Preventing Loss of Winter Hardiness in Turfgrass on Golf Greens and Fairways in the Late Winter

by

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ABSTRACT

Preventing Loss of Winter Hardiness of Turfgrass on Golf Greens and Fairways in the Late Winter

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Plant growth regulators (PGRs) such as gibberellic acid (GA_3), abscisic acid (ABA) and trinexapac-ethyl (TE) can be applied to turfgrasses to promote winter hardiness. Lethal temperature of 50% mortality measurements revealed that application of these PGRs had no effect on the acclimation status of creeping bentgrass and annual bluegrass for the months of January, February and March. Fall PGR application decreased acclimation status for annual bluegrass, whereas ABA and GA_3 applications increased acclimation status for creeping bentgrass in April. Treatment with exogeneous ABA increased endogenous ABA levels in annual bluegrass. A simulated warming event in a growth chamber in late winter resulted in a loss of acclimation status for both grasses compared to a control. GA_3 increased annual bluegrass photosynthesis and TE decreased creeping bentgrass photosynthesis during the warming event. PGRs are beneficial for maintaining the acclimation status of creeping bentgrass, while reducing the acclimation status of annual bluegrass.

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LIST OF ABBREVIATIONS

AB – annual bluegrass

ABA – abscisic acid

C – Celsius

CB – creeping bentgrass

DW – dry weight

FW – fresh weight

GA_3 – gibberellic acid

g – gram(s)

g – gravitational force

hr – hour

K – potassium

LT_{50} – lethal temperature of 50% survival

mg – milligram(s)

mL – millilitre(s)

N – nitrogen

NCER – net carbon exchange rate

ng – nanogram(s)

nM - nanomolar

µL – microlitres(s)

µmol – micromole(s)

P – phosphorus

PPFD – photosynthetic photon flux density

PGR – plant growth regulator

PGRs – plant growth regulators

ppm – parts per million

RA – re-acclimated

s – second(s)

TE – trinexapac-ethyl

TNC – total non-structural carbohydrates

GENERAL INTRODUCTION

Turfgrasses on golf courses in Ontario must have the ability to survive sub-0^{o}C temperatures and maintain acclimation status through warming events in the late winter that leave plants susceptible to freezing events (Tompkins et al., 2000). Annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis stolonifera* L.) are the two predominantly used turfgrass species on golf greens in southern Ontario. These two species have different abilities to acclimate to cold temperatures and resist de-acclimation during warming events (Tompkins et al., 2000). Global climate change may cause higher temperatures in the late winter and early spring which cause premature de-acclimation of turfgrasses and significantly decreasing the plants cold tolerance (Hoglind et al., 2013).

Plant growth regulators (PGRs) have been proposed as a method to prevent premature losses of acclimation status of turfgrasses during warming periods. Two plant hormones involved in winter hardiness are abscisic acid (ABA) (Chen and Gusta, 1983) and gibberellic acid (GA_3) (Reid at el. 1974). Plants with high ABA levels have greater cold tolerance than plants with low ABA levels. In contrast, plants with high GA_3 levels tend to have relatively low cold tolerance.

Trinexapac-ethyl (TE) is a plant growth regulator that inhibits GA_3 synthesis and has been demonstrated to increase the stress resistance of turfgrass under shade (Goss et al., 2002), drought and heat (McCann and Huang, 2007) and freezing (Fagerness et al., 2002). Trinexapac-ethyl application increases a protein associated with cold acclimation when even applied at non-acclimating temperatures (Hwang et al., 1999).

The first objective of the study was to demonstrate the loss of winter hardiness of the two turfgrass species during from the winter period leading into the de-acclimation period. Creeping bentgrass was expected to have a higher peak cold tolerance than annual bluegrass in the months of January and February but once subjected to de-acclimating temperatures in March and April, creeping bentgrass' cold tolerance advantage over annual bluegrass was expected to decline. The second objective of the study was to determine the effect of the foliar application of plant growth regulators and hormones to prevent loss of acclimation of annual bluegrass and creeping bentgrass in the late winter and early spring. The application of ABA and TE was expected to increase cold tolerance of the turfgrasses, while GA3 was expected to lower the cold tolerance of turfgrasses. The third objective was to evaluate short term change in winter hardiness of annual bluegrass and creeping bentgrass through a simulated warming event in late February and early March. Annual bluegrass was expected to de-acclimate and lose cold tolerance at a faster rate than creeping bentgrass after the simulated warming event.

CHAPTER ONE – LITERATURE REVIEW

1.1 WINTER HARDINESS

Although not universally defined, winter hardiness can be described as a measure of a plant's ability to withstand the conditions associated with winter without injury or death. Plants that are exposed to winter conditions in northern temperate climates such as southwestern Ontario endure numerous stresses such as freezing temperatures, ice encasement, desiccation and disease (Hon et al., 1995). An important component of winter hardiness in plants is the acclimation to cold temperatures resulting in cold hardiness (Gusta and Wisnieski, 2013). The degree of cold hardiness levels is season dependent (Qian et al. 2001), with the greatest variation in cold hardiness for a species is observed in late winter/early spring when temperatures oscillate above and below freezing while the plant is emerging from dormancy (Tompkins et al., 2000). Dormancy is the physiological condition obtained in before the onset winter is a decline in plant growth and metabolic activity, and the plant maintains this slower metabolic state until temperature and photoperiod are conducive for growth in the spring. Once a plant has emerged from dormancy its ability to survive low temperatures is greatly diminished (Gusta and Fowler, 1976; Fowler and Gusta, 1977)

1.2 COLD ACCLIMATION OF COOL-SEASON PLANTS

Cold acclimation, also known as cold hardening, is the process where a plant undergoes physiological changes to survive cold temperatures (Kalberer, 2006). Cold acclimation begins in the fall with low temperatures (between 0^{o}C and 5^{o}C) and the daily photoperiod is reduced (Beck et al., 2004). Temperature is more important than photoperiod for cold acclimation of cool-season plants (Beck et al., 2004; Chen and Li, 1980). Acclimation of plant tissues to

temperatures just above 0°C increases survival to a successive temperature drop below freezing (Chen and Li, 1980). Cold acclimation is initiated when plants are exposed to temperatures between 0°C and 5°C. For many herbaceous plants one day of exposure to approximately 0°C is sufficient to trigger winter acclimation, regardless of photoperiod (Livingston, 1996). There is concern that global warming could result in historically high temperatures in the fall may prevent the occurrence of proper acclimation (Thorsen and Hoglind, 2010). In general, winter hardy cultivars of a species acclimate faster in the fall and de-acclimate more slowly in the late winter/early spring than less hardy cultivars (Eagles and Williams, 1992). In some cases, plants that are considered more winter hardy at the apex of winter de-acclimate faster in the late winter and early spring than plants that are considered less winter hardy at the apex of winter (Jorgensen et al., 2010).

Exposure to low temperatures initiates a series of physiological and biochemical adjustments within the plant (Thomashow, 2010). Hormonal adjustment occurs as abscisic acid (ABA) begins to accumulate and gibberellic acid (GA_3) levels decrease (Li et al., 2002). Photosynthetic activity is maintained during cold acclimation by an increase in Calvin cycle enzyme activity (Strand et al., 1999). Changes in cellular solutes associated with cold acclimation include the accumulation of amino acids, soluble proteins, and non-structural carbohydrates (Patton et al., 2007). The accumulation of cryoprotective solutes during cold hardening is considered the main determinant of cold hardiness (Kalberer, 2006). The physiological changes made during acclimation prevent intracellular ice formation when exposed to temperatures below freezing (Ruelland et al., 2009). As temperatures drop below

0°C the cell undergoes freeze induced dehydration, which may help prevent ice from forming intracellularly (Pearce, 1988).

Maximum cold tolerance in grasses is acquired by the mid-winter months of January and February, depending on when temperature and environmental conditions have stabilized (Qian et al., 2001, Ball et al., 2002; Dionne et al., 2001). Cold tolerance slowly declines under consistent snow cover and below 0°C temperatures (Tompkins et al., 2000). When temperatures increase above 10 °C cold tolerance declines at an accelerated rate to levels apparent during pre-acclimation (Qian et al 2001; Ball et al., 2002). Alterations in the levels of cryoprotective compounds such as fructose, glucose, sucrose, and raffinose follow a similar trend to that of cold tolerance over winter. The level of cryoprotective solutes peak mid-winter when conditions are consistently below 0°C and levels decline slowly until temperatures are above 0°C when cryoprotective solute concentrations drop to levels observed before acclimation (Ball et al., 2002).

1.3 PHYSIOLOGICAL FACTORS INVOLVED IN COLD HARDINESS

1.3.1 Proteins and Amino Acids

Soluble proteins accumulate as temperatures approach 0°C and are correlated with plant survival at low temperatures (Gusta and Weiser, 1972). Protein content in chilling tolerant olive trees is higher in the cold acclimated plants than the non-acclimated plants (Hashempour et al. 2014). The difference in protein content between cold acclimated plants and non-acclimated plants is cultivar-dependent. In asparagus, hardier cultivars synthesize more proteins during subfreezing acclimation than non-hardy cultivars (Landry and Wolyn, 2012).

Soluble protein accumulation during acclimation appears to be less important than proline and carbohydrate accumulation as differences in soluble protein between cold acclimated and non-acclimated plants were of less magnitude than that of proline and carbohydrates (Alberdi et al., 1993).

Dehydrins are soluble proteins that accumulate in response to low temperature and stresses that cause cellular dehydration (Close, 1997; Kosova et al., 2008). Dehydrins serve a cryoprotective function by stabilizing the cell in response to dehydration stress caused by extracellular ice (Wisnieski et al., 1999; Close, 1997). Dehydrin levels are high in plants in cold acclimating conditions (Trischuk et al., 2014). In a study by Cansev et al. (2009), dehydrin accumulation is directly correlated with cold hardiness in January in the almost every olive tree cultivar that were analyzed. In general, plants that were able to survive the lowest temperatures have greatest levels of dehydrins (Trischuk et al., 2014; Cansev et al. 2009).

Plants can synthesize antifreeze proteins that are capable of inhibiting ice crystal formation within the cell (Janska et al., 2010). Antifreeze proteins not only change the freezing point of a solution but also change the morphology of the ice crystals (Hon et al., 1994). These antifreeze proteins accumulate during cold acclimation and contribute to a plant's freezing tolerance (Marinates et al., 1993; Hon et al, 1994; Antikainen & Griffith, 1997). Protein extracts from cold acclimated rye leaves have greater anti-freeze activity than non-cold acclimated leaves (Marinates et al., 1993). The physical removal of apoplastic proteins from cold-acclimated leaves is associated with an increase in electrolyte leakage from the cell indicating

the importance of these proteins in maintaining osmotic gradients as well (Marentes et al., 1993)

The amino acid proline is an indicator of stress resistance in plants subjected to low water availability (Tatar and Gevrek, 2008), salt stress (Huang et al., 2013) and freezing temperatures (De Ronde et al., 2004). An increased proline concentration is a physiological response to low temperature stress (Alden and Hermann, 1971; Levitt, 1980; Lalk and Dorffling, 1985). During dehydration caused by freezing stress, it is suggested that proline protects the cell in a variety of ways (Alves and Setter, 2004) including: stabilization of membrane proteins (Schmidt et al., 2016), limiting dehydration stress as an osmotic adjuster (Huang et al, 2015; Delauney and Verma,1993), and scavenging free radicals (Prasad et al., 1994; Kaul et al., 2008). Proline concentration is higher in January during freezing temperatures of winter than it is in November and March when temperatures were above 10^{o}C at the time of harvesting (Zhang et al., 2016).

1.3.2 Carbohydrates

The cold acclimation phase leads to a large accumulation of soluble carbohydrates and other non-structural carbohydrates (Ostrem et al., 2011). Total non-structural carbohydrates (TNC) are associated with the stress tolerance of a plant, including its freezing tolerance (Watschke et al., 1972; Fry et al., 1993; Patton et al., 2007). TNCs have cryoprotective properties that prevent crystallization of water within the cell (Uemura and Steponkus, 1999; Ostrem et al., 2011). Soluble sugars such as sucrose also play a role in scavenging of reactive oxygen species (Tarkowski and Van den Ende, 2015). Sugars can also stabilize membranes and

preserve protein structure, such as catalase, when subjected to freezing temperatures (Caffrey et al., 1988, Carpenter and Crow, 1988; Carpenter et al., 1986). Plants which are more winter hardy are speculated to accumulate sugars during the acclimation phase whereas less hardy plants are unable to do so as effectively (Gusta and Wisniewski, 2013).

Fructose polymers, known as fructans, accumulate in many cool-season plants during acclimation (Panjtandoust and Wolyn, 2016; Espevig, 2011). Fructans are the dominant reserve carbohydrate in cool-season grasses found in northern temperate climates (Ojima and Isawa, 1968). Starch also accumulates in cool-season grasses but not as much as fructans (Jensen et al., 2014). Fructans are cryoprotective compounds that stabilize the cell membrane under desiccation stress (Livingston et al. 2009). Fructans accumulate as temperatures decrease and approach 0^{o}C but their levels decline once temperatures fall below 0^{o}C, and the levels of simple sugars are slightly increased due to the conversion of fructans to sugars at sub-0°C temperatures (Hoffman et al., 2014a). Fructans remain the dominant carbohydrate reserve during this period despite some conversion to simple sugars. Plants with high freezing tolerance accumulate more fructans than plants with low freezing tolerance (Suzuki and Nass, 1988). Van den Ende et al. (2016) suggest fructans may be important for regrowth during the spring.

1.3.3 Cell Membrane and Fats

Cold acclimation causes desaturation of membrane lipids (Wang, 1990). The absolute amount of membrane lipids remains unchanged during cold acclimation but the proportion of unsaturated fats to saturated fats in the phospholipid membrane is increased (Degenkolbe et al., 2012). Lipid desaturation is found to be positively correlated with freezing tolerance

(Ishizaki-Nishizawa, 1996). Arabidopsis mutants that are incapable of desaturating membrane lipids undergo more damage than mutants able to desaturate lipid membranes when exposed to chilling stress (Miquel et al., 1993).

1.3.4 Water content and osmotic adjustment

Osmotic adjustments made by plants in response to winter acclimation are postulated to contribute to winter hardiness by increasing the concentration of solutes within the cell (Green and Raztlaff, 1975). Increased solute levels help maintain cell water content. Acclimated plants have lower osmotic potential within the leaves than non-acclimated plants, preventing electrolyte leakage (Lalk and Dorffling, 1985; Burchett et al., 2006). During the cold acclimation phase the plant will begin to release calcium ions in the cytosol (Ruelland et al., 2002).

The total water residing in tissue is inversely related to winter hardiness (Kacperska-Palacz and Egierszdorff, 1972). However, there are cases where a perennial ryegrass cultivar that has a higher relative water content is more winter hardy than a cultivar with lower relative water content (Chang et al., 2017); it is worth mentioning that the authors of this paper concluded that the water withholding capacity of perennial ryegrass cultivars contributed to freezing tolerance, although they do not present any other evidence in the study as to why they made this conclusion.

1.3.5 Extracellular and Intracellular Ice Nucleation

Freezing injury of plants has been extensively reviewed (Levitt, 1980; Uemura et al., 2006; Steponkus, 1984). Intracellular ice formation is the most lethal form of ice formation within the plant as it can puncture cell membranes (Levitt, 1980; Taiz and Zeiger, 2010). Cold

acclimation is necessary for preventing intracellular ice formation (Steponkus et al., 1984). Intracellular ice can develop within cold acclimated plants, but only during rapid temperature drops that are atypical of what occur in the field (Steponkus et al., 1984). In acclimated plants ice will form in the extracellular space causing a drop of water potential outside of the cell resulting in dehydration stress (Pearce, 1988; Uemura et al., 2006). Ice formation in the extracellular space is postulated to be a survival strategy in plants to tolerate freezing temperatures, as the dehydration stress imposed by extracellular ice is much less lethal than membrane rupturing caused by intracellular ice (Pearce, 1988).

1.4 ASSESSING ACCLIMATION STATUS

The acclimation status of a plant is typically assessed through what is known as an LT_{50}, a test that subjects plants to cold temperatures to estimate the temperature of 50% survival (Andrews, 1974). The LT_{50} test begins with a short period of acclimation to a subfreezing temperature, typically -2^{o}C for 8 to 12 hours (Hulke et al., 2008). Temperature is reduced in a step wise manner, typically at a rate of -2^{o}C per hour and is held for an hour at each one of the target temperatures. Plants are sampled at each temperature, are then thawed at 2-4 ^{o}C for at least 12hrs. Survival is assessed at each one of the target temperatures using a variety of methods. For turfgrass, assessing LT_{50} at the conclusion of the freezing test is conducted in one of three ways: electrolyte leakage assay (Tompkins et al, 2000), canopy imaging LT_{50} (Kimball et al., 2017) and individual/small population plant regrowth LT_{50} (Espevig et al., 2014). Percent survival is assumed to follow a sigmoidal curve and LT_{50} is predicted through a probability analysis (Andrews et al., 1974).

There are two types of LT_{50} tests, an LT_{50} test of cold tolerance and an LT_{50} test for freezing tolerance. A cold tolerance/hardiness test assesses the tolerance of a plant to cold temperatures in the absence of ice encasement (Tompkins et al., 2000). For freezing tolerance tests, water is sprayed onto the plants during freezing or plants are wrapped in moist paper towel for ice nucleation on the exterior of the plant (Hoffman et al., 2014, Espevig et al., 2014). Due to the short nature of these tests, which are typically completed in less than 30 hr, the effect of ice nucleation during a freezing tolerance test is minimal. Death due to ice encasement takes at least 45 days in annual bluegrass*, Poa annua* L., while in creeping bentgrass, *Agrostis stolonifera* L., it will take even longer to see lethal effects (Castonguay, 2009).

