA) content using the method of Dhindsa et al. (1981) with modifications. A 1.0-mL enzyme solution was added to 2 mL of reaction solution containing 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The solution was heated in a water bath at 95°C for 30 min, quickly cooled on ice, and centrifuged at 10,000 gn for 30 min. The absorbance readings were taken at 532 and 600 nm. The nonspecific absorbance at 600 nm was subtracted from absorbance at 532 nm and MDA content was calculated using the adjusted absorbance and extinction coefficient of 155 mM–1cm-1 (Heath and Packer 1968).

Apoplastic protein content

Measuring apoplastic protein concentration can indicate plant cold and freeze tolerance, as more proteins in the apoplast could reduce ice crystal formation, particularly if they have antifreeze properties(Griffith et al., 1992). Apoplastic proteins, assumed to play a role in cellular antifreeze mechanisms, were extracted as by methods in Hon et al. (1994). After harvesting and separating leaf and crown tissue, tissues were rinsed with deionized water and then vacuum-infiltrated with a 20 mM ascorbic acid and 20 mM $CaCl_2$ solution. This was then centrifuged at 900 g to recover the protein content. The total apoplastic protein content was measured using a Bradford (1976) method assay utilizing bovine serum albumin as the standard protein.

Experimental Design and Statistical analysis

Field plots were arranged as a completely randomized design with four replications. The growth chamber experiment was conducted as a completely randomized block design with ice treatment

as the main block. Chemical treatment and sampling time were completely randomized within the main block. The experiment was repeated using the same growth chamber. All data were subjected to analysis of variance (ANOVA) using SAS 9.4 (SAS institute Inc., Cary, NC) mixed model procedure. For analyzing the data, time (year and week) was a fixed factor in the model. As ANOVA determined no yearly interaction, data from multiple years was pooled together.

Results

MDA content

Prior to ice cover at 0 d, ethephon treatments had greater MDA when compared to the untreated control in leaf tissue. After 20 and 40 d of ice-covered annual bluegrass ethephon treatment had 154 and 79% more lipid peroxidation when compared to the untreated control respectively in leaf tissue (Figure 8A:). After 20 and 40 d of non-ice-covered annual bluegrass, ethephon treatment had 110 and 62.8% more malondialdehyde content when compared to the untreated control respectively in leaf tissue (Figure 8B:). In crown tissue under ice and no ice cover, ethephon treatment had greater malondialdehyde content when compared to the untreated control on 0, 20 and 40 d (Figure 8C and 8D:).

Catalase activity

In leaf tissue of ABG, AVG treatment increased CAT after 40 and 80 d of non-ice-covered samples by 17.9 and 34.1% respectively when compared to the untreated control (Figure 9B:). Retain and ACC treated plants were not significantly different in CAT activity than the AVG treatment. In leaf tissue that was covered by ice, AVG treatment increased CAT activity by 40.6% after 80 d when compared to the untreated control (Figure 9A:). In crown tissue, AVG

and Retain treated ABG had greater CAT when compared to the untreated control after 40 d of no ice cover (Figure 9D:). CAT was 74.5% greater in samples treated with AVG when compared to the untreated control after 40 d without ice cover. After 80 d with ice cover, Retain treated ABG had 34.9% more CAT activity when compared to the untreated control (Figure 9C:).

Superoxide dismutase activity

Prior to ice treatment, ethephon treatment had less superoxide dismutase activity when compared to the untreated control in leaf tissue of ABG. After 40 and 80 d of ice cover, SOD activity of ABG was 32 and 38% less for ethephon treatment respectively when compared to the untreated control (Figure 10A:). After 20 d with no ice cover, ethephon treatment had 27.9% less SOD activity when compared to the untreated control while AVG treatment had 16.7% more SOD activity in leaf tissue (Figure 10B). After 80 d with no ice cover ethephon treatment had 40.9% less SOD activity in leaf tissue when compared to the untreated control. In crown tissue after 40 and 80 d of ice cover, ACC treatment had 26.6% and 19.2% greater SOD activity when compared to the untreated control (Figure 10C:). While the AVG treatment after 80 days of ice cover had 42.9% less SOD activity. After 80 d of no ice cover, AVG had 31% more SOD activity when compared to the untreated control while ethephon treatment had 37.4% less SOD activity in crown tissue (Figure 10D:).

Peroxidase activity

Ethephon treatment decreased peroxidase when compared to the untreated control prior to ice cover (0 day) by 24.2% in leaf tissue. After 20 days of ice cover, ethephon treatments had 28.3% less peroxidase activity when compared to the untreated control in leaf tissue (Figure

11A:). After 80 days of ice cover, ethephon treatment had 32.3% less POD activity when compared to the untreated control while the AVG treatment had 26.4% higher POD activity in leaf tissue when compared to the untreated control. Retain treated ABG as had higher POD activity when compared to the untreated control at 80 d of ice cover in leaf tissue. In crown tissue prior to ice cover ethephon treatment had 26.6% less POD activity. Ethephon treatment in crown tissue after 20 and 80 d of ice cover had 42.3 and 52% less POD activity respectively when compared to the untreated control (Figure 11C:).

Ascorbate peroxidase activity

Few significant differences were detected among treatments of APX activity. In leaf tissue, AVG treatment increased APX activity after 80 d by 40 and 160% in ice cover and non-ice-covered treatments, respectively (Figure 12A:). In crown tissue, AVG treated plots had greater APX activity than controls after 80 d in non-ice covered ABG after 40 and 80 d (Figure 12D:).