One of the criticisms of the LT_{50} test is that reproducing the status of a plant under field conditions cannot be achieved because of the short-term nature of LT_{50} tests (Gusta, et al., 2000). Short term tests demonstrate death due to low-temperature injury where ice forms in the intracellular and/or extracellular space which can puncture cell membranes as opposed to desiccation (Taylor and Olsen, 1985), low temperature disease, or anoxia. Breeders of winter wheat select for cultivars with low LT_{50} (Fowler et al., 1981). LT_{50} tests have high repeatability which is lacking in field tests because of variables such as temperature and precipitation impact the physiological status of the plant and cannot be controlled for in the field. LT_{50} has been positively correlated with survival under prolonged freezing in winter wheat (Skinner and Garland-Campbell, 2008). LT_{50} tests are not designed to simulate field conditions, LT_{50} tests are meant to assess the cold tolerance of a plant or several plants at a point in time. Assessing acclimation status is arguably more important than the other three types of winterkill since ice nucleation which punctures the cell wall once a plant has de-acclimated is the cause of

winterkill in the late winter and early spring. LT_{50} can be used as an effective indicator of acclimation status since LT_{50} is lower in acclimated plants than non-acclimated plants (Hoffman et al., 2014a).

1.5 COLD DE-ACCLIMATION OF COOL-SEASON PLANTS

De-acclimation or dehardening is the process by which plants lose winter hardiness that was developed in the fall (Kalberer et al., 2006). Cold acclimation has been extensively researched but cold de-acclimation has not been studied as thoroughly. De-acclimation is a major concern for many perennial crops such as winter wheat (Gusta and Fowler, 1976) and Timothy grass (Jorgensen et al., 2010). Cold de-acclimation resistance is a crucial component of winter survival in winter cereals (Rapacz et al., 2017). Plants begin to de-acclimate in the spring when subjected to increasing temperatures typically above 0^oC (Chen and Li, 1980). Photoperiod slightly impacts de-acclimation however temperature is the most influential factor, as much greater differences in acclimation status occur due to temperature increase as compared to an increase in photoperiod (Rapacz, 2002). During de-acclimation, tissues begin to thaw, cells begin to take up water and metabolic activity starts again. Cryoprotective solutes which were accumulated during cold acclimation decline greatly during de-acclimation (Zuther et al. 2015). Gibbelleric acid, GA_3, concentration is increased during de-acclimation (Barnes and Wilson, 1986). Despite the role of ABA in cold signalling, ABA levels appear to have no effect on the rate of growth during de-acclimation (Rapacz et al., 2003). De-acclimation has been demonstrated to occur more rapidly (days to weeks) than acclimation (weeks to months) to cold temperatures (Pomeroy et al. 1975; Chen and Li, 1980; Gay and Eagles, 1991). For certain plants, such as *Solanum commersonnii* Dunal., de-acclimation can occur in just one day if

temperatures are warmed sufficiently (Chen and Li, 1980). Kalberer et al. (2007) suggest that de-acclimation resistance and re-acclimating ability are very important for winter survival. A freezing event can be lethal to a plant once a plant has de-acclimated (Limin and Fowler, 1985). A problem with regards to selecting species and cultivars for cold tolerance is that maximum cold tolerance is not necessarily correlated to de-acclimation resistance (Jorgensen et al., 2010; Tompkins et al., 2000).

Global climate change may potentially result in growing seasons that extend into the late fall and early winter, which could be detrimental to the plants ability to properly acclimate (Hoglind et al., 2013). Zuther et al. (2015) suggest that maximum mid-winter hardiness is much less of a concern than premature de-acclimation because plants do not typically die midwinter. Global climate change causes the temperate regions to have winters with warmer average temperature and irregular temperature patterns (Gu et al., 2008; Belanger et al., 2002). Milder winters result in a reduction in protective snow cover leaving the plants susceptible to sudden freezing injury after de-acclimation (Belanger et al., 2002; Pagter and Williams, 2011).

It is possible for plants to regain lost cold hardiness in a very limited capacity once de-acclimated when subjected to cold acclimating conditions but attaining previous levels of cold hardiness is rarely achieved (Espevig et al. 2014; Gusta and Fowler, 1976; Trischuk et al., 2014). In a study conducted by Pomeroy et al. (1975) winter wheat and winter barley were were exposed to acclimation temperatures of 2^{o}C during the day and -2^{o}C at night for 6 weeks in a growth cabinet. At the completion of the acclimation phase the plants were then subjected to de-acclimation temperatures of 20^{o}C days and 15^{o}C nights. Plants in the Pomeroy et al. (1975)

study that underwent de-acclimation temperatures for more than a week were unable to regain their initial cold hardiness when placed back under acclimation temperatures.

1.6 WINTER HARDINESS OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS

Creeping bentgrass is considered a much more winter hardy grass than annual bluegrass (Christians, 2016). Tompkins et al. (2000) demonstrated that at peak acclimation creeping bentgrass can survive much lower temperatures (LT_{50} of -30°C) than annual bluegrass (LT_{50} of -14°C). The advantage in cold tolerance for creeping bentgrass over annual bluegrass is diminished in the spring when temperatures rise above 0°C; moreover, creeping bentgrass cold hardiness levels are less than those of annual bluegrass at temperatures that are consistently greater than 10°C (Tompkins et al., 2000). A growth chamber study by Hoffman et al. (2014b) demonstrated similar LT_{50} among both of these grasses after a 5-day de-acclimation period at 12°C. Anaerobic conditions associated with ice encasement and impermeable covers must also be considered in addition to cold hardiness when considering winter hardiness of a species (Castonguay et al., 2009). Annual bluegrass is more susceptible to anoxic conditions associated with ice encasement than creeping bentgrass.

Creeping bentgrass's ability to survive low temperature extremes is likely due to the vegetative status of the tillers and stolons. At the typical cut height of a golf green, creeping bentgrass does not produce seed and remains predominately in the vegetative state. Annual bluegrass can produce seed at very low mowing heights suggesting that the tillers of this grass are more likely to be in a reproductive state leaving them susceptible to low temperature injury (Malyshev & Henry, 2012).

1.7 TURF CONDITIONS ASSOCIATED WITH PUTTING GREENS

Turfgrass on golf greens undergo additional abiotic stresses other than those associated with winter. Golf greens that are maintained at low height of cut (<3.2mm) are typically encircled by trees imposing shade stress (Goss et al., 2002). Mowing at low heights of cut limits the ability of a turf plant to conduct photosynthesis and maintain carbohydrate reserves (Huang et al., 2006). High traffic and poor light conditions associated with shade also negatively affect the plant's ability to accumulate carbohydrates (Bell, 2011). Failure to accumulate carbohydrate reserves hinders the ability of a plant to tolerate stress, recover from injury, and successfully emerge from dormancy (Watschke et al., 1972; Christians, 2016). Golf greens are more likely to succumb to winterkill than fairways because of the increased stress associated with golf greens.

1.8 WINTERKILL OF COOL-SEASON GRASSES ON PUTTING GREENS

A common cause of winterkill on golf greens is temperature fluctuations in the late winter/early spring when turfgrass is exposed to warm conditions resulting in de-acclimation (Dionne et al., 1999). The de-acclimation of turfgrass can be fatal when the warming conditions are then followed by a rapid decrease in soil temperature, resulting in lethal freezing temperatures at the crown level (Dionne et al., 1999). Winter kill is a problem in areas where very little snow is present to insulate the turfgrass from fluctuating and freezing air temperatures that cause injury. Maintenance of snow cover on golf greens is vital for maintaining cold hardiness of turfgrass into the spring (Tompkins et al., 2000). The number of days with adequate snow cover in temperate areas is steadily declining which is a concern for golf courses which do not have the capacity to place covers over their greens (Hoglind et al., 2013).

1.9 PLANT HORMONES AND FUNCTION

The eight main plant hormones are auxins, cytokinins, gibberellins, ABA, ethylene, salicylic acid, jasmonates and brassinosteriods. Auxin regulates many processes such as cell division, cell expansion, apical dominance and differentiation of the root system (Gallavotti, 2013; Wang and Irving, 2011). Cytokinins are believed to regulate cell division and differentiation, delay chlorophyll breakdown and induce stem morphogenesis (Mok and Mok, 2001; Kulaeva and Prokoptseva, 2004). Gibberellins are responsible for stem growth, flowering, and emergence from seed dormancy (Wang and Irving, 2011). Abscisic acid is an antagonist of gibberellins (Gaspar et al., 1996). Abscisic acid is known as stress hormone which regulates stomatal closure, maintains seed dormancy, and slows cell elongation (Wang and Irving, 2011; Gaspar et al., 1996). Ethylene is a gaseous biological signalling molecule that works in conjunction with other plant hormones to induce fruit ripening, defoliation, abscission of flowers and fruits, and initiating adventitious roots (Kulaeva and Prokroptseva, 2004; Bakshi et al., 2015). Salicylic acid has been identified as plant defense hormone to signal disease resistance once a plant has been infected by a pathogen (Vlot et al., 2009). Salicylic acid is also active in regulation of flowering, inhibition of germination, and responses to abiotic stress (Gaspar et al., 1996: Vlot et al., 2009). Jasmonates are primarily involved in abiotic and biotic stress signalling and have an antagonistic relationship with salicylic acid (Robert-Seilaniantz et al., 2011). For healthy plant tissues, jasmonates regulate carbon partitioning, senescence and reproductive development (Browse, 2009). Brassinosteroids are plant steroid hormone involved in developmental processes such as cell division, cell elongation, reproductive development and leaf senescence (Choudhary et al. 2012).

1.9.1 Abscisic Acid

Abscisic Acid (ABA) is a known growth inhibitor that regulates many important cold acclimation genes (Janska et al, 2009; Gusta et al., 2005). Increased ABA levels are a typical response to temperatures approaching 0^{o}C (Dorffling et al., 1990; Li et al., 2002). When plants are subjected to temperatures of 0^{o}C and below, there is a significant increase in the ABA to gibberellin ratio (Zhang et al., 2012). ABA is a signalling hormone for cold acclimation (Chen and Gusta, 1983). An ABA-deficient Arabidopsis mutant is unable to successfully undergo cold acclimation (Heino et al., 1990). Hardy varieties of winter wheat accumulate ABA more rapidly than non-hardy varieties (Lalk and Dorffling, 1985). Plants with higher ABA levels in the leaves and stolons contain higher cold tolerance than leaves and stolons containing lower ABA levels (Zhang et al., 2008). Although ABA is implicated in dormancy and cold tolerance, application of ABA and its analogs can decrease the amount of days it takes for plants to flower (Wilen et al., 1994).

Exogenously applied ABA can reduce freezing damage to leaves (Rikin and Richmond, 1979). Chen et al. (1979) concluded that exogenous application of ABA can induce cold acclimation. ABA can induce cold acclimation at non-acclimating temperatures (Chen and Gusta, 1983). Plants sprayed with ABA also have reduced GA_3 levels (Waldman et al. 1974). The reduction in GA_3 resulting from ABA application is also evident at non-acclimating temperatures. However, this reduction in GA_3 is not always the case, as exogenous application of ABA increases total gibberellin content and the bioactive gibberellin GA_1 in oriental melon (Kim et al., 2016). Increased ABA concentration is correlated with an increase in proline concentration (Rajagopal and Andersen., 1978).

ABA has been shown to increase stress tolerance of turfgrass subjected to non-freezing stress. For example, Kentucky bluegrass treated with ABA had less electrolyte leakage, increased turf quality and increased photochemical efficiency when subjected to drought (Wang et al., 2003). ABA treatments significantly decreased the death rate of bermudagrass when subjected to drought stress measured by reduce electrolyte leakage and increased antioxidant enzyme activity (Lu et al., 2009).

1.9.2 Gibberellic Acid

Gibberellins are hormones responsible for regulating growth of plants including seed germination (Debeaujon and Koornneef, 2000), stem elongation (Lockhart, 1957), meristematic tissue development and development of floral organs (Wilson et al, 1992). A bioactive gibberellin, GA_3 increases cell length, cell width and internodal length (Potter et al., 1993). Endogenous GA_3 content declines in wheat plants at temperatures approaching and below 0^oC (Reid et al., 1974). Cotton plants overexpressing a protein, GhDREB1, that downregulates GA_3 showed greater cold tolerance than the wild type (Shan et al., 2007). When exogenous GA_3 is applied it negatively affects the plants ability to respond to drought and salt stress (Qin et al., 2011). The loss of responsiveness to stress is likely due to an upregulation of not only GA_3 but other bioactive gibberellins. Exogenous application of GA_3 breaks plant dormancy and de-acclimates hardened plants (Suttle. 2004; Barnes & Wilson, 1986) and delays the onset of dormancy (Dipaola et al., 1981). As the level of endogenous GA_3 increases there is a substantial loss of cold hardiness and a compromised ability to survive prolonged periods at low temperature (Roberts, 1970).

Inhibiting GA_3 synthesis has been implicated in increasing winter hardiness. It has been demonstrated that other types of stress tolerance can be increased by use of GA_3 inhibitors (Vettakkorumakankav et al. 1999). Exogenous application of paclobutrazol, an early stage GA_3 inhibitor, increased heat stress tolerance for wheat seedlings (Kraus and Fletcher, 1994). In winter wheat and winter canola, the fall application of GA_3 inhibitors can increase cold tolerance as well as ice encasement tolerance, but has had inconsistent results (Morrison and Andrews, 1992; Gusta et al., 1993). Inhibiting GA_3 synthesis is likely important for improving a plant's ability to resist de-acclimation in late winter.

1.10 PLANT GROWTH REGULATORS

1.10.1 Trinexapac-ethyl

Trinexapac-ethyl (TE) is a plant growth regulator (PGR) commonly used on golf courses that belongs to a group of compounds known as acylcyclohexanediones. TE reduces shoot growth by preventing GA_3 synthesis through inhibiting the hydrolyzation of a non-biologically active gibberellin, GA_{20}, into a biologically active form, GA_1 (Adams et al., 1992; Rademacher, 1992). The inhibition of GA_3 synthesis by TE prevents cell elongation and increases cell density of turfgrass leaves (Ervin and Koski, 2001). TE is used in turfgrass management to reduce clipping yield (Lickfeldt et al., 2001), increase tiller density (Beasley et al., 2005), stolon number (Fagerness et al., 2002), root biomass under water deficit (Zhang et al., 2017) and improve turf quality (Ervin and Koski, 1998). TE improves the stress resistance of turfgrass to conditions such as shade (Goss et al. 2002), heat and drought (McCann and Huang, 2007), and freezing (Fagerness et al., 2002). The application of TE to Kentucky bluegrass increases a protein associated with cold acclimation (Hwang et al., 1999). GA_3 inhibitors increase carbohydrate

partitioning to the crown in turfgrass, which suggests TE could increase crown survival overwinter (Hanson and Branham, 1987)

TE increases TNCs within turfgrass in stressed environments. Creeping bentgrass treated with TE in a drought environment has more soluble sugars than TE, drought and control treatments alone (Bian et al., 2009). Zoysiagrass undergoes an increase in TNC when TE was applied under shade conditions (Qian and Engelke, 1999).

TE has little effect on TNC for multiple turfgrass species in non-stressed environments. TE reduces clipping yield but does not increase TNC content in tall fescue (Richie et al., 2001). Han et al. (2004) showed very little change in TNC for creeping bentgrass from 4 to 12 weeks after treatment and no change in TNC after 12 weeks. TNC is also unaffected in Kentucky bluegrass pots maintained in a greenhouse (Ervin and Koski, 2002). In contrast to the two previous studies, one study did show an increase in TNC concentration in three turfgrass species four weeks after initial treatment in a non-stressed environment (Ervin and Zhang, 2007).

TE can increase other indicators of winter hardiness in stressed environments. Spring barley treated with TE in a drought environment undergoes small increases in ABA concentration in the stem (Bingham and McCabe, 2006). ABA is increased in barley seedlings by 46% after application of TE (Adams et al., 1992). Prohexadione-calcium, an acylcyclohexanedione that uses the same mode of action as TE, increases ABA concentrations in wheat and oilseed rape (Grossman et al., 1994). Freezing tolerance is increased by a late season

application of TE and maintained for up to 4 weeks (Fagerness et al., 2002). However, 8 weeks after TE treatment increased freezing tolerance is not apparent.

The effect of TE on proline content is uncertain. Bian et al. (2009) demonstrated that TE has no effect on proline content in both stressed and non-stressed environments. A more recent study showed that TE increased proline content of perennial ryegrass under drought compared to the drought control and non-stressed control (Mohammadi et al., 2017)

1.11 PROJECT RATIONALE

Applying PGRs with the purpose of preserving the acclimation status of turfgrass has become a topic of interest lately within the scientific community (Laskowski et al., 2018). Primo Maxx, a late stage GA_3 inhibitor, has been marketed as a method to increase the cold tolerance of plants by increasing non-structural carbohydrate reserves. Using PGRs to increase winter hardiness has been studied in winter cereals in the past but very little literature exists in turfgrass. Altering the ABA to GA_3 ratio within a plant has been hypothesized as a method to increase winter hardiness in winter cereals. Apart from PGR treatments, the acclimation status of creeping bentgrass and annual bluegrass has never been tracked over the course of a winter in the same study from January to April in a similar manner to that performed by Qian et al. (2001) with buffalograss.

1.12 HYPOTHESIS

Acclimation status acquired in the fall begins to decline as the winter persists into the spring. A warming period can cause an even greater loss of acclimation status than the standard transition from winter to spring. It is hypothesized that winterkill of turfgrasses can be influenced through fall application of PGRs by impacting ABA to GA_3 ratio in the late winter and early spring.

1.13 OBJECTIVES

• Demonstrate the decline in winter hardiness of cool-season turfgrasses over the winter period.

• Determine the effect of foliar application of plant growth regulators and hormones to prevent loss of acclimation status of turfgrass.

• To evaluate short term changes in winter hardiness of annual bluegrass and creeping bentgrass through a simulated warming event.

CHAPTER TWO - METHODS

2.1 FIELD EXPERIMENT – WINTER 2017-2018

A field experiment on a golf green was established in November of 2017 that included 4 PGR treatments and 2 species treatments were arranged with all factorial combinations in a randomized complete block design with 4 blocks for a total of 32 experimental units. The green was fertilized to a rate of 2.4 kg/100m^2 of N, 0.38 kg/100m^2 of P, and 3.7 kg/100m^2 of K for the year prior to establishing plots. A pot-in-pot method was used to overcome the issues associated with removing turfgrass cores over the winter period. Turfplugs, 65mm in diameter, of annual bluegrass (*Poa annua* L.) and creeping bentgrass cv. 'A4' (*Agrostis stolonifera* L.) were removed from a sand based USGA specification golf green in Guelph ON (43° 32' 59.7"N, 80° 12' 53.9"W). Air temperature was obtained from Environment Canada weather station located at the Guelph Turfgrass Institute (Figure 2.1A). Soil temperature was monitored with three datalogger's (Specware WatchDog B-Series Button Logger) placed at crown level (~1cm depth) (Figure 2.1B). In each case, the turfplug was cut to 50mm in length and placed in a 65mm diameter pot to be flush with the surface of the pot. The pots containing a turfplug were then placed into another pot that was installed in the rootzone of the experimental golf green flush with the surface of the green (43° 32' 59.7"N, 80° 12' 53.9"W). Each experimental unit had eight sets of nine pots to be extracted at each date for a total of 72 pots. Turfplugs in six pots were used for cold tolerance tests and the turplugs in three pots were reserved for biochemical analysis. On November 29 and December 3 of 2017, PGRs were applied using a bicycle sprayer for a total of four treatments: an unsprayed control, Sigma-Aldrich recommended rate of GA_3 (2 mg/m^2), Primo Maxx suggested rate of TE (4.4 mg/m^2), and ABA rate (11.1 mg/m^2) as used by Li

et al. (2017) (Table 2.1). Four sets of turfplugs from this experimental set up were used for a monthly assessment of winter hardiness. Two sets of turfplugs were used for simulated warming event and 2 turfplugs sets were used for the controls of simulated warming event (Table 2.2).

Two separate and independent experiments were conducted in the winter of 2017-2018 to assess the effect of fertilizer on winter hardiness. Only cold tolerance was assessed for these experiments and there were no significant differences among fertilizer treatments in both experiments (see Appendix A).

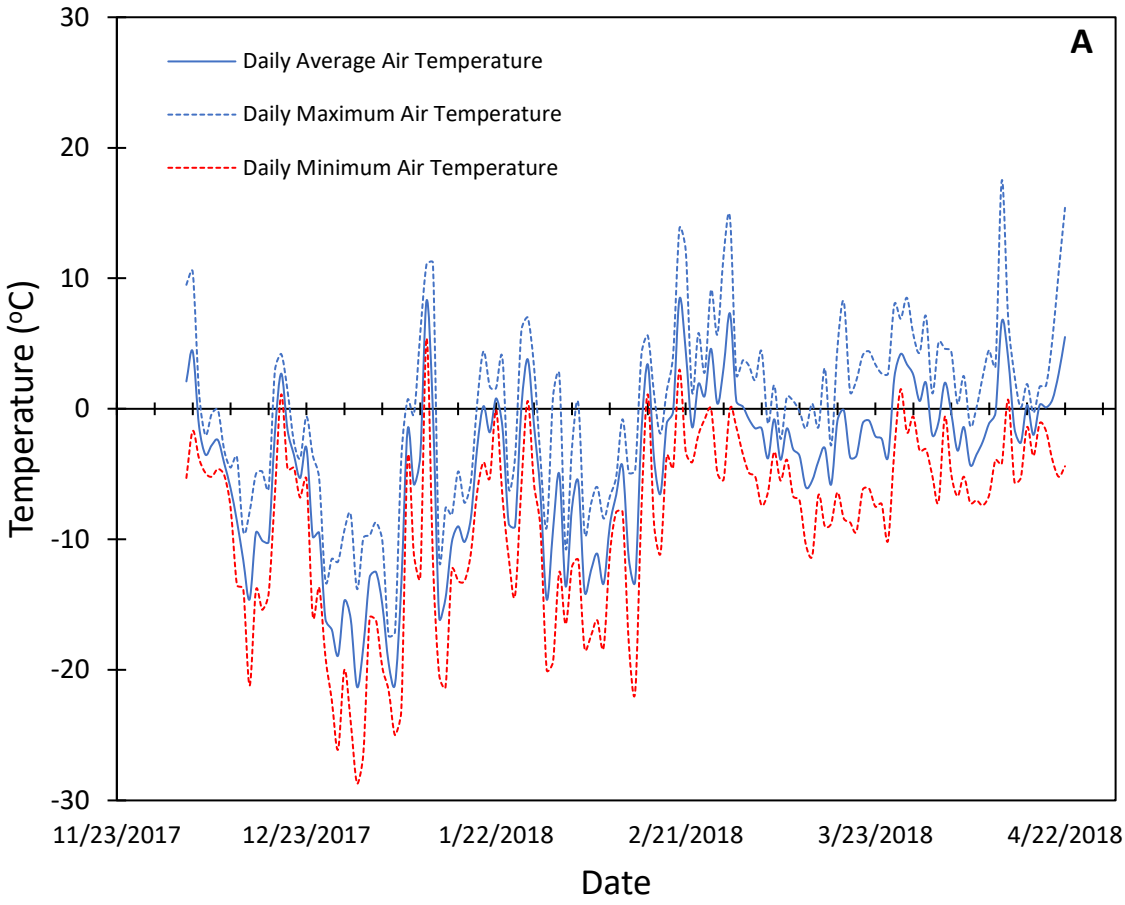
Table 2.1: PGR treatments applied to annual bluegrass and 'A4' creeping bentgrass plots. All PGR treatments applied in a spray volume of 50 mL/m^2 to a USGA golf green in November of 2017.

Treatment	Rate
Abscisic acid (ABA)	**11.1 mg/m^2**
Trinexapac-ethyl (TE)	**4.4 mg/m^2***
Gibberellic acid (GA_3)	**2.0 mg/m^2**
Control	**Untreated**

*Equivalent to Primo Maxx label rate for creeping bentgrass putting greens (3.8 mL/100 m^2)

2.1.1 Monthly Assessment of Winter Hardiness of Field Acclimated Turfgrass

In 2018, a set of nine turfplugs was removed from each treatment on January 13, February 15, March 21, and April 22. Six turfplugs were used for LT_{50} to assess cold tolerance as an indicator of acclimation status and three turfplugs were designated for biochemical analysis.


A
Daily Average Air Temperature
Daily Maximum Air Temperature
Daily Minimum Air Temperature
Temperature (°C)
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20
10
0
-10
-20
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11/23/2017
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2/21/2018
3/23/2018
4/22/2018
Date

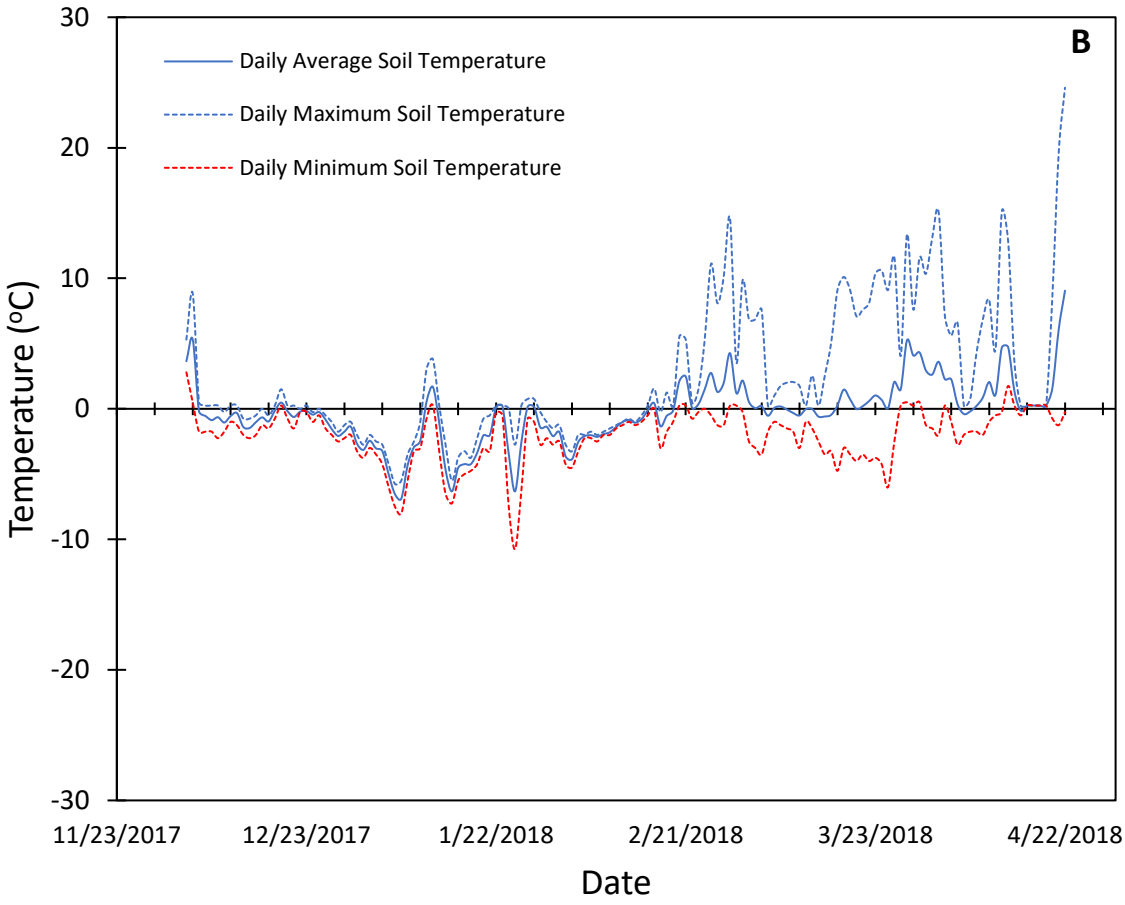


Figure 2.1: Air temperature (A) and soil temperature (B) at 1 cm depth measured every hour with a B-Series WatchDog data logger for the USGA specification golf green at the Guelph Turfgrass Institute in Guelph, Ontario from December 4, 2017 to April 22, 2018.

2.1.2 Simulated De-acclimation Event in Growth Chamber of Field Acclimated Turfgrass

Simulated thaw/refreeze event in a Conviron PGW36 controlled environmental growth chamber was conducted for field grown plants that underwent exogenous application of PGR. Due to the number of samples two independent cycles of simulated warming in the growth chamber were performed. Cycle 1 was conducted using samples from blocks 1 and 2 on February 24, 2018 and Cycle 2 was conducted with blocks 3 and 4 on March 8, 2018 (Table 2.2). On each of these dates a set of turfplugs was extracted for a simulated warming period in a

growth chamber and then at the end of the warming period were returned the field and cold tolerance was assessed 1 month thereafter to assess at the ability to re-acclimate (RA) and labeled Cycled 1/RA and Cycled 2/RA. Upon removal from the field, samples were placed in the growth chamber for 5 days (10^{o}C day/ 4^{o}C night) with a 10-hour photoperiod and photosynthetic photon flux density of 275 µmol m^{-2} s^{-1} achieved with incandescent and florescent lightbulbs. A control for Cycled 1, Cycled 2, Cycled 1/RA and Cycled 2/RA was placed in a freezer set to -2^{o}C over the same 5 days in the dark. At the end of the 5-day de-acclimation period, Cycled 1, Non-Cycled 1, Cycled 2, and Non-Cycled 2 were assessed for cold tolerance and destructively harvested for crown tissue. Cycled RA 1 and Non-Cycled RA 1 were extracted on March 31, 2018 and assessed for cold tolerance and destructively harvested for crown tissue, whereas Cycled RA 2 and Non-Cycled RA 2 were extracted on April 10, 2018 and assessed for cold tolerance and destructively harvested for crown tissue.

Table 2.2: Extraction dates for turfplugs subjected to the following cycled treatments: 5 days of simulated warming in a growth chamber (Cycled 1 and Cycled 2), 5 days in a freezer at -2°C (Non-Cycled 1 and Non-Cycled 2), five days of simulated warming in a growth chamber and then placed back in the field for about one month (Cycled RA 1 and Cycled RA 2), five days of simulated warming in a growth chamber and five days in a freezer at -2°C and then placed back into the field for one month (Non-Cycled RA 1 and Non-Cycled RA 2). Plant growth regulator treatments (n=2) of GA_3 (2 mg/m^2), trinexapac-ethyl (4.4 mg/m^2), ABA (11.1 mg/m^2) and an untreated control were applied to the plots of each cycle treatment in November of 2017.

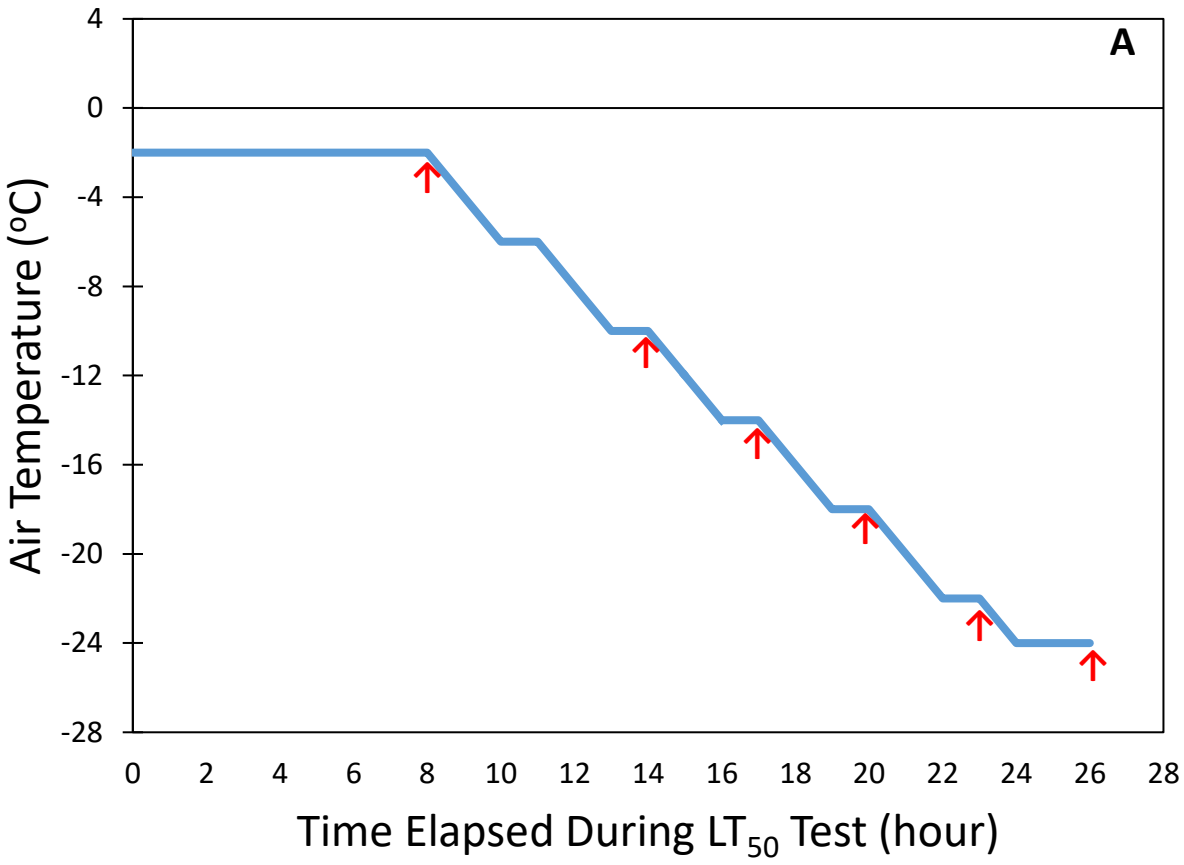
Pooled Cycled Treatments	Cycled Treatments	Date Removed from Field For Cycling	Date of LT_{50} Assessment
Cycled	**Cycled 1**	**February 24, 2018**	**March 1, 2018**
	Cycled 2	**March 8, 2018**	**March 13, 2018**
Non-Cycled	**Non-Cycled 1**	**February 24, 2018**	**March 1, 2018**
	Non-Cycled 2	**March 8, 2018**	**March 13, 2018**
Cycled RA	**Cycled RA 1**	**February 24, 2018**	**March 31, 2018**
	Cycled RA 2	**March 8, 2018**	**April 8, 2018**
Non-Cycled RA	**Non-Cycled RA 1**	**February 24, 2018**	**March 31, 2018**
	Non-Cycled RA 2	**March 8, 2018**	**April 8, 2018**

2.2 ASSESSMENT OF WINTER HARDINESS

2.2.1 Cold Tolerance (LT_{50})

LT_{50} is the temperature corresponding at which 50% of plants survive. Cold tolerance was assessed at each of the designated dates (Table 2.2). Six turfplugs were removed from each experimental unit and temperature equilibrated in a freezer set at -2°C for 8 hours (Figure 2.2). After 8 hours, one turfplug was removed, and the temperature was decreased at a rate of -2°C per hour and stabilized every -4°C increment for one hour: -6, -10, -14, -18, -22, -24°C. One turfplug from each treatment replicate was removed at each of the following target temperatures: -2, -10, -14, -18, -22, -24°C. Once a turfplug was removed from the freezer it was placed in a 4°C refrigerator for at least 24hr.