Apoplastic proteins

Apoplastic protein concentrations increased in leaf and crown tissue over time. In leaf tissue, apoplastic protein concentration increased until about 40 d of low temperature treatment. In crown tissue, apoplastic protein concentrations increased up to 80 d of low temperature treatment. After 20, 40 and 80 d at -4^{o}C, Retain and AVG treated plants had greater apoplastic protein concentrations when compared to the untreated control in leaf tissue (Figure 13A:). In crown tissue on day 80, all treatments applied to ABG had greater apoplastic protein concentrations when compare to the untreated control. For example, Retain treated ABG had

40% greater apoplastic protein concentration when compared to ethephon treated ABG (Figure 13B:).

Fatty acids

Six fatty acids, including unsaturated and saturated, were detected in leaf, crown and root tissues of annual bluegrass plants. The saturated fatty acids included palmitic acid (16:0) and stearic acid (18:0). The monounsaturated fatty acids were palmitoleic acid (16:1) and oleic acid (18:1) and the polyunsaturated fatty acids were linoleic acid (18:2) and α-linolenic acid (18:3). The fatty acid hexadecadienoic acid (16:2) was not observed in measurable quantities. Significant effects from PGR treatments were observed on fatty acid composition of ABG crown membrane profiles and were consistent for each time point of sampling (0, 20, 40, or 80 d of low temperature or ice treatment). Ice treatment duration and ice treatment did not change fatty acid composition within a given treatment. Statistical analysis indicated differences among sampling day resulting in data being presented separately by sampling day. Ice cover treatment was not statistically significant resulting in data being pooled together for ice cover treatment.

Prior to ice cover and freezing, 0 d leaf, crown and root tissue fatty acids were analyzed. Overall, ethylene inhibition treatments decreased saturated fatty acid concentrations and increased unsaturated fatty acid concentrations in leaf and crown tissue. In leaf tissue, Retain treated ABG had increased oleic acid content when compared to the untreated control (Table 6:). In crown tissue at 0 d, Retain treated ABG had lower palmitic acid and stearic acid when compared to the untreated control. For example, Retain treated ABG had 16% lower palmitic acid and 19% lower stearic acid content in crown tissue on 0 d when compared to the untreated

control (Table 5:). In root tissue, both ethephon and ACC treated ABG had lower linoleic acid contents when compared to the untreated control (Table 6).

Ethylene inhibitory treatments increased unsaturated fatty acid concentrations in leaf tissue and decreased saturated fatty acid concentrations in crown tissue when compared to the untreated control when measured at 20 d in the low temperature chamber (Table 5 and 6:). Retain treated ABG had greater oleic acid content then the untreated control in leaf tissue (Table 6:). In crown tissue, Retain treated ABG had 13% less palmitic acid content when compared to the untreated control (Table 6:). In root tissue, AVG treated ABG had less stearic acid content when compared to the untreated control. Retain and AVG treated ABG had 25 and 20% less palmitic acid content when compared to the untreated control, respectively in root tissue (Table 5:). In crown tissue, Retain treated ABG had 18% less palmitic acid content and 24% greater palmitoleic acid content when compared to the untreated control after 40 d of low temperature treatment (Table 6). Retain and AVG treated ABG had lower palmitic acid after 80 d of low temperature treatment in both leaf and crown tissue when compared to the untreated control (Table 5:). Retain also had 33 and 19% greater linoleic acid content in leaf and crown tissue when compared to the untreated control respectively and 13% greater linolenic acid content in crown tissue. In root tissue, Retain had 39% and AVG treated ABG had 32% greater oleic acid content when compared to the untreated controls (Table 6:).

Effective ethylene treatment of ABG significantly altered fatty acid composition of leaf, crown, and root tissue. Ethephon treated ABG exhibited lower unsaturated fatty acid concentrations when compared to the untreated control plants after 20 d under low temperature treatment. For example, ethephon treatment had 17% less palmitoleic acid and 23% less oleic acid content when compared to the untreated control in crown tissue (Table 6:). Ethephon

treatment had 39% greater stearic acid when compared to the untreated control after 40 d of low temperature treatment in leaf tissue (Table 5:). Ethephon treated ABG had 32% less oleic and linolenic acid content when compared to the untreated control in root tissue (Table 6:). Ethephon treated ABG had 48% greater stearic acid content in leaf tissue and 18% greater stearic acid content in crown tissue when compared to the untreated controls after 80 d of low temperature treatment (Table 5:).

Discussion

Reducing unwanted flowering and seed heads in annual bluegrass with ethephon is a turfgrass management practice, particularly in southern regions of the US (Askew, 2017). This practice may be detrimental to annual bluegrass growing in colder regions, as some minor, yet still significant reductions in ice recovery were detected in Laskowski and Merewitz (2019) following fall ethephon treatments. This decrease in survival of winter conditions could be associated with the decreased antioxidant activity, increased lipid peroxidation, and higher levels of saturated fatty acids found here in ethephon treated annual bluegrass.

How ethylene and effective ethylene treatments may affect fatty acid changes during cold acclimation is not well understood or may be different in various plant species. Munshaw et al. (2010) found that ethephon treatment in the fall had no effect on freeze tolerance of bermudagrass (*Cynodon* spp.), there were no effects on lipid unsaturation levels, and no changes in the level of linolenic acid due to ethephon treatment were detected. Linolenic acid may be an important fatty acid for grass cold tolerance, as it was found to be differential in bermudagrass varieties differing in cold tolerance (Cyril et al., 2002). Here, for annual bluegrass, ethephon treated plants had greater saturated fatty acid content when compared to the untreated controls.

Similar results were found for *Chlorella vulgaris* in response to ethephon treatment (Kim et al., 2016). Harber and Fuchigami (1989) and Lyons and Pratt (1964) discuss findings indicating that increased ethylene levels resulted in greater levels of fatty acid unsaturation. Further testing of ethephon under ice conditions in the field may be warranted to determine whether there are any changes in winter sensitivity following fall applications of ethephon. Additionally, more research on how ethylene is associated with fatty acid changes, perhaps fatty acid desaturase enzyme activity, in grass species under stress conditions is needed.

Ethylene inhibition improved turfgrass recovery from simulated winter conditions (Laskowski and Merewitz, 2019). Ethylene inhibition treatment caused changes in fatty acid content, as some increases in unsaturated fatty acid concentrations and some saturated fatty acids decreases were detected. Retain treated annual bluegrass had greater linolenic concentrations when compared to the untreated controls after 80 d of ice or no ice conditions. Alterations in linolenic acid may not always be required for cold tolerance (Roche, 1979), but it does seem to play an important role in some grass species responses to cold (Cyril et al., 2002). Compared to fruit tissue, more research is needed on leaf and crown tissue responses in fatty acid changes due to ethylene inhibition treatments and how the treatments may promote abiotic stress tolerance.

In addition to fatty acid changes, ethylene inhibition treatments of annual bluegrass caused greater protein content of the apoplast in leaf tissues compared to controls. Plants have several defenses against cellular ice formation, such as the movement of water from intracellular to inter- or extracellular (apoplastic) areas to reduce the formation of intracellular ice crystals (Atici and Nalbantoglu, 1999). Proteins, some possessing antifreeze activity, may accumulate in the apoplast, which would further reduce ice crystal formation. Antifreeze proteins can bind to the surface of ice crystals and reduce the rate at which ice may grow in plant tissues (Griffith et

al., 1992). Our results are contrary to what researchers have found in winter rye leaves where ethylene promoted apoplastic protein content and ethylene inhibitory treatments had lower apoplastic protein concentrations (Yu et al., 2001). We did not find any major, consistent differences in apoplastic protein content due to ethylene regulatory treatments for crown tissues, which is a primary overwintering structure for perennial turfgrasses. It is unclear why our results contrast with the results in wheat, but further investigation into apoplastic survival mechanisms, or lack thereof, of annual bluegrass may be warranted.

Plant growth or cellular survival at low temperatures can cause oxidative stress or the production of stressful levels of reactive oxygen species (ROS) (Okuda et al., 1991), which can lead to lipid peroxidation (Thomashow, 1999). In annual bluegrass under ice and no ice cover, ethylene treatments were associated with greater levels of lipid peroxidation. Only under no ice conditions, ethylene inhibition treatments reduced lipid peroxidation of crown and leaf tissues. The presence of ice or no ice seemed to play a role in how lipid peroxidation responded to ethylene regulatory treatments, but the total amount of lipid peroxidation was not different between ice and no ice conditions. This could be related to the amount of available oxygen, as oxygen may be more limited under ice conditions.

To combat lipid peroxidation and other oxidative stress damages, the health of a plant tissue's antioxidant system is important for winter survival. SOD, POD was consistently reduced in ethephon treated leaf and crown tissues under ice and in low temperature conditions with no ice cover. APX and CAT were less effected by ethylene treatment. Ethylene inhibitors may have indirectly or directly promoted preservation of antioxidant enzymes. SOD and POD activity were preserved closer to day 0 control levels to a greater extent in plants treated with AVG and APX and CAT activity was promoted by AVG treatment. Tasgen et al. (2006) investigated the

response of several antioxidant enzymes to cold treatment of winter wheat and found CAT to decline and peroxidase to increase with cold treatment. It is possible that maintenance of or promoting the levels of these antioxidants played a significant role in the survival of annual bluegrass to these simulated winter conditions.

In conclusion, ethephon treatment of annual bluegrass may be detrimental to spring recovery by increasing lipid peroxidation, increasing saturated fatty acid content, and decreasing antioxidant activity when compared to untreated annual bluegrass after winter conditions. Products that are ethylene inhibitors could potentially be beneficial to annual bluegrass survival during the winter and may act via increases in antioxidant activity and increases in plant cell membrane unsaturated fatty acid contents when compared to untreated annual bluegrass. To utilize ethylene inhibition as a turfgrass winter preparatory management strategy, more research into application timing and frequency may be desirable. As simulated winter conditions were used here, testing these treatments in the field may be important future research.

Overall conclusions and future directions

There are many aspects of turfgrass growth and management that can affect turfgrass survival of cold/ice conditions. The plant's ability to cold acclimate properly through changes in growth habit, hormone concentrations, accumulation of metabolites, carbohydrate storage, and changes to cell membrane fatty acid composition can all affect how a plant may survive winter. Putting green surfaces which are the most susceptible to winter related injury can experience ice cover which can lead to anoxic conditions, crown hydration injury, and the buildup of toxic gases can be detrimental to plant cell membranes. Current cultural practices can be labor intensive, costly, and damaging to putting greens to alleviate stress of winter. The research contained in this thesis explored whether commonly used chemicals and plant growth regulators could be used within winter preparatory turf management practices to reduce winter damage to annual bluegrass and aimed to determine physiological responses to ice and chemical treatments.

The research has shown that chemical applications of various plant protectants and plant growth regulators can positively influence annual bluegrass survival of ice encasement and low temperature. Civitas, mefluidide and propiconazole treatments increased annual bluegrass regrowth following prolonged ice encasement and may have been related to beneficial shifts in FA ratios to favor unsaturated FA content. It was not clear whether the most commonly used PGR, TE, altered winter survival. Some decreases in survival were found due to TE treatment but not in a consistent manner and not on all dates evaluated.