To assess survival, individual plugs from each treatment were sectioned into quarters and 5 tillers from each quadrant were placed into trays of sand. These trays were then placed on a misting bench for a 7-day regrowth period and then assessed for survival. Survival of each tiller was based upon a binary assessment of dead or alive, whereas the tiller was classified as alive if it regenerated green tissue after the 7-day regrowth period. If there was any question whether the plant was alive, the plant was pulled on to see if root tissue had regenerated. If the tiller regenerated root tissue it was considered alive. See statistical analysis section (3.1.1) in methods for how LT_{50} was calculated.


A
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-28
Air Temperature (°C)
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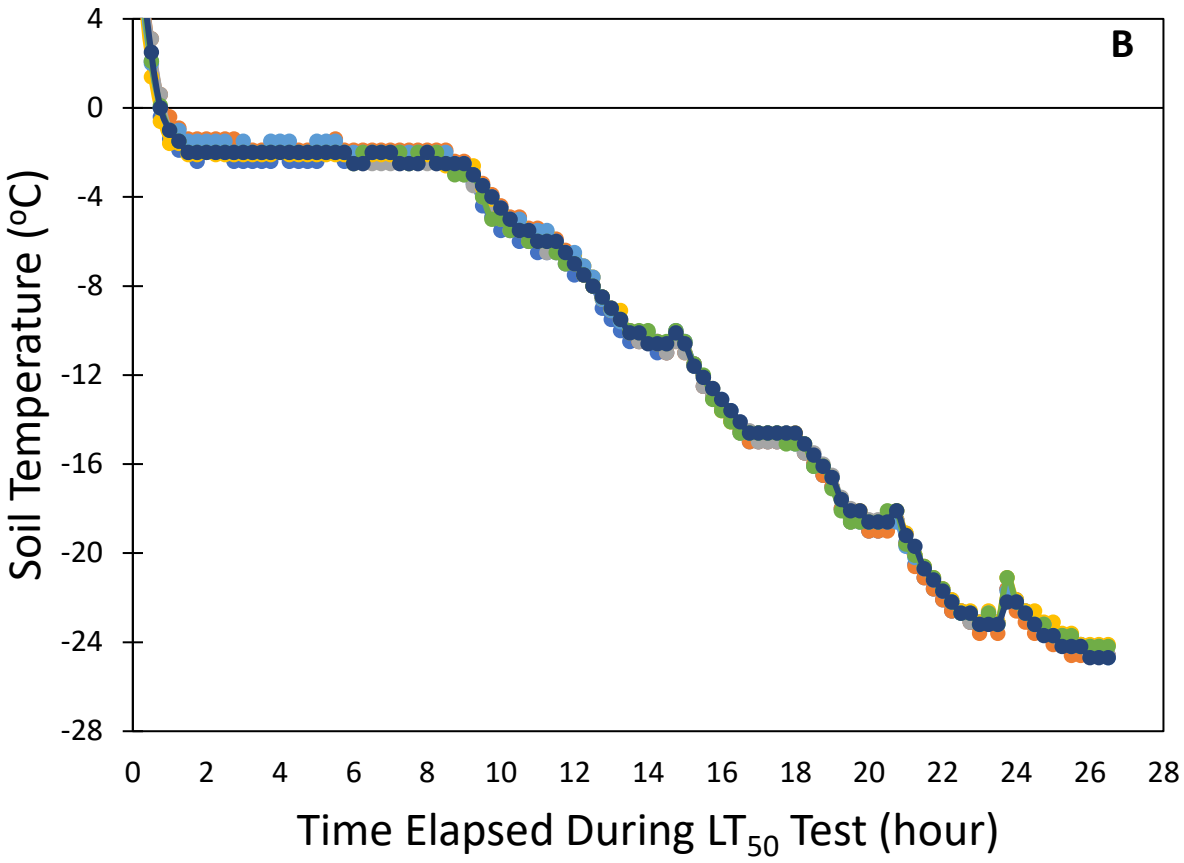


Figure 2.2: Temperature series program used to conduct freezer tests evaluating the LT_{50} of turfplugs (A) and soil temperature from seven B-Series WatchDog data loggers placed a 1 cm depth in turfplugs at different locations during a live freezer test (B).

2.2.2 Net Carbon Exchange Rate

Net carbon exchange rate (NCER) was assessed for cycled treatments samples using an infrared gas analyzer (Li-6400; LI-COR Inc., Lincoln, Nebraska, USA) while they were in a modified 6400-17 Whole Plant Arabidopsis Chamber under the following growth parameters: 400 μmol CO_2, CO_2 mol^{-1} air flow rate 500 and a PAR 500 μmol m^{-2} s^{-1}). NCER was assessed

during the period with the lights on as well as in the dark after 2 and 4 days in the growth chamber.

2.2.3 Crown Tissue for Biochemical Analysis:

The three turfplugs designated for biochemical analysis were incubated in a 4^{o}C fridge for 12 hours. Thereafter, leaf tissue was trimmed from turfplugs with a Wahl Precision hair trimmer. The turfplugs were cut 1 cm below soil level to remove crown tissue from the roots. Crown tissue was washed thoroughly with cold water to remove any soil from the crown tissue. The crown tissue from three plugs was pooled together and divided into two separate sub samples. One sub sample was designated as fresh material and the other was designated for freeze drying. These sub samples were individually wrapped in tin foil and frozen in liquid nitrogen and stored at -80^{o}C for biochemical analysis.

The freeze-dried sub sample was used to quantify proline. In August 2018, these sub samples were placed in a LabConco -50 ^{o}C freeze drier for at least 24 hrs to be lyophilized. When the samples were removed from the freezer drier they were ground to a fine powder in a Black and Decker coffee grinder and passed through a 60-mesh sieve.

2.2.4 Phytohormone Analysis

ABA and GA_3 were extracted and quantified using a method developed by Merewitz et al. (2015). Previously frozen crown tissue was pulverized under liquid nitrogen with a mortar and pestle. Frozen crown tissue powder (~100 mg) was transferred to a 1.5 mL microfuge tube and mixed with 1 mL of extraction buffer (80:20 v/v methanol: water, 0.1% formic acid, 0.1 g/L butylated hydroxytoluene) and then vortexed for 15 s. The extraction buffer also included an

internal standard of 100 nmol of ABA-d6 (Toronto Research Chemicals, North York, Canada). An internal standard for deuterated GA_3 was not available. The samples were then placed on a shaking bed at 250 rpm at 4°C for 18 hr and then centrifuged at 12,000 x *g* for 15 min at 4°C. A 10 μL injection of the supernatant was injected into a Supelco Ascentis Express C18 column (2.1 mm x 50 mm, 2.7 μm particle size; Sigma-Aldrich, St. Louis, MO, USA) attached to a UPLC system in tandem with mass spectrometry (Quattro Premier XE, Waters, Milford, MA, USA). The extract was eluted at a flow rate of 0.400 mL per minute with solvent A (water + 0.1% formic acid) and solvent B (acetonitrile) as follows; 99% A and 1% B, 0.00-0.50 min; 99%-30% A and 1%-70% B, 0.50-3.00 min; 30%-0% A and 70%-100% B, 3.00-3.01min; 0% A and 100% B, 3.00-3.50 min; 0%-99% A and 100%-1.0% B, 3.50-3.51; 99% A and 1% B, 3.51-5.00 min. The mass spectrometer was set to the following parameters electrospray was set to negative ionization mode, a desolvation temperature of 350°C, desolvation gas flow of 800 L/hr, and a cone gas glow of 40 L/hr. ABA was determined with precursor ions and product ions of m/z 263 and m/z 153, whereas GA_3 was determined by m/z 345 and m/z 239 respectively. Phytohormones were estimated from a standard curve of ABA and GA_3 (Millipore Sigma Canada Co., Oakville, Canada) in the range of 0 nM to 2000 nM that contained an internal standard of 100 μM ABA-d6. ABA and GA_3 content were calculated on a fresh weight basis.

2.2.5 Proline Determination

Proline content was determined using the method described in Bates et al. (1973) with modifications made by Patton et al. (2007).50 mg of crown tissue was mixed with 1 mL of 3% (w/v) 5-sulfosalicylic acid (Millipore Sigma Canada Co., Oakville, Canada) in a 1.5 mL microfuge

tube, vortexed for 15s, and then incubated in a 70°C water bath for 20 min. The tubes were subsequently placed on an orbital shaker for 15 min prior to centrifugation at 14,000 x *g* for 10 min. For proline determination, a 100 μL aliquot of the supernatant was combined with 900 μL of double-deionized water, 1 mL acid ninhydrin, and 1 mL of glacial acetic acid. Thereafter, tubes were vortexed for 15 s and then transferred to a 100°C oven for 1h. Tubes were transferred to an ice bath at the end of the incubation period until cooled., followed by the addition of 4 mL of toluene. Phase separation was performed by vortexing for 20 s; 3 mL of the upper layer was transferred to spectrophotometric cuvettes and the absorbance read at 520 nm (Evolution 60S UV-Visible Spectrophotometer, Thermo Fisher Scientific, Waltham, MA.). Toluene was used as a blank. To estimate the proline content in crown tissue, absorbance values for the crown tissue extracts were compared to a known range (0 to 62.5 ug per mL) of L-proline (Millipore Sigma Canada Co., Oakville, Canada), and expressed on a dry weight basis.

2.3 Statistical Analysis

Cold tolerance was analyzed in R statistical program using a binomial model and a link-logistical function to predict temperature of 50% survival (LT_{50}). Block was considered a random effect. Date and PGR treatments were considered fixed effects. Species and date were analyzed separately except for a monthly comparison of LT_{50} values with all PGR treatments pooled.

All other statistical analysis was completed with SAS 9.4 (SAS Institute Inc, Cary, NC) using the function Proc Glimmix (Generalized Linear Mixed Model) for NCER, proline content, ABA content, and GA_3 content. Data was screened for normality and homogeneity of residuals before analysis was conducted. Block was considered a random effect. Date and PGR

treatments were considered fixed effects. Species were analyzed separately for NCER, proline content, ABA content, and GA_3 content an alpha of 0.05 was set for the type 1 error rate of all statistical analysis. Least Square means were computed for all dependent variables for PGR treatments and harvest dates, as well as the interaction between those two dependent variables. Means were compared pairwise with a Tukey HSD test.

Two independent cycling events were sampled with two replications each for samples that underwent a two-independent simulated de-acclimation events in the growth chamber. A t-test found no differences between the individual cycling dates for LT_{50} and biochemical analysis. Since no differences were found, samples from the two cycling events were pooled with the corresponding de-acclimation treatment to result in 4 replications per PGR treatment, for example Cycled 1 and Cycled 2 were pooled together as Cycled (Table 2.2).

CHAPTER THREE - RESULTS

3.1 ASSESSING IMPACT OF PLANT GROWTH REGULATORS ON COLD TOLERANCE OF FIELD ACCLIMATED TURFGRASS

3.1.1 Cold Tolerance (LT_{50})

Creeping bentgrass maintained a low LT_{50} from January through early April, and the LT_{50} increased with warmer temperatures in mid to late April (Figure 3.1). Annual bluegrass had an increase in LT_{50} from January to February, with LT_{50} at its lowest in March although all of these LT_{50} were between -16 and -20^{o}C (Figure 3.1). Annual bluegrass was more sensitive to the warming period in late March and early April than creeping bentgrass and LT_{50} increased from March to early April.

Fall application of PGRs had no effect on LT_{50} over the winter, but during the de-acclimation phase in late March and April both species began to show differences in LT_{50} among PGR treatments (Supplemental Figure B3.1). All PGR applications increased the risk of freezing injury for annual bluegrass in the month of April by increasing LT_{50} in the range that crowns could experience in April (Figure 3.2). Application of ABA and GA_3 decreased LT_{50} for creeping bentgrass as compared to the control and TE for the month of April.

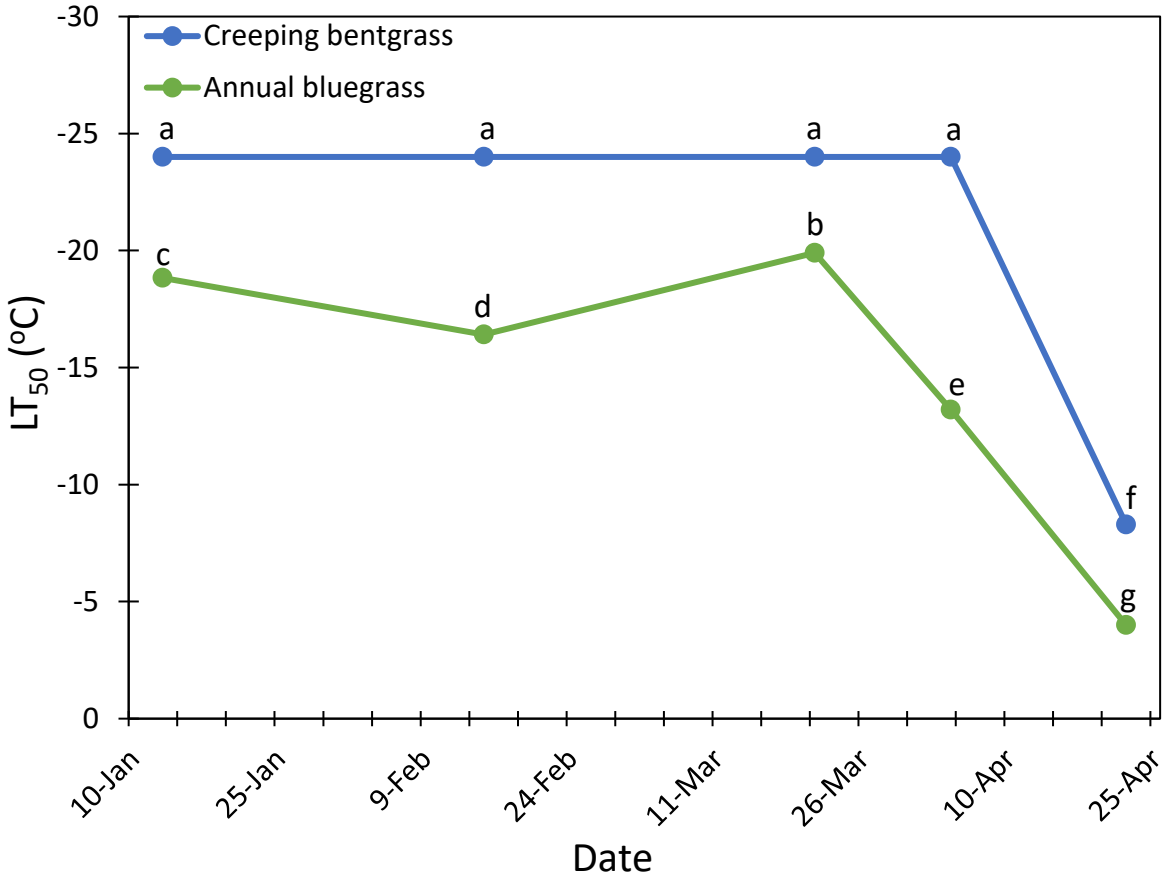


Figure 3.1 Assessment of monthly acclimation status by lethal temperature of 50% survival (LT_{50}) to a minimum temperature of -24°C of annual bluegrass and creeping bentgrass with all PGR treatments pooled for a field experiment at the Guelph Turfgrass Institute in Guelph, Ontario. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means ($n = 16$) pairwise comparisons with least square means.

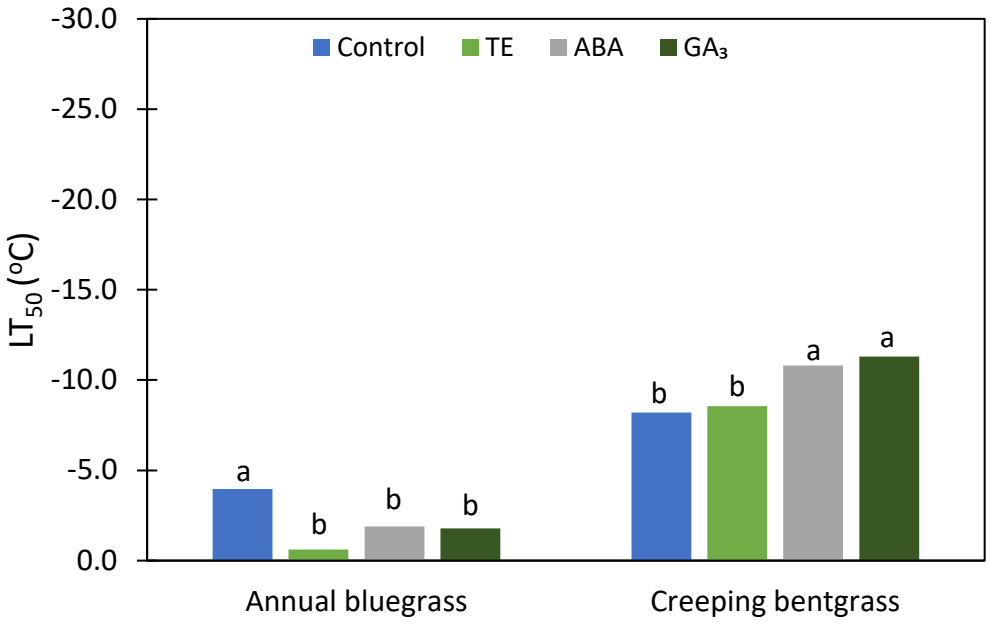


Figure 3.2 Assesment of acclimation status on April 22 by lethal temperature of 50% survival (LT_{50}) for annual bluegrass and creeping bentgrass after fall application of abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Different lowercase letter indicate significant differences ($P \leq 0.05$) for means ($n = 4$) across individual dates based on a pairwise comparisons with least square means. No signficant differences were observed on January, February and March sampling dates.