The ethylene biosynthetic pathway may be a good target to modify annual bluegrass tolerance of ice encasement conditions. Natural ethylene evolution and ethephon applications during fall acclimation could be detrimental to annual bluegrass winter survival and spring regrowth, depending on the severity of winter conditions. Ethephon treated annual bluegrass

plants were found to have decreased regrowth under ice and non-ice-covered conditions. Treatment of annual bluegrass with ethylene inhibitory products, such as ReTain, may be a viable way of increasing annual bluegrass spring regrowth in low temperatures if ice cover persists in the winter. The mechanisms associated with this enhancement of survival still need further elucidation; however, we found several physiological processes altered following fall-applied ethylene regulatory practices and ice encasement conditions.

The mechanisms associated with ethylene-associated enhancements in ice tolerance were associated with antioxidants, fatty acids, and content of apoplastic proteins. We found that ethephon treatment of annual bluegrass may be detrimental to spring recovery by increasing lipid peroxidation, increasing saturated fatty acid content, and decreasing antioxidant activity when compared to untreated annual bluegrass after winter conditions. Products that are ethylene inhibitors could potentially be beneficial to annual bluegrass survival during the winter and may act via increases in apoplastic proteins, changes in antioxidant activity and increases in plant cell membrane unsaturated fatty acid contents when compared to untreated annual bluegrass.

Future work should evaluate all these chemical treatments under ice in field conditions, determine potential effects on de-acclimation, evaluate application timings and frequencies to a greater extent, and be tested on other turfgrass species that are sensitive to cold or winterkill stresses such as perennial ryegrass. Additionally, investigating the direct mechanism of these treatments on enhanced regrowth may be warranted such as whether other physiological factors, like shifts in carbohydrate storage, could have played a role in annual bluegrass acclimation.

APPENDIX

Figure 8: *Malondialdehyde (MDA) content of annual bluegrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4 ºC in leaf tissue under (A) ice cover or (B) no ice cover and in crown tissue under (C) ice cover or (D) no ice cover.* Means from both 2016 and 2017 are pooled together. Bars with different letters are significantly different ($P \leq 0.05$) due to treatment within a given day.

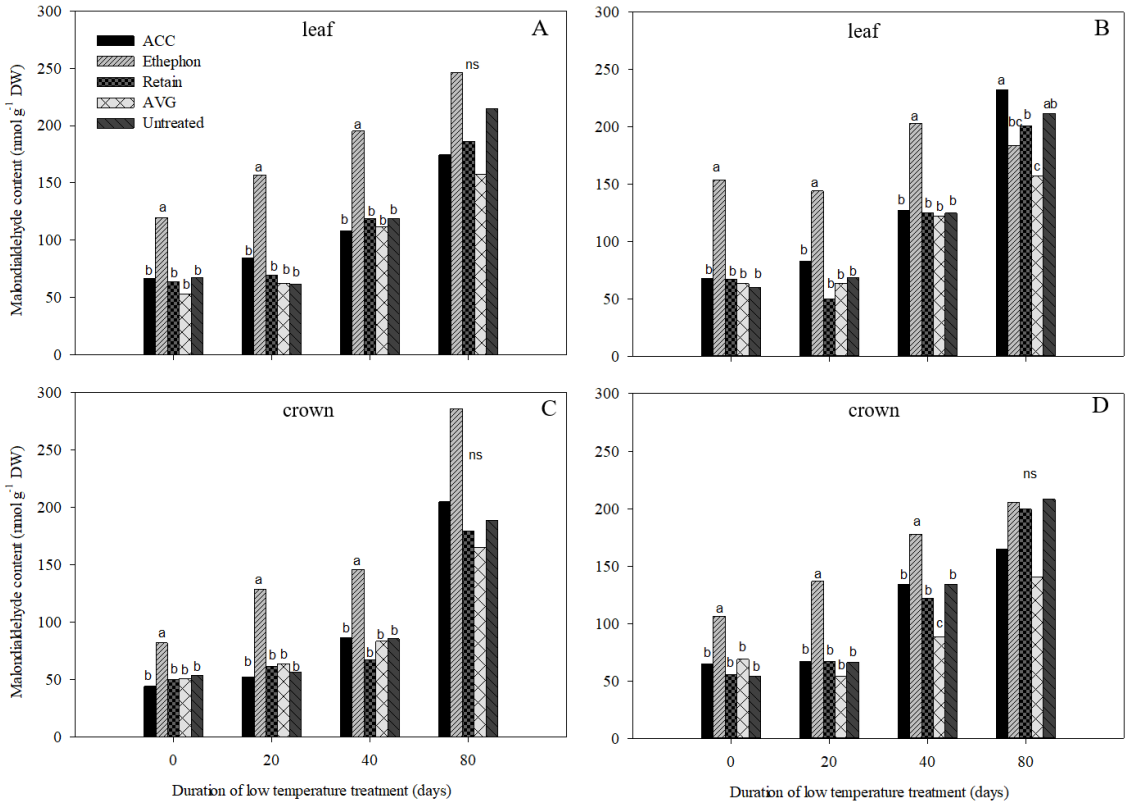

Figure 9: *Catalase (CAT) activity of annual bluegrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in (A) leaf tissue under ice cover or (B) no ice cover and in (C) crown tissue under ice cover or (D) no ice cover.* Means from both 2016 and 2017 are pooled together. Bars with different letters are significantly different ($P \leq 0.05$) due to treatment within a given day.