3.1.2 Biochemical Analysis

Fall application of ABA increased endogenous ABA levels for annual bluegrass in the months of January and February relative to all other PGR treatments when temperatures were consistently below 0^{o}C (Figure 2.1 and Figure 3.3). Fall application of PGRs had no effect on endogenous levels of ABA for annual bluegrass in the months of March and April relative to the control when temperatures began to increase (Supplemental Figure B3.2A). PGR treatments had no effect on endogenous levels of ABA for CB for all months relative to the control (Supplemental Figure B3.2B).

Plant growth regulator treatments had no effect on endogenous levels of GA_3 for creeping bentgrass on all dates relative to the control (Supplemental Figure B3.3). Fall application of GA_3 resulted in increased levels of endogenous levels of GA_3 relative to the control annual bluegrass in the month of January. (Figure 3.4).

Fall application of ABA resulted in greater proline levels in annual bluegrass than the control and GA_3 on January 13th after being subjected to consistent temperatures below 0^{o}C (Figure 3.5A). Proline content was higher in TE than the GA_3 treatment for creeping bentgrass in the month of January, but no PGR treatment was found to be different than the control (Figure 3.5B). PGRs did not impact proline content in both grasses for the months of February and March (Supplemental Figure B3.4). Trinexapac-ethyl treated plants had greater proline levels than the control and ABA treatment for annual bluegrass in the month of April when average soil temperatures at crown level rose above 0^{o}C, whereas all PGRs had no effect on creeping bentgrass for the month of April.

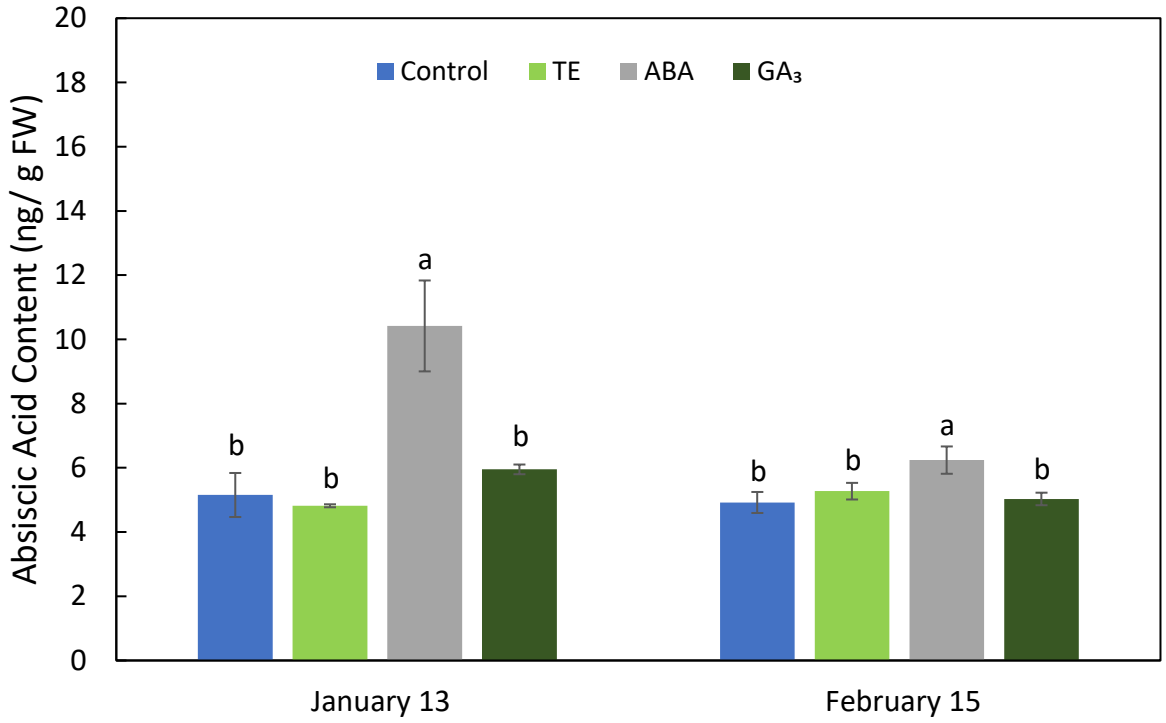


Figure 3.3 Monthly assessment of endogenous levels of abscisic acid content (ng/g FW) within the crown tissue of annual bluegrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means ($n = 4$) within individual dates based on a Tukey's HSD test. No significant differences were apparent for annual bluegrass during the March and April sampling dates. There were no significant differences for creeping bentgrass on all sampling dates.

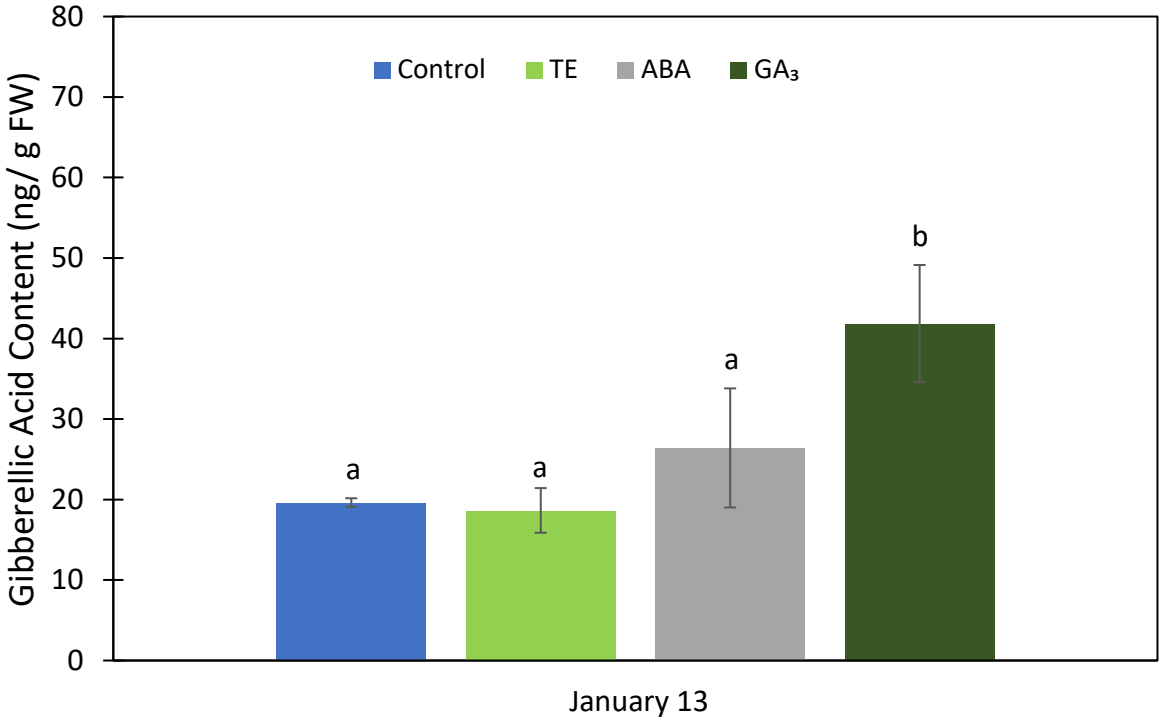
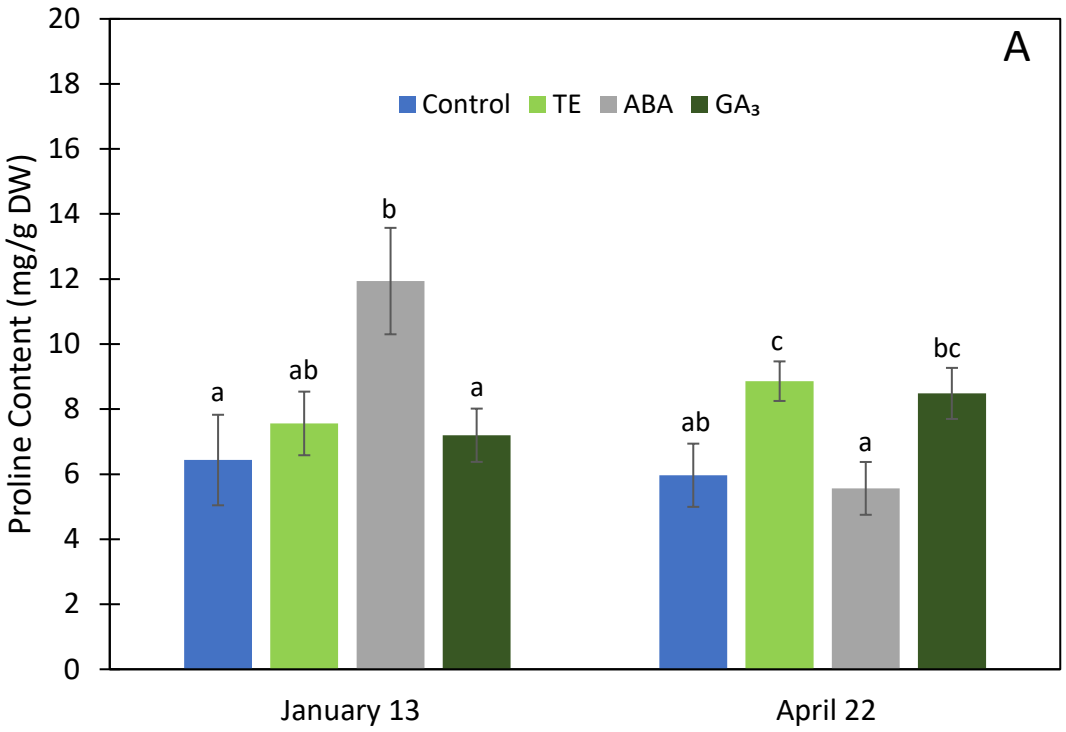


Figure 3.4 Assessment of endogenous levels of gibberellic acid content (ng/g FW) within the crown tissue of annual bluegrass on January 13 after fall application of plant growth regulator (PGR) treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means ($n = 4$) based on a Tukey's HSD test. Significant differences between PGR treatments were only observed on the January sampling date for annual bluegrass. No significant differences between PGR treatments were found for creeping bentgrass on all dates.


A
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
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ab
c
a
bc
January 13
April 22

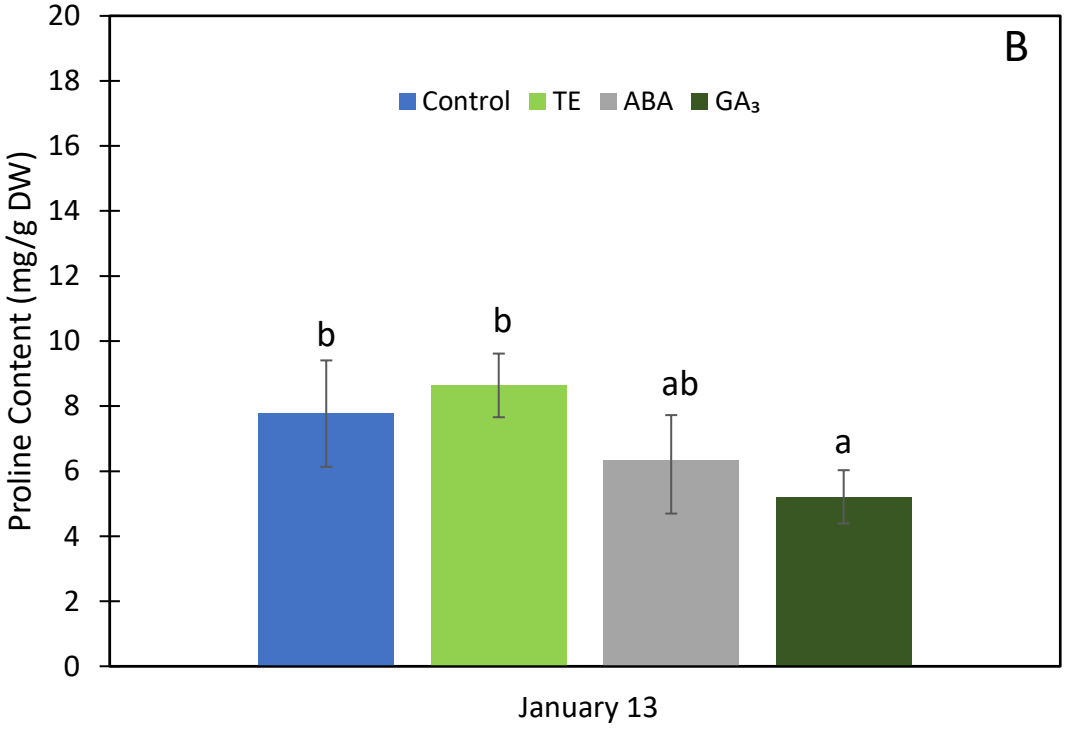

B
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
20
18
16
14
12
10
8
6
4
2
0
b
b
ab
a
January 13

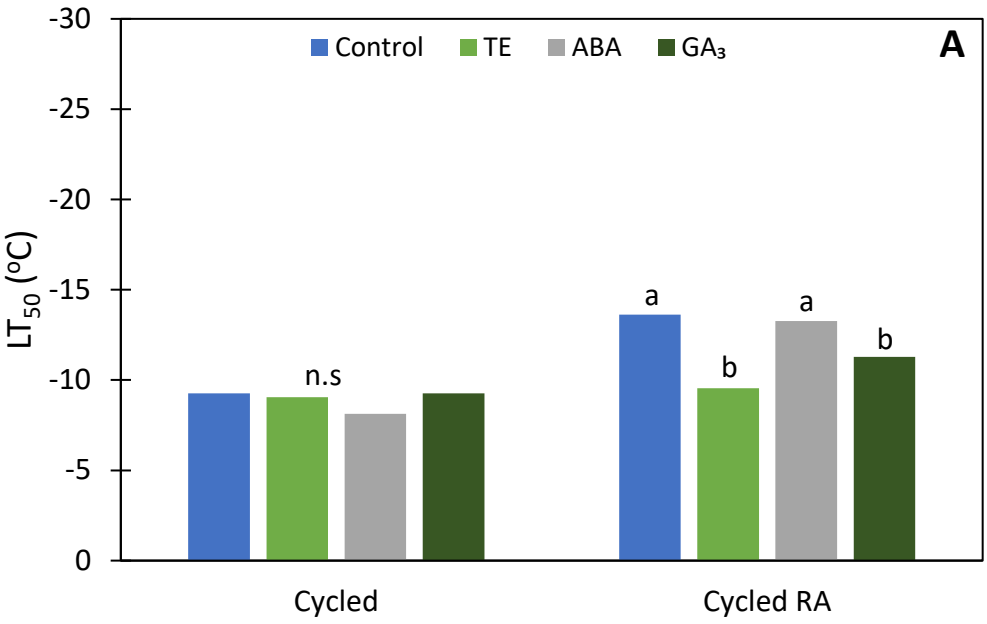
Figure 3.5 Monthly assessment of endogenous levels of proline content (mg/g DW) within the crown tissue of (A) annual bluegrass and (B) creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means ($n = 4$) within individual dates based on a Tukey's HSD test. No significant differences were observed for annual bluegrass on the February and March sampling dates. Significant differences were observed for creeping bentgrass on the January sampling date.

3.2 ASSESSING IMPACT OF PLANT GROWTH REGULATORS ON COLD TOLERANCE OF FIELD ACCLIMATED TURFGRASS SUBJECTED TO A SIMULATED DE-ACCLIMATION EVENT IN A GROWTH CHAMBER

3.2.1 Cold Tolerance (LT_{50})

Fall application of PGRs had no effect on LT_{50} of annual bluegrass during the simulated warming period for the cycled treatment (Figure 3.6A). Trinexapac-ethyl and GA_3 resulted in decreased LT_{50} of creeping bentgrass for cycled treatments relative to the untreated control and ABA (Figure 3.6B). For the cycled RA treatment, trinexapac-ethyl and GA_3 had the opposite effect for annual bluegrass during RA, resulting in increased LT_{50} relative to the untreated control and ABA (Figure 3.6A). PGR treatment had no effect on the ability of creeping bentgrass to regain LT_{50} after de-acclimation and being returned to the field relative to the control, ABA application did result in lower LT_{50} relative to GA_3 (Figure 3.6B).

A simulated de-acclimation event resulted in a loss of cold hardiness as observed by an increase in LT_{50} relative to the plants that remained in a freezer as a control (Figure 3.7). Annual bluegrass and creeping bentgrass plugs that were subjected to the de-acclimation event (Cycled) and returned to the field had similar LT_{50} to plugs that did not experience de-acclimation (Supplemental Figure B3.5).


A
Control
TE
ABA
GA₃
LT50 (°C)
-30
-25
-20
-15
-10
-5
0
n.s
a
b
a
b
Cycled
Cycled RA

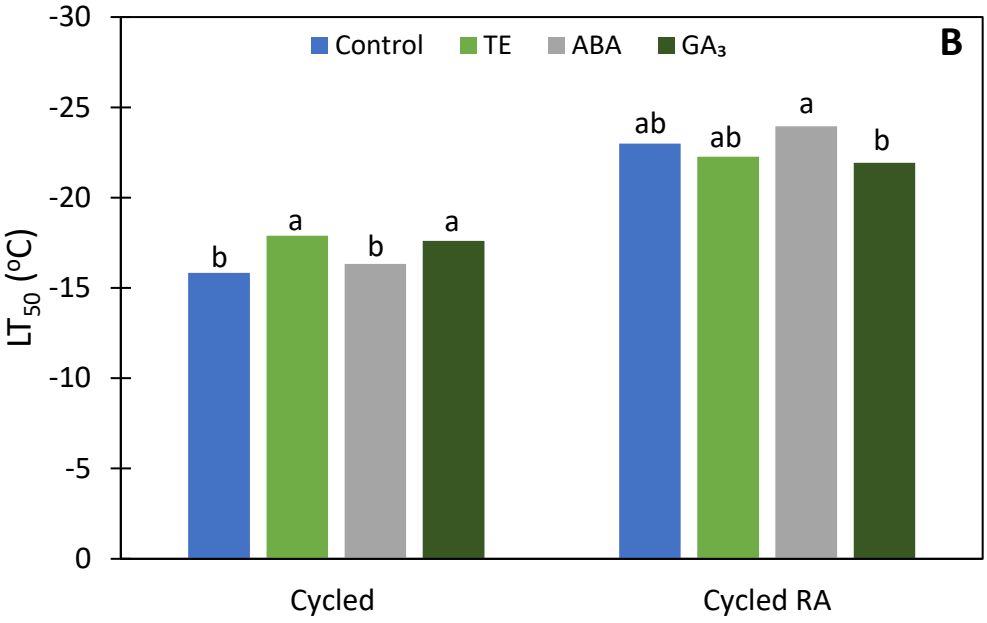

B
Control
TE
ABA
GA₃
LT50 (°C)
-30
-25
-20
-15
-10
-5
0
b
a
b
a
ab
ab
a
b
Cycled
Cycled RA

Figure 3.6 Assessment of acclimation status by lethal temperature of 50% survival (LT_{50}) of annual bluegrass (A) and creeping bentgrass (B) to a minimum temperature of -24°C after the fall application of abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3) directly following a simulated thawing event in a growth chamber (Cycled) and after about 1 month in the field after a simulated thawing event in a growth chamber (Cycled RA). Different lower-case letters indicate significant differences ($P \leq 0.05$) for means ($n = 4$) within individual dates based on a pairwise comparisons with least square means.

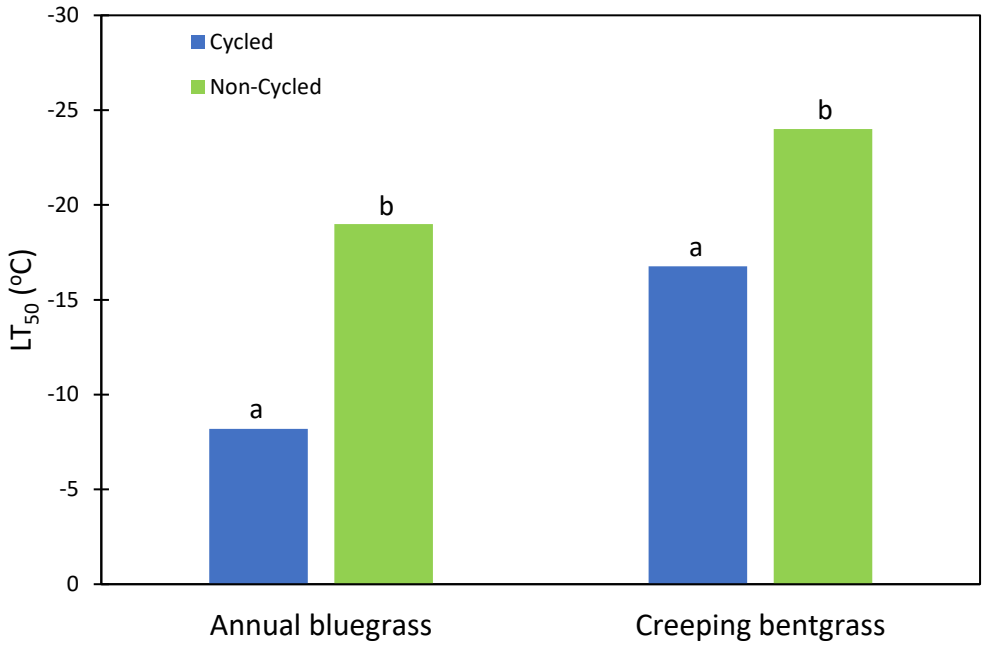


Figure 3.7 Assessment of acclimation status by lethal temperature of 50% survival (LT_{50}) for field acclimated annual bluegrass and creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). PGR treatments were pooled for analysis. Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber (Cycled), five days of simulated warming in a growth chamber and five days in a freezer at -2°C (Non-Cycled). All PGR treatments were pooled for each individual species. Different lower-case letters indicate significant differences ($P \leq 0.05$) for means ($n = 16$) within de-acclimation treatment based on a Tukey's HSD test.

3.2.2 Biochemical Analysis

Regardless of grass type, there were no differences among PGR treatments for endogenous levels of ABA, GA_3, and proline during the simulated warming event (cycled) or during the re-acclimation treatment (cycled RA) (Supplemental Figure B3.9, Supplemental Figure B3.10, Supplemental Figure B3.11).

The simulated de-acclimation event resulted in lower ABA levels in the cycled treatment compared to the non-cycled control both grasses (Figure 3.8). The ABA levels in Cycled RA was no different in ABA content than its the non-cycled control for both species (Supplemental Figure B3.6). The warming period imposed by the cycled treatment resulted in higher GA_3 level than the non-cycled control for annual bluegrass (Figure 3.9). For creeping bentgrass the warming period of the cycled treatment had no effect on GA_3 content. Annual bluegrass and creeping bentgrass plugs subjected to the Cycled RA treatment were no different in GA_3 content than non-cycled RA control (Supplemental Figure B3.7).

The de-acclimation treatment had no effect on proline content for both grasses compared to the non-cycled control (Supplemental Figure B3.8). Proline content in annual bluegrass was not affected by 5 days of de-acclimation in the growth chamber and re-acclimation in the field relative to the freezer control (Figure 3.10). Proline content was not different between re-acclimation treatments for creeping bentgrass.

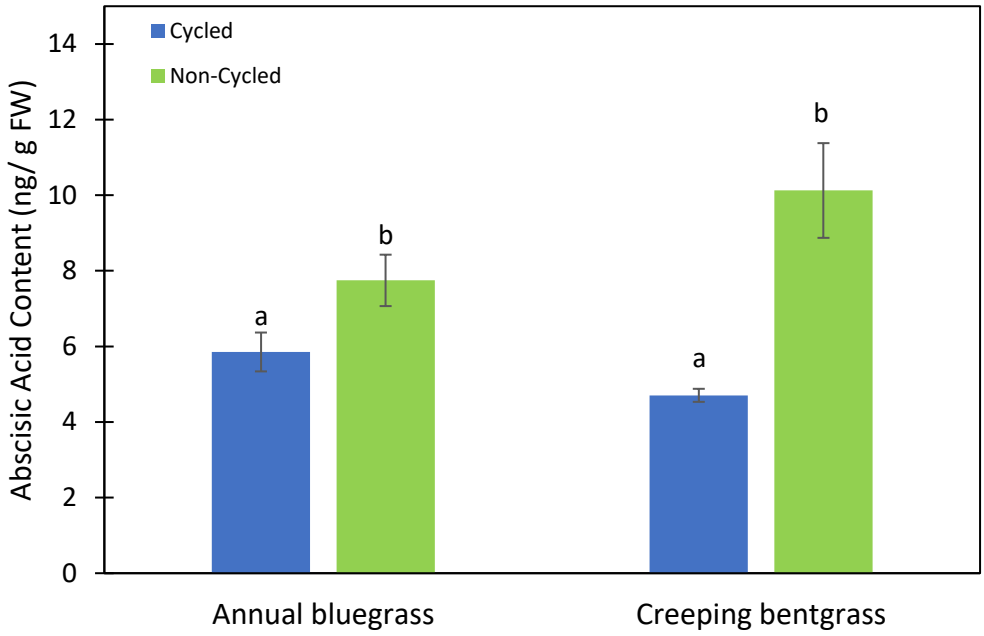


Figure 3.8 Abscisic acid content (ng/g FW) in the crown tissue of field acclimated annual bluegrass and creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber (Cycled), five days of simulated warming in a growth chamber and five days in a freezer at -2°C (Non-Cycled). All PGR treatments were pooled for each individual species. Error bars represent the standard error for means of each de-acclimation treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means (n = 16) within de-acclimation treatment and species based on a pairwise comparisons with least square means.

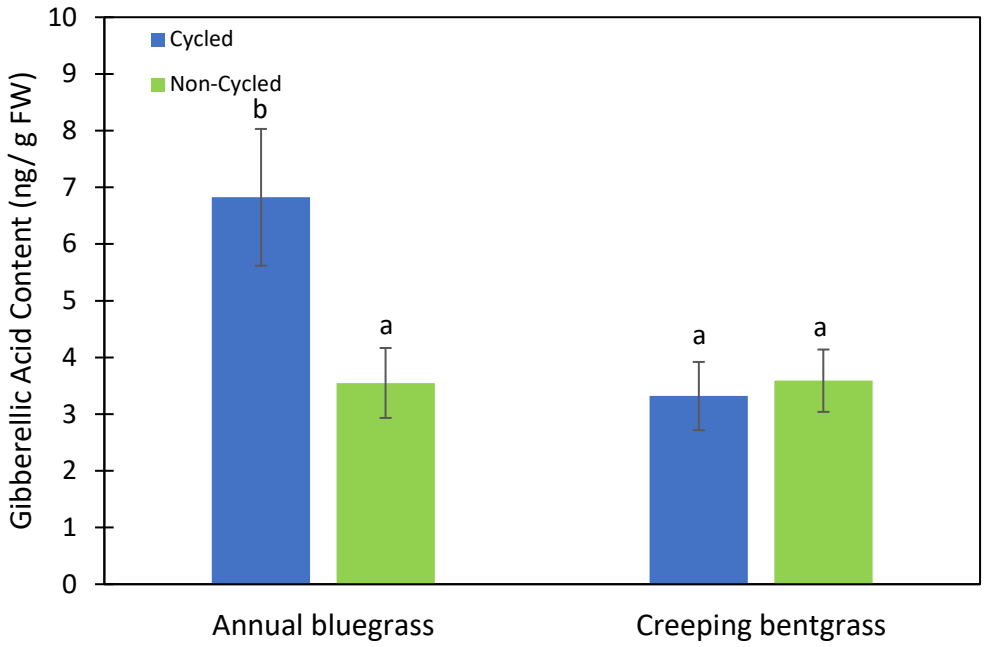


Figure 3.9 Gibberellic acid content (ng/g FW) in the crown tissue of field acclimated annual bluegrass and creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber (Cycled), five days of simulated warming in a growth chamber and five days in a freezer at -2°C (Non-Cycled). Error bars represent the standard error for means of each de-acclimation treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means (n = 16) across de-acclimation treatment and species based on a Tukey's HSD test.

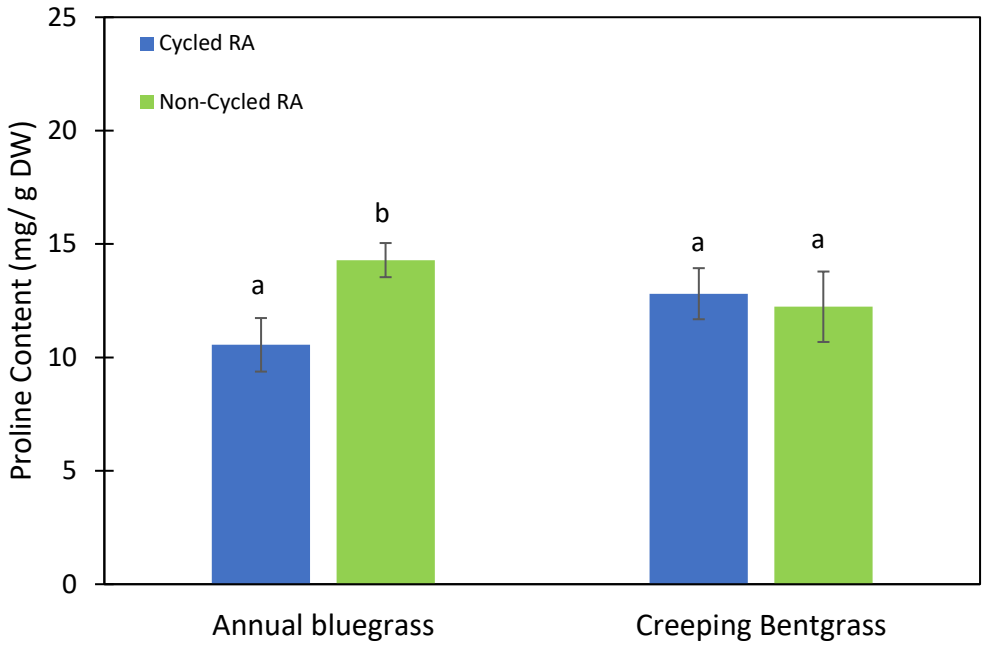


Figure 3.10 Proline content (mg/g DW) in the crown tissue of field acclimated annual bluegrass and creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2°C and then placed back into the field (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31st and April 8. Error bars represent the standard error for means of each de-acclimation treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means ($n = 16$) across de-acclimation treatment and species based on a Tukey's HSD test.

3.2.3 Net Carbon Exchange Rate (Photosynthesis and Respiration)

Fall application of TE resulted in greater NCER in the presence of light than the control and all other PGR treatments for annual bluegrass after 2 and 4 days of a simulated warming event (Figure 3.11A and 3.12A). Trinexapac-ethyl resulted in lower NCER in the presence of light than the control and all other PGR treatments for creeping bentgrass at both 2 and 4 days of a simulated warming event (Figure 3.11B and Figure 3.12B).

Fall application of TE to annual bluegrass resulted in a NCER in the dark than GA_3 after 2 days of simulated warming, but neither PGR treatment was different compared to the control (Figure 3.13). After 4 days of simulated warming there was no difference in NCER in the dark among PGR treatments for annual bluegrass (Supplemental Figure B3.13A). PGR treatment had no effect on NCER in the dark for creeping bentgrass (Supplemental Figure B3.12, Supplemental Figure B3.13B).

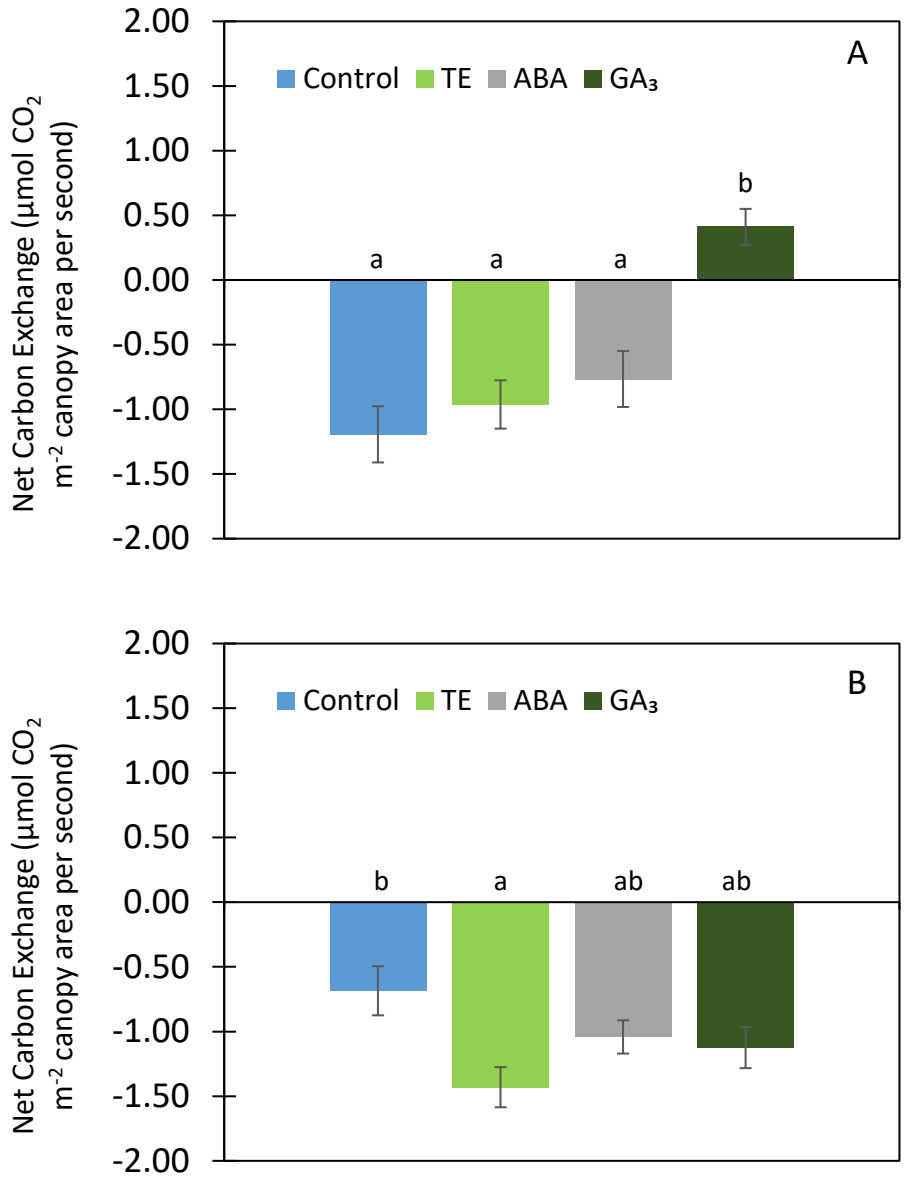


Figure 3.11 Assessment of net carbon exchange in the presence of light (photosynthesis) after two days in a growth chamber with 10°C day and 4°C night for annual bluegrass (A) and creeping bentgrass (B) treated with the following plant growth regulators: abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3). Each bar represents three pots measured for each field plot that were treated as subsamples for the four replicate field plots. Error bars represent the standard error for the four replicate field plots for each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means (n = 4) based on a Tukey's HSD test.