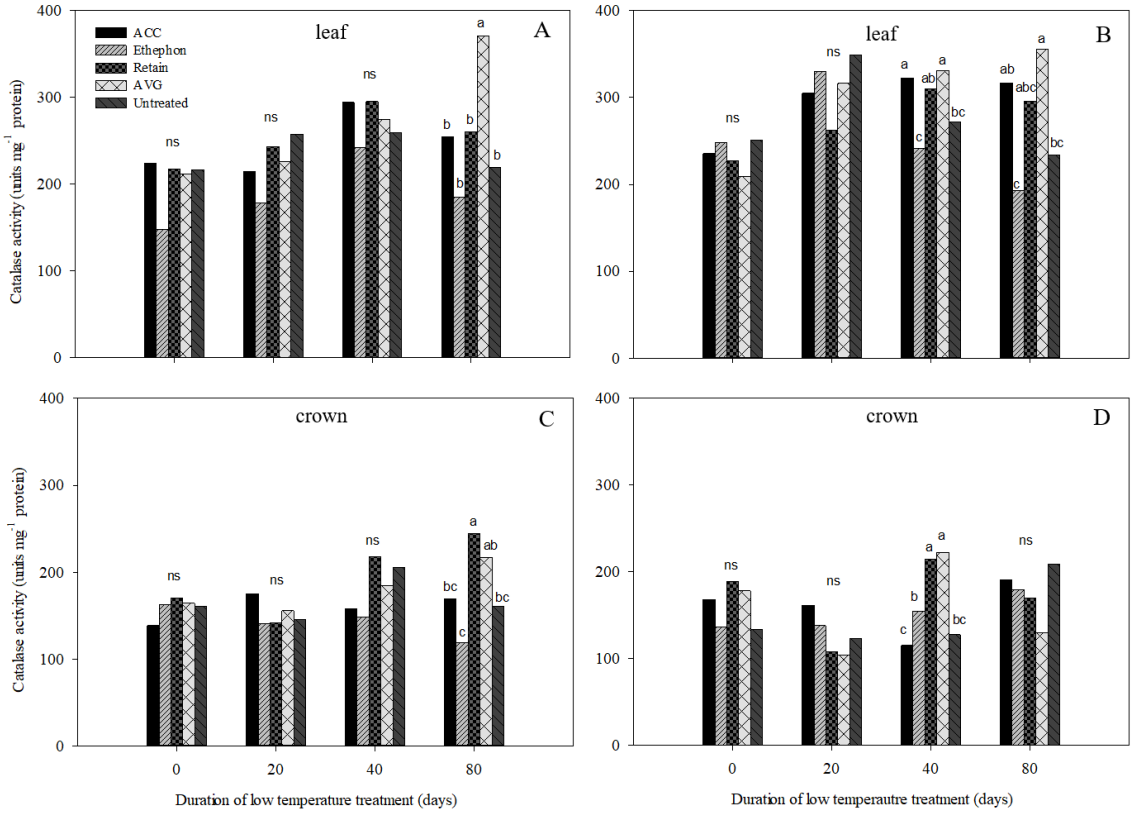

Figure 10: *Superoxide dismutase (SOD) activity of annual bluegrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in leaf tissue under (A) ice cover or (B) no ice cover and in crown tissue under (C) ice cover or (D) no ice cover.* Means from both 2016 and 2017 are pooled together. Bars with different letters are significantly different ($P \leq 0.05$) due to treatment within a given day.