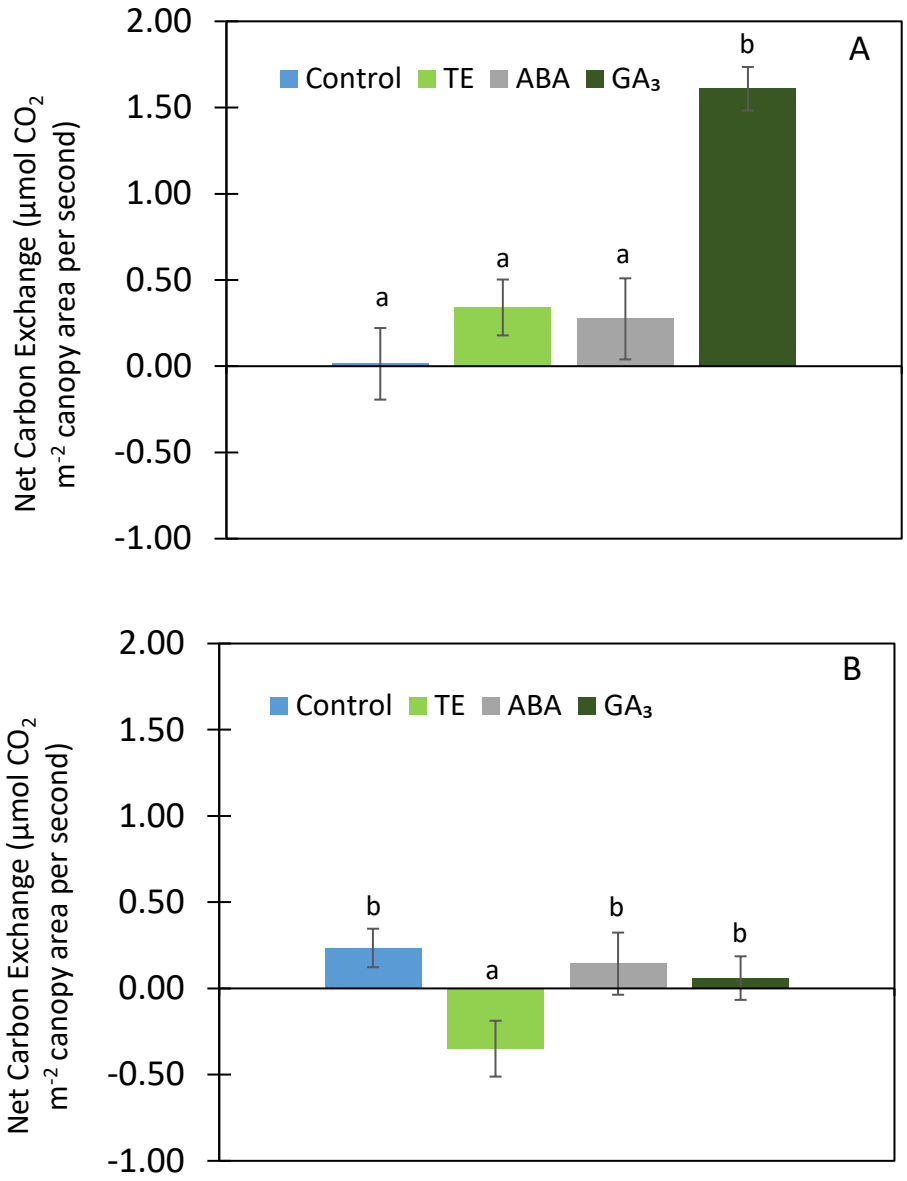


Figure 3.12 Assessment of net carbon exchange in the presence of light (photosynthesis) after four days in a growth chamber with 10°C day and 4°C night for annual bluegrass (A) and creeping bentgrass (B) treated with the following plant growth regulators: abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3). Each bar represents three pots measured for each field plot that were treated as subsamples for the four replicate field plots. Error bars represent the standard error for the four replicate field plots for each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means (n = 4) based on a Tukey's HSD test.

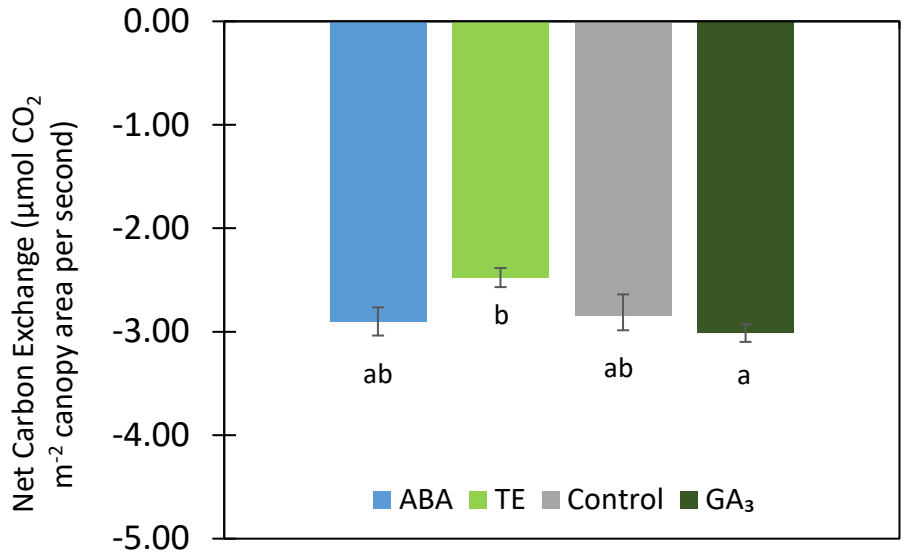


Figure 3.13 Assessment of net carbon exchange in the absence of light (dark respiration) for annual bluegrass after two days in a growth chamber with 10°C day and 4°C night for annual bluegrass treated with the following plant growth regulators: abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3). Each bar represents three pots measured for each field plot that were treated as subsamples for the four replicate field plots. Error bars represent the standard error for the four replicate field plots for each plant growth regulator treatment. Different lower-case letter indicates significant differences ($P \leq 0.05$) for means ($n = 4$) based on a Tukey's HSD test. No significant differences were found for creeping bentgrass.

CHAPTER FOUR - DISCUSSION

4.1 ASSESSING IMPACT OF PLANT GROWTH REGULATORS ON COLD TOLERANCE OF FIELD ACCLIMATED TURFGRASS

The present study demonstrated that PGR application in the fall had a positive effect on cold tolerance in creeping bentgrass and a negative effect on this trait in annual bluegrass. Gibberellic acid inhibitors have had a varying effect on the cold tolerance of different species and even cultivars of a species (Gusta, et al., 1993). In addition to variation in cold tolerance among species and cultivars, GA_3 inhibitors also have had contrasting effects on cold tolerance in an individual species depending on the year (Morrison and Andrews, 1992). The results of the present study demonstrate the negative effects of TE on the winter hardiness of annual bluegrass and is in agreement with Lashkowski et al. (2018) where TE application resulted in decreased survival under ice encasement in a growth chamber. In the absence of ice, Lashkowski et al. (2018) found TE to be no different than the control for both years. Application of TE is beneficial for the freezing survival of the C4 turfgrass bermudagrass, a species that has stolons and rhizomes similar to the stoloniferous CB (Fagerness et al, 2002; Richardson, 2002). Trinexapac-ethyl has been shown to initiate the translation of proteins associated with cold acclimation at non-acclimating temperatures in Kentucky bluegrass (Hwang et al., 1999). The current study demonstrated that application of TE had a positive or no effect on the cold tolerance of CB.

PGR rebound associated with GA_3 inhibitors has been demonstrated in turfgrass science literature (Fagerness and Yelverton, 2000; Kreuser and Soldat, 2011). The PGR rebound effect is due to an accumulation of a non-bioactive gibberellin, GA_{20}, which is subsequently converted to

bioactive gibberellin GA_1. The rebound effect is characterized by a reduction in growth after initial application of the PGR, and then a substantial increase in growth resulting in greater plant dry weight in the PGR treated turf (Fagerness and Yelverton, 2000) and a decrease in non-structural carbohydrates (Han et al., 1998). The present study has shown how annual bluegrass is more responsive to increases in temperature and the application of GA_3. Annual bluegrasses response to warm ups may explain how the present study observed a rebound effect within this species and not in creeping bentgrass in April. The rebound phase can leave a plant susceptible to cold by lowering carbohydrate reserves. It is likely that creeping bentgrass also had an accumulation of GA_{20} but had not metabolized as much of the TE as annual bluegrass over the winter period.

The loss of cold tolerance of annual bluegrass associated with ABA application was not expected based on previous research (Flores et al., 1988; Chen and Gusta, 1983; Churchill et al., 1998). The exogenous application of ABA increases the cold tolerance of plants grown at non-acclimating temperature in addition to increasing the cold tolerance of winter wheat (Chen and Gusta, 1983). ABA can increase cold tolerance of some species, but it also may induce flowering in other species (Wilen et al., 1994). Annual bluegrass is a species that flowers at low mowing heights and some biotypes have been known to have a vernalization requirement; thus, application of ABA could be detrimental to winter hardiness (Johnson and White, 1997). Once a vernalization requirement has been met, there is a significant loss of cold hardiness (Fowler et al., 1996) and ABA application may reduce the time needed to satisfy the vernalization requirement (Wilen et al., 1994).

The application of GA_3 is associated with loss of cold tolerance in several plants (Rikin et al., 1975; Roberts et al., 1970; Irving and Lanphear, 1968). Exogenous GA_3 application results in higher endogenous GA_3 levels and lower ABA levels in *Camellia oleifera* Abel. (Wen et al., 2018). Altering the ABA to GA_3 ratio in favour of GA_3 is associated with loss in winter hardiness (Roberts et al., 1970; Wang et al., 2015). The current research suggests annual bluegrass is more responsive to GA_3 application than creeping bentgrass as evidenced by the increase in photosynthetic rate when GA_3 is applied to the former. Application of gibberellins can promote flowering in winter wheat and winter barley that have vernalization requirements possessing the VRN1 gene (Pearce et al., 2013). VRN1 is a primary vernalization regulator for cool-season grasses in the Poaceae family (Ergon et al., 2016; Pearce et al., 2013). Many ecotypes of annual bluegrass have a vernalization requirement and can flower at low heights of cut, whereas creeping bentgrass is not known to flower at the standard cut height of a fairway or golf green so the vernalization requirement is unknown (Johnson and White, 1997). The requirement for vernalization for annual bluegrass and lack of vernalization requirement for creeping bentgrass could explain the differences in response to GA_3 between the two species.

In this study, there were no differences for creeping bentgrass among PGR treatments for January, February, and March because the freezer being used for cold tolerance testing only had the capacity to reach -24°C, making it impossible determine exact LT_{50} values below -24°C. Determining LT_{50} values below -24°C is probably not important because crown temperatures will probably not drop below -24°C temperature even in very cold regions (Qian et al., 2001). Other winter hardiness research by Tompkins et al. (2004) and Gusta et al. (1976) only measured cold tolerance during the de-acclimation period in their studies. Creeping bentgrass

has an LT_{50} below -30°C (Espevig et al., 2014). In this study, the limitations of the low temperature equipment prevented the delivery of freezing temperatures required to adequately assess the in LT_{50} in PGR treatments for creeping bentgrass throughout the winter, however differences in LT_{50} among PGR treatments in January and February are not as important as differences in LT_{50} during de-acclimation in the months of March and April.

Plant growth regulators such as TE can reduce mowing frequency in the fall. The decision to apply TE in the fall should depend on what the dominant turfgrass species is on the greens and fairways of a golf course. The current study suggests turfgrass managers should not apply TE in the fall to annual bluegrass dominated stands as it will leave their turf stand susceptible to winterkill. The hypothesis that PGRs would have a protective effect of maintaining acclimation status of creeping bentgrass was supported by this study but, the effect was probably not great enough to warrant applying a PGR in the fall to reduce winter kill.

An ice storm occurred between April 8 and April 22 that may have caused injury to de-acclimated annual bluegrass in the field plots. The month of April had relatively high LT_{50} values for annual bluegrass across all PGR treatments in comparison to other research in April with similar soil temperatures. Tompkins et al. (2000) reported LT_{50} values of -12°C for annual bluegrass in late April, whereas this study revealed an LT_{50} of -1.9°C when all PGR treatments were combined. Moreover, annual bluegrass grown at non-acclimating temperatures of 20°C has an LT_{50} of -8.2°C (Hoffman et al., 2014b) and -9.3°C (Hoffman et al., 2014a). Samples of annual bluegrass harvested on March 31 and April 8 that served as controls for the simulated de-acclimation events had a combined LT_{50} of -12.7°C across all PGR treatments which is much

closer to the values reported by Tompkins et al. (2000) during the month of April. For April the present study had entire reps of AB that had no survival even at -2°C, which suggests the plants were already dead before being subjected to the freezing test.

The difference in cold tolerance between PGR treated and untreated annual bluegrass was significant for annual bluegrass in the month of April. The LT_{50} of the PGR treatments pooled was -1.9°C while it was -3.9°C for the control. Between April 8th and April 22nd soil temperatures dropped below -1.9°C a total of four times which maybe have caused significant injury to the PGR treated turf, while never dropping below -3.9°C meaning the control annual bluegrass was likely unharmed. A limitation of our LT_{50} evaluation is that -6°C was not used as a target temperature for our assessment. Most of the LT_{50} for annual bluegrass in April fell within the range of -2°C and -10°C and not having a target temperature between those two values leaves some uncertainty with regards to the LT_{50} estimate.

4.2 ASSESSING IMPACT OF PLANT GROWTH REGULATORS ON COLD TOLERANCE OF FIELD ACCLIMATED TURFGRASS SUBJECTED TO A SIMULATED DE-ACCLIMATION EVENT IN A GROWTH CHAMBER

In this study the fall application of TE reduced the photosynthetic rate of creeping bentgrass after a 4-day de-acclimation event in a growth chamber which is not consistent with the response to TE for this species in other research (Krishnan and Merewitz, 2015; McCann and Huang, 2007). TE increases photosynthesis under stress are typically a drought and heat response. Previous research in Dr. Lyons' lab demonstrated that TE treated turfgrass had no differences in photosynthetic rate compared to the control following acclimation in growth chamber (Kerr, 2014). An extensive literature search yielded no research that shows the de-

acclimation response of TE treated turfgrass. It is important to understand that heat and drought studies measure a plants photosynthetic rate as a plant goes into dormancy while in the present research measures a plants photosynthetic rate as it emerges from dormancy.

GA_3 application in the fall significantly increased the photosynthetic rate after 4 days in the growth chamber for annual bluegrass but not creeping bentgrass. Similarly, GA_3 application increased the photosynthetic rate in several species such as tomato plants (Arteca and Dong, 1981), mustard (Khan, 1996; Khan et al., 1996), black cumin (Shad, et al., 2007) & sweet sorghum (Nimir, 2017). There are very few cases in which GA_3 application does not upregulate photosynthetic rate and these cases do not deal with plants emerging from a dormant state (Tsai & Arteca, 1985). GA_3 has also been used to delay dormancy of winter wheat planted late in the fall season (Pavlista et al., 2014) and to break seed dormancy (Henry & Brennan, 1988). GA_3 application is associated with increasing anti-dormancy mechanisms such as photosynthetic rate and Rubisco activity (Yuan and Xu, 2001). There are cultivars of wheat that are insensitive to GA_3 and cannot have seed dormancy broken via exogenous GA_3 application (Tuttle et al, 2015). The present research demonstrates a lack of sensitivity to GA_3 application by creeping bentgrass and sensitivity to GA_3 by annual bluegrass.

In this study, the LT_{50} values after a simulated warming event are likely the best indicator of how PGRs affect acclimation while being subjected to de-acclimation temperatures as the LT_{50} values did not fall below -24^{o}C. All the LT_{50} values obtained from the simulated de-acclimation event were within the measurable limits of -10^{o}C and -24^{o}C of the cold tolerance

tests. This allowed the present study to distinguish differences in cold tolerance levels among PGR treatments for both species.

Previous de-acclimation experiments on annual bluegrass and creeping bentgrass did not include field acclimated plants that overwintered (Hoffman et al., 2014ab). The results of our experiment agree with those of Espevig et al. (2014) demonstrating that creeping bentgrass has greater capacity to re-acclimate than annual bluegrass when subjected to reacclimating temperatures. The present study revealed an increase in GA_3 content in annual bluegrass but not in creeping bentgrass during a simulated de-acclimation event in a growth chamber. This supports the understanding that annual bluegrass is much more responsive to de-acclimation events than creeping bentgrass (Hoffman et al., 2014; Espevig et al., 2014).

Greenhouse grown, and growth chamber acclimated creeping bentgrass were acclimated using a 6-week acclimation regime were considerably de-acclimated after being subjected after 5 days at 12^{o}C (Hoffman et al., 2014a). The LT_{50} of creeping bentgrass was not different than that of AB experiencing the same conditions in the same study. The plants used by Hoffman et al. (2014a) were chamber acclimated, which likely indicates the plants did not reach maximum hardiness levels (Espevig et al. 2014). The present study used a similar de-acclimation regime to Espevig et al. (2014) of 10^{o}C/4^{o}C day/night for 5 days and observed differences in the LT_{50} of creeping bentgrass and annual bluegrass that were different by almost 10^{o}C. In this study, the difference in LT_{50} between these grasses is likely due to the fact that they were exposed to 14-15 weeks of outdoor winter conditions as field acclimated overwintering plants were removed from the field in late February and early March. Espevig et

al. (2014) used field acclimated creeping bentgrass removed from the field in late November for their experiment which reached an LT_{50} <-30 for both years which is likely close to the actual of LT_{50} creeping bentgrass in the present study. The LT_{50} in the present study could not be determined because the freezer used for the experiment could not exceed -24°C. The creeping bentgrass used in Espevig et al. (2014) study was removed from the field in November and cycled as a de-acclimation treatment and reached an LT_{50} of -25°C and -22°C respectively at the end of the treatments. The creeping bentgrass in the Espevig et al. (2014) study demonstrated more de-acclimation resistance because they were removed from the field before being subjected to the stresses of winter and an expanded photoperiod, whereas the plants in the present study had been subjected to the stresses of winter were more likely to come out of dormancy.