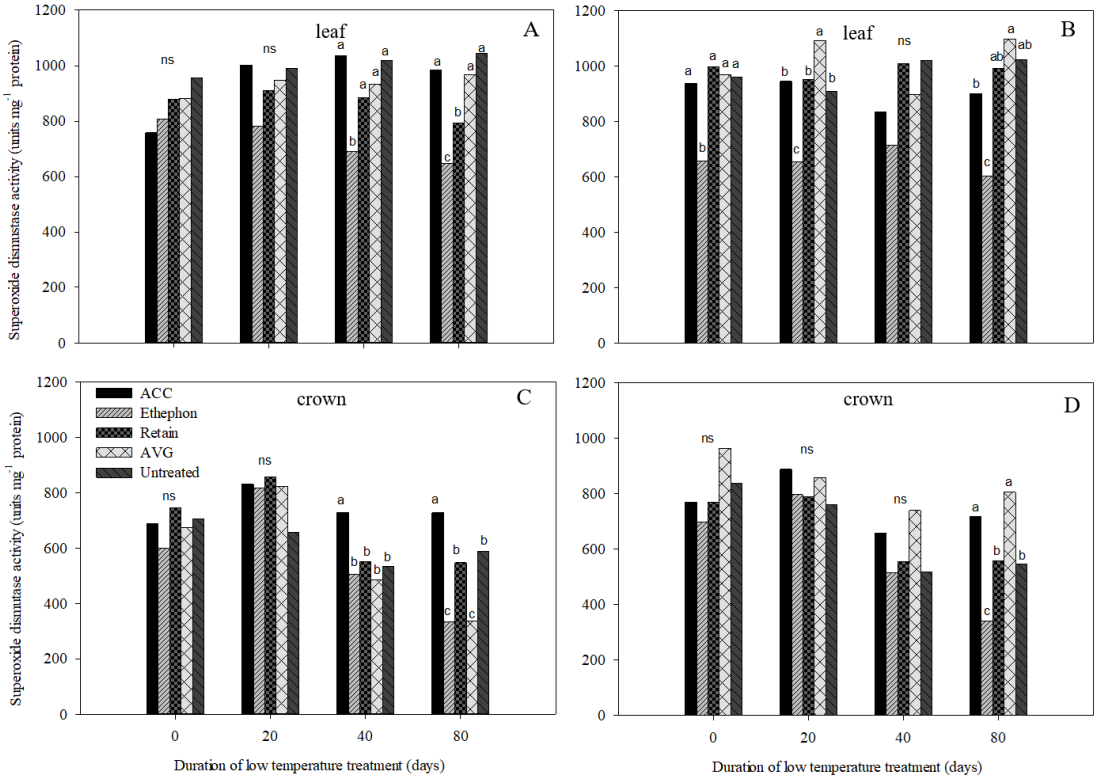

Figure 11: *Peroxidase activity of annual bluegrass (Poa annua) treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in leaf tissue under (A) ice cover or (B) no ice cover and in crown tissue under (C) ice cover or (D) no ice cover.* Means from both 2016 and 2017 are pooled together. Bars with different letters are significantly different ($P \leq 0.05$) due to treatment within a given day.

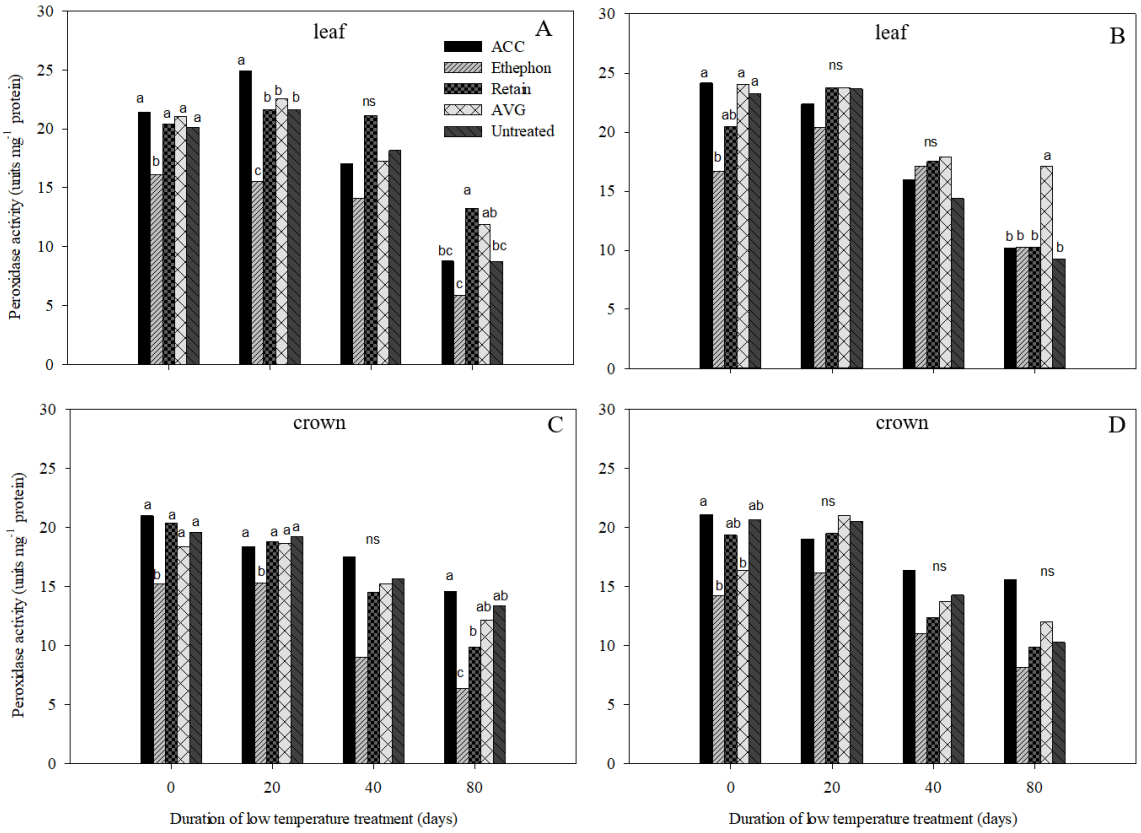

Figure 12: *Ascorbate peroxidase activity of annual bluegrass (Poa annua) treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in leaf tissue under (A) ice cover or (B) no ice cover and in crown tissue under (C) ice cover or (D) no ice cover.* Means from both 2016 and 2017 are pooled together. Bars with different letters are significantly different ($P \leq 0.05$) due to treatment within a given day.

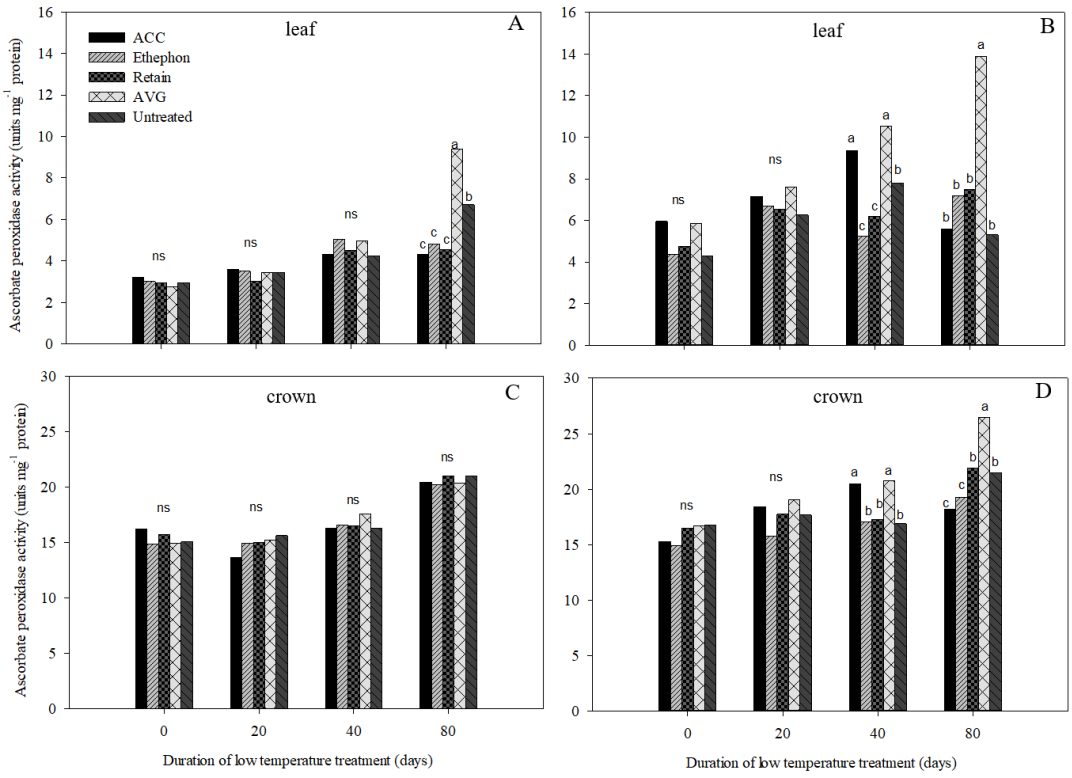

Figure 13: *Apoplastic protein concentration of annual bluegrass (Poa annua) treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated after 0, 20, 40 and 80 days at -4ºC in (A) leaf tissue and in (B) crown tissue.* Means from both 2016 and 2017 and from ice cover treatments are pooled together. Least significant difference (LSD) values are indicated by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment.

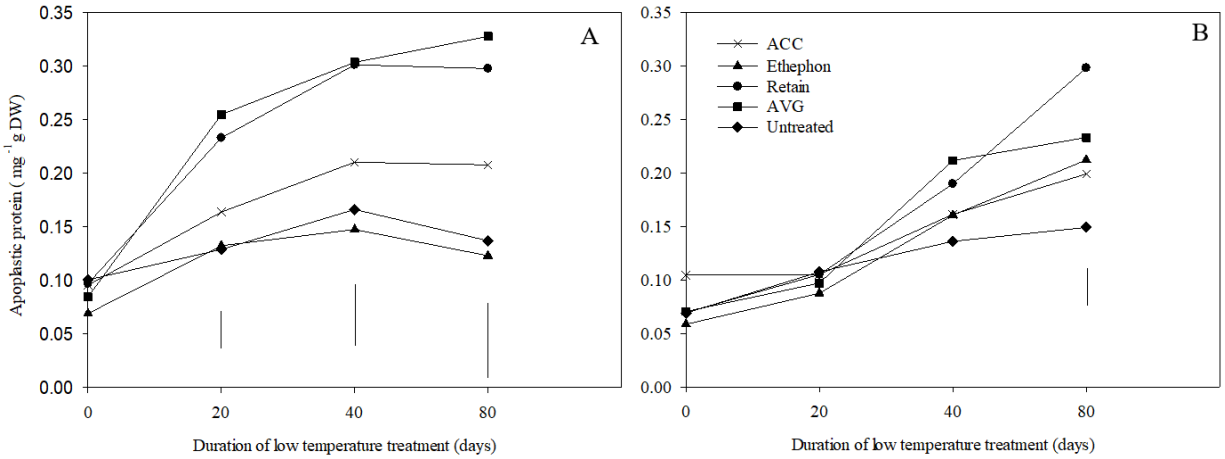

Table 6: *Changes in the saturated fatty acid contents of A) leaf B) crown and C) root tissue of annual bluegrass treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated and then exposed to 0, 20, 40, or 80 days in a low temperature growth chamber (-4 °C).* Means from both 2016 and 2017 are pooled together. Ice and no ice cover treatment means are pooled together. Within each column for each fatty acid, means followed by the same letter are not significantly different ($P \leq 0.05$). Columns with no letters indicate no significant differences among chemical treatments.

	Leaf				**Crown**				**Root**			
	Stress treatment period (d)				**Stress treatment period (d)**				**Stress treatment period (d)**			
	0	**20**	**40**	**80**	**0**	**20**	**40**	**80**	**0**	**20**	**40**	**80**
16:0	Molar percentage (mol %)											
ACC	14.2	18.1	21.3	19.5 a	41.6 a	41.9 a	40.4 bc	39.1 bc	27.5	30.5	32.6 a	31.0
Ethephon	10.3	16.6	19.2	25.0 a	37.8 ab	39.9 a	45.8 a	43.2 ab	27.3	29.1	30.7 abc	28.7
Retain	14.0	16.6	17.8	16.2 b	32.3 b	36.9 b	36.4 c	35.5 c	25.4	25.5	24.6 c	27.3
AVG	11.9	16.9	17.5	14.6 b	42.7 a	40.7 a	39.5 bc	35.1 c	28.7	27.6	26.4 bc	25.5
Untreated	11.9	17.6	21.8	23.6 a	38.5 ab	42.4 a	44.4 ab	42.0 ab	28.9	30.0	32.9 a	31.6
18:0												
ACC	3.8 ab	4.0	3.2 b	2.8 bc	21.6 a	19.1	36.2 a	21.1 bc	3.7	7.1 a	6.4	5.8
Ethephon	3.2 b	4.3	5.1 a	5.5 a	22.1 a	13.6	25.4 b	28.5 a	3.3	6.4 a	5.7	6.6
Retain	2.5 b	5.0	3.7 ab	3.4 b	17.2 b	13.8	25.7 b	19.1 c	3.1	6.3 a	5.8	5.7
AVG	3.2 b	4.6	3.6 ab	3.6 b	19.3 ab	13.3	23.6 bc	22.3 bc	2.3	5.8 a	6.2	7.0
Untreated	2.3 b	4.0	3.1 b	2.9 bc	21.4 a	13.0	24.3 bc	23.3 b	3.1	6.5 a	6.7	7.3

Table 7: *Changes in the unsaturated fatty acid contents of leaf, crown and root tissue of annual bluegrass (Poa annua) treated with 1-aminocyclopropane-1-carboxylic acid (ACC), ethephon, Retain, aminoethoxyvinylglycine (AVG), or untreated and then exposed to 0, 20, 40, or 80 days in a low temperature growth chamber (-4 °C).* Means from both 2016 and 2017 are pooled together. Ice and no ice cover treatment means are pooled together. Within each column for each fatty acid, means followed by the same letter are not significantly different ($P \leq 0.05$). Columns with no letters indicate no significant differences among chemical treatments.

	Leaf				Crown				Root			
	Stress treatment period (d)				Stress treatment period (d)				Stress treatment period (d)			
	0	20	40	80	0	20	40	80	0	20	40	80
16:1ɳ	Molar percentage (mol %)											
ACC	4.7	4.9	6.2	5.6 ab	2.1	11.4c	1.8 bc	2.0	3.5	0.8	0.9	0.9
Ethephon	3.4	4.5	5.3	5.1 b	2.0	10.4 d	1.8 bc	1.8	3.6	0.7	0.8	1.0
Retain	5.1	4.2	6.3	6.4 a	1.4	12.3 ab	2.3 b	2.0	2.6	0.9	1.0	0.9
AVG	5.0	3.7	4.5	5.1 b	2.3	11.9 bc	1.6 c	1.5	3.2	0.7	0.6	0.8
Untreated	4.3	3.6	5.0	4.5 b	1.9	12.6 a	1.7 c	1.6	3.4	0.8	0.7	0.8
18:1												
ACC	1.8 ab	2.0 bc	2.2	1.8	2.4	2.6 ab	2.7	2.2	10.8	11.0	9.7 a	9.9 a
Ethephon	1.9 ab	2.4 ab	2.3	2.2	2.3	2.1 b	2.3	2.7	8.4	11.5	6.8 b	9.3 ab
Retain	2.7 a	2.7 a	2.9	2.5	3.2	2.7 ab	2.3	2.6	12.1	11.2	10.4 a	11.8 a
AVG	2.2 a	1.6 c	1.9	1.1	3.3	3.4 a	3.4	3.4	9.3	11.5	11.0 a	9.7 ab
Untreated	1.8 ab	2.0 bc	2.4	1.8	2.8	2.7 ab	2.7	2.6	7.7	7.6	11.7 a	7.2 b
18:2												
ACC	12.0	15.0	16.5	16.3 a	19.5	21.0 a	21.2 a	20.5 ab	32.5 c	34.7	34.0	35.9
Ethephon	15.3	14.7	9.5	9.95 b	22.6	19.7 ab	15.0 b	16.7 c	33.6 bc	34.6	36.3	34.9
Retain	16.3	14.3	14.2	15.8 a	22.1	19.7 ab	16.7 ab	20.7 a	34.4 bc	35.2	34.4	36.0
AVG	13.6	14.4	13.8	7.1 b	19.6	18.7 ab	15.6 b	11.6 d	35.7 b	37.1	36.2	36.2
Untreated	15.4	13.1	9.4	10.6 b	19.3	17.1 b	15.3 b	16.8 b	34.7 bc	39.1	36.7	36.6
18:3												
ACC	44.2	44.7	46.9	43.8	14.0	14.7	13.8	13.3 cd	15.1	14.2	14.6 a	14.6
Ethephon	44.7	47.8	49.3	48.2	13.5	12.8	12.6	11.2 d	15.2	16.3	10.4 b	13.4
Retain	44.5	45.9	47.0	42.8	14.9	15.9	15.1	17.2 a	13.6	12.1	15.1 a	12.0
AVG	45.5	47.1	47.2	48.3	15.9	15.4	14.4	15.0 abc	14.8	15.3	15.1 a	15.6
Untreated	45.3	46.4	46.7	50.7	15.8	14.2	15.5	14.9 bc	14.9	14.7	15.3 a	14.3

Table 8: *Analysis of variance for main treatment factors and interactions of percent regrowth, fatty acid analysis, ascorbate peroxidase, superoxide dismutase, peroxidase, and CAT activity, malondialdehyde content and apoplastic protein concentrations of annual bluegrass (Poa annua) under effective ethylene and ethylene inhibition treatments and ice or no ice cover treatments in East Lansing, MI during 2016 and 2017.*

			Fatty acids content		APX		SOD		POD		CAT		MDA		Antifreeze proteins	
Effect	Percent regrowth		Leaf	Crown	Leaf	Crown	Leaf	Crown	Leaf	Crown	Leaf	Crown	Leaf	Crown	Leaf	Crown
Rep																
Chemical treatment (T)		***	***	***	***	***	***	**	***	**	**	***	***	**	***	**
Ice cover treatment (I)		*	ns	ns	*	*	*	**	**	***	**	**	***	***	ns	ns
T x I		nsł	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Date (D)		***	***	***	**	**	**	**	***	*	**	*	***	**	**	**
T x D		***	**	***	**	*	***	*	**	***	**	***	***	***	**	*
I x D		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
T x I x D		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

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