Annual bluegrass has little re-hardening capacity as freezing tolerance is not regained after de-acclimation for 12 days at 10°C, followed by re-hardening in a growth chamber at 2°C for 23 days (Espevig et al., 2014). In contrast to this, the present study demonstrated that annual bluegrass fully regained cold tolerance after a 5-day de-acclimation treatment under 10°C day and 4°C night prior to transfer back to the field for re-acclimation. The difference in re-hardening capacity of annual bluegrass between this study and the Espevig et al. (2014) research is likely a consequence of the de-acclimation treatment. Specifically, 12 days at 10°C is likely sufficient to completely bring annual bluegrass out of dormancy, whereas the 5-day de-acclimation treatment in this study was unable to affect this grass' re-acclimation capacity.

4.3 DIFFERENCES IN ACCLIMATION STATUS BETWEEN TURFGRASS SPECIES

The differences observed between species could be determined by the reproductive or vegetative state of the plant. Plants that are in a vegetative state of growth have a lower LT_{50} than plants in a state of reproduction (Mahfoozi et al., 2001). The physiological cost associated with sexual reproduction would be detrimental to the cold tolerances as the plants ability to allocate physiological important cryoprotective compounds such as non-structural carbohydrates to vegetative structures would be compromised (Zhang and Jiang, 2000). Reproductive growth would reduce the ability of a plant to compete in golf green or fairway setting where the plant is undergoing stress from traffic and mowing. It is hard to generalize about the sexual status of annual bluegrass due to the vast number of biotypes that exist and the diversity therein (Huff and Mantia, 2011). Most biotypes of annual bluegrass produce viable seeds at very low mowing heights, less than 3 mm and even at 6 mm, indicating that the plant is undergoing reproductive development over vegetative development (Lush, 1988). Creeping bentgrass does not produce seed at the height of cut of golf greens and fairways in the same manner as annual bluegrass (Lush, 1988). Creeping bentgrass selects exclusively for vegetative development of stolons at low mowing heights. Creeping bentgrass has been demonstrated to be able to acclimate to winter temperatures much better than annual bluegrass; in addition, creeping bentgrass is able to regain acclimation status better than annual bluegrass (Espevig et al., 2014).

Our study used gibberellic acid as a positive control to see the full spectrum of effects of the ABA to GA_3 ratio. Gibberellic acid has been used as a counter treatment to the overapplication of GA_3 inhibitors in bermudagrass stands (Cavanaugh, 2014). There is potential

to use PGRs as an annual bluegrass control as the results of the present study demonstrated that PGR treatments render annual bluegrass susceptible to low temperature injury after de-acclimation.

5.0 CONCLUSIONS AND SIGNIFICANCE

The research is vital to the golf industry in Canada due to significant loss in revenue and repairs to greens. We predict that we can prevent premature loss of winter hardiness due to temperature fluctuations through hormone manipulation. Our research will assist golf course superintendents improve the economic sustainability of their operation by providing a mechanism to impact winter survival when winterkill is most common. Another implication of our study is that findings can be applied to other sectors of the turfgrass industry and other plant production systems that suffer from winterkill associated with de-acclimation such as winter wheat and winter rye (Fowler and Gusta, 1977). Conducting freezing tolerance tests requires many plants per experimental unit and winter cereals are relatively large plants and take up a lot of physical space compared to turfgrass species. The present study allows for a better understanding of how plant growth regulators effect winter hardiness during de-acclimation that can potentially be applied to physically bigger species of the Poaceae family.

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APPENDICES

Appendix A

Supplemental Materials and Methods

Effect of Late Season N Application on Cold Tolerance

On May 15, 2017, 1.5 m x 1 m plots of creeping bentgrass were established on a USGA specification green for a total of 20 experimental units. Initial fertilization application was made on May 23rd. Monopotassium phosphate (0-52-34) dissolved in water was applied as a liquid application to achieve a rate of 0.38 kg/100m^2 total P, which also supplied 0.25 kg/100 m^2 K. An additional 0.25 kg/100m^2 K was applied in the form of potassium sulfate (0-0-53) salt for a total potassium application of 0.50 kg/100m^2 for that date. Two more potassium sulfate salt applications of 0.50 kg/100 m^2 were made on August 2nd and October 3rd for a total potassium rate of 1.5 kg/100m^2 for the year. Nitrogen was applied in the form of (46-0-0) microprill urea every two weeks over the course until the specified date of last N application (See Table 1), at which date a "dormant nitrogen" application of 0.25 kg/100m^2 N was applied. The nitrogen rate was intended to be 0.21 kg/100m^2 of N per application, this number was accidently divided by 7 in the spreadsheet. 0.03 kg/100 m^2 was applied every two weeks until the final application date. The experiment was arranged in a randomized complete block design with four replicates (see Table 2).

Plugs 3.8mm in diameter were pulled from the plots on January 29 and May 10, 2018 to be subjected to a cold tolerance test. Before being placed into the freezer, plugs were cut to 1.9

cm in length and placed in conetainer racks. Six turfplugs were removed from each experimental unit and one turfplug was removed at each one of the target temperatures: -2, -6, -10, -14, -18, -22^{o}C. Plugs placed into a freezer for 8 hours at -2^{o}C to equilibrate. At the completion of 8 hours, one turfplug was removed and the temperature was then decreased at a rate of -2^{o}C per hour and stabilized every -4^{o}C increment for one hour: -6, -10, -14, -18, and -22^{o}C. Once a turfplug was removed from the freezer it was placed in a 4^{o}C refrigerator for at least 24 hr. Pictures were taken 24 hr and then 1 week after the cold tolerance test was completed.

Supplemental Table A2.1 N rates and dormant application specified for each N treatment.

Treatment	Date of Last N Application	N Over the Year	Dormant N Applciation	Total N Applied
N-Sept	Sept 15th, 2017	0.28	0.25	0.53
N-Oct	October 16th, 2017	0.34	0.25	0.59
N-Nov	November 17th, 2017	0.40	0.25	0.65
Control	November 17th, 2017	0.40	0	0.40
N-Dec	December 4th, 2017	0.46	0.25	0.71

Effect of Yearly K Application on Cold Tolerance

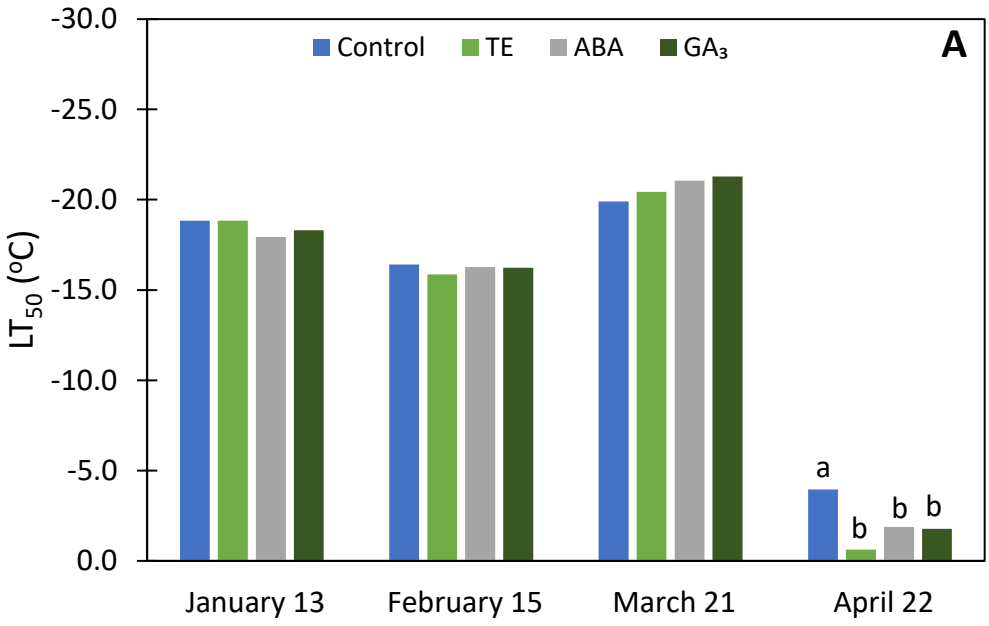
On May 15, 2017, 1.5 m x 1 m plots of creeping bentgrass were established on a USGA specification green for a total of 16 experimental units. Initial fertilization application was made on May 23rd. Monoammonium phosphate (12-61-0) dissolved in water was applied in a liquid application to achieve a rate of 0.38 kg/100m^2 total P, which also supplied 0.11 kg/100m^2 N. After initial application of N, N was supplied with (46-0-0) micro prill urea at a rate of 0.12 kg/100m^2 N per app every two weeks until December 4th, 2017 for a total N yard of 1.5 kg/100m^2 total N for the year. Potassium was applied in three equal apps K depending on the rate for the treatment in the form of potassium sulfate (0-0-53) on May 23, August 2 and October 3. Potassium rates for the year were 0, 150 kg/ha K, 300 kg/ha K and 450 kg/ha K

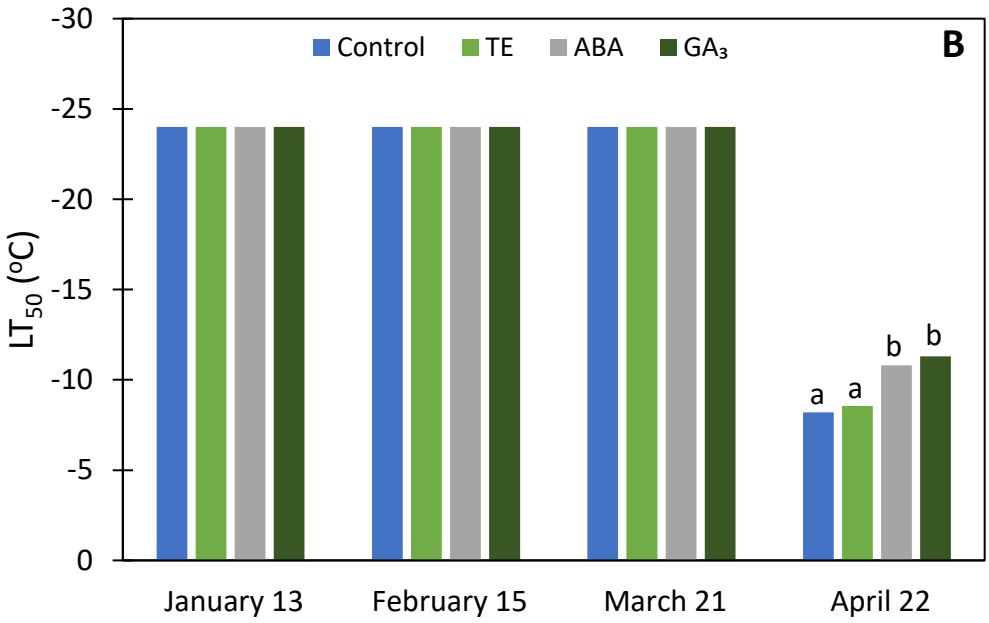
Plugs 3.8 mm in diameter were pulled from the plots on May 10th to be subjected to a cold tolerance test. Before being placed into the freezer, plugs were cut to 1.9cm in length and placed in conetainer racks. Six turfplugs were removed from each experimental unit and oneturf plug was removed at each one of the target temperatures: -2, -6, -10, -14, -18, -22^oC. Plugs placed into a freezer for 8 hours at -2^oC to equilibrate. At the completion of 8 hours one turfplug was removed and the temperature was then decreased at a rate of -2^oC per hour and stabilized every -4^oC increment for one hour: -6, -10, -14, -18, and -22 ^oC. Once a turfplug was removed from the freezer it was placed in a 4^oC refrigerator for at least 24 hr. Pictures were taken 24 hr and then 1 week after the cold tolerance test was completed to assess survival.

Supplemental Table A2.2 Yearly potassium treatment rates.

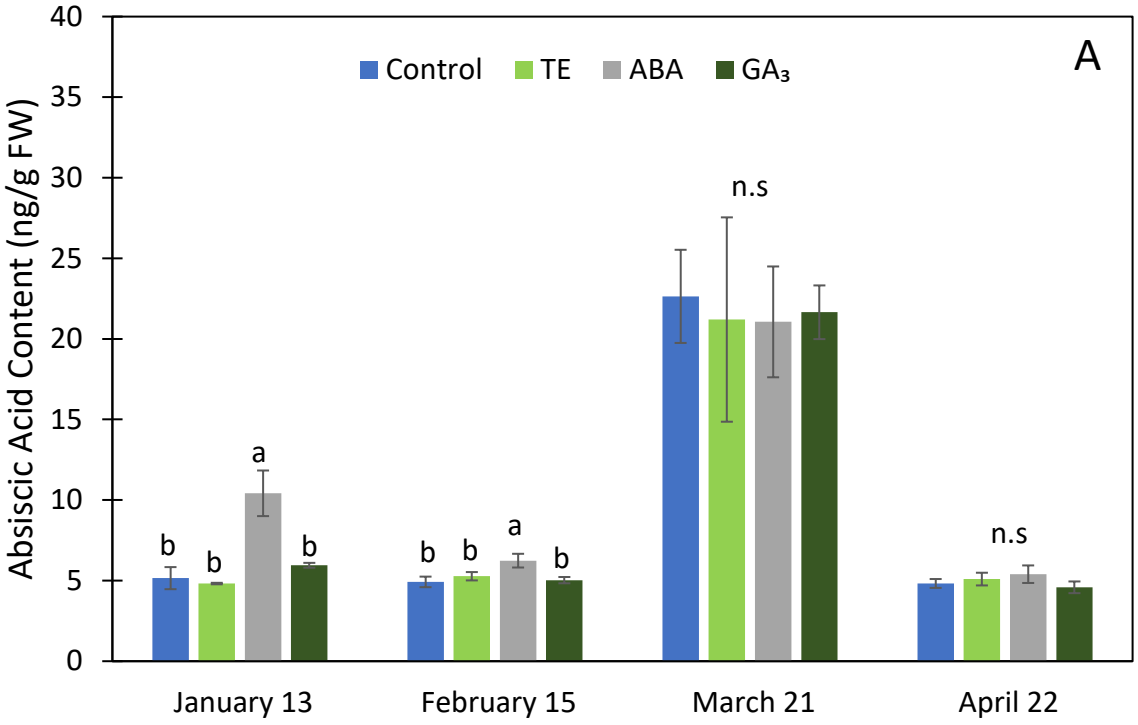
Potassium Rate (kg/ha)
0
150
300
450

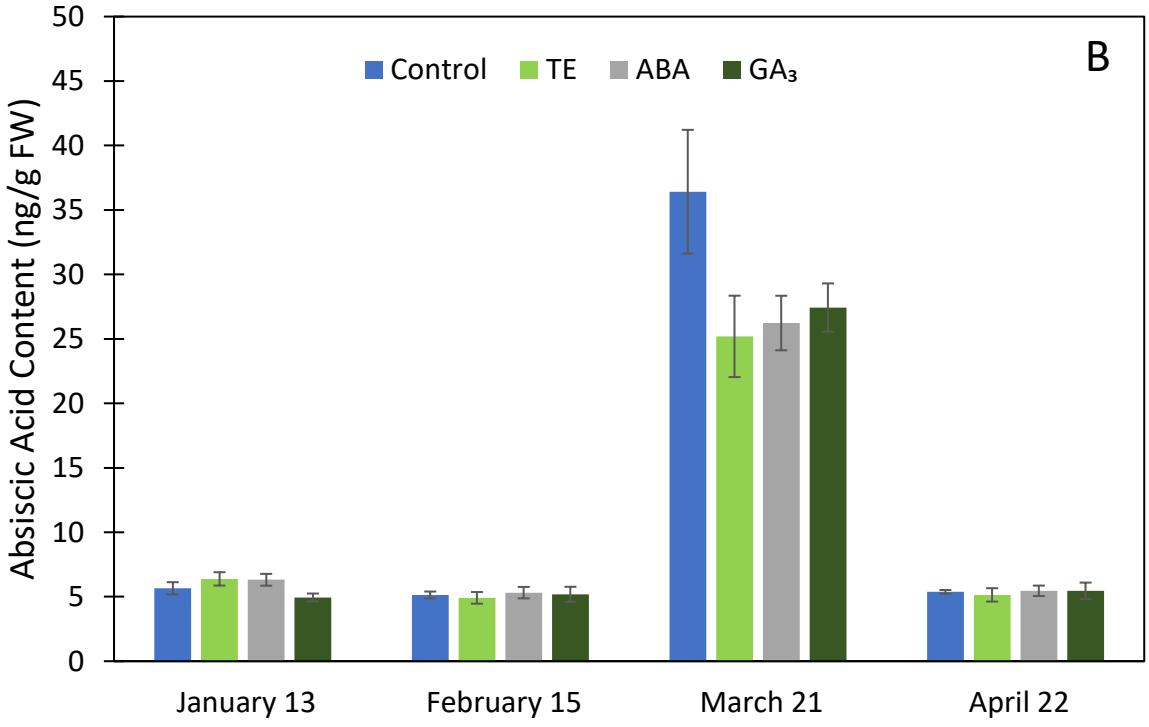
APPENDIX B


A
Control
TE
ABA
GA3
LT50 (°C)
-30.0
-25.0
-20.0
-15.0
-10.0
-5.0
0.0
a
b
b
b
January 13
February 15
March 21
April 22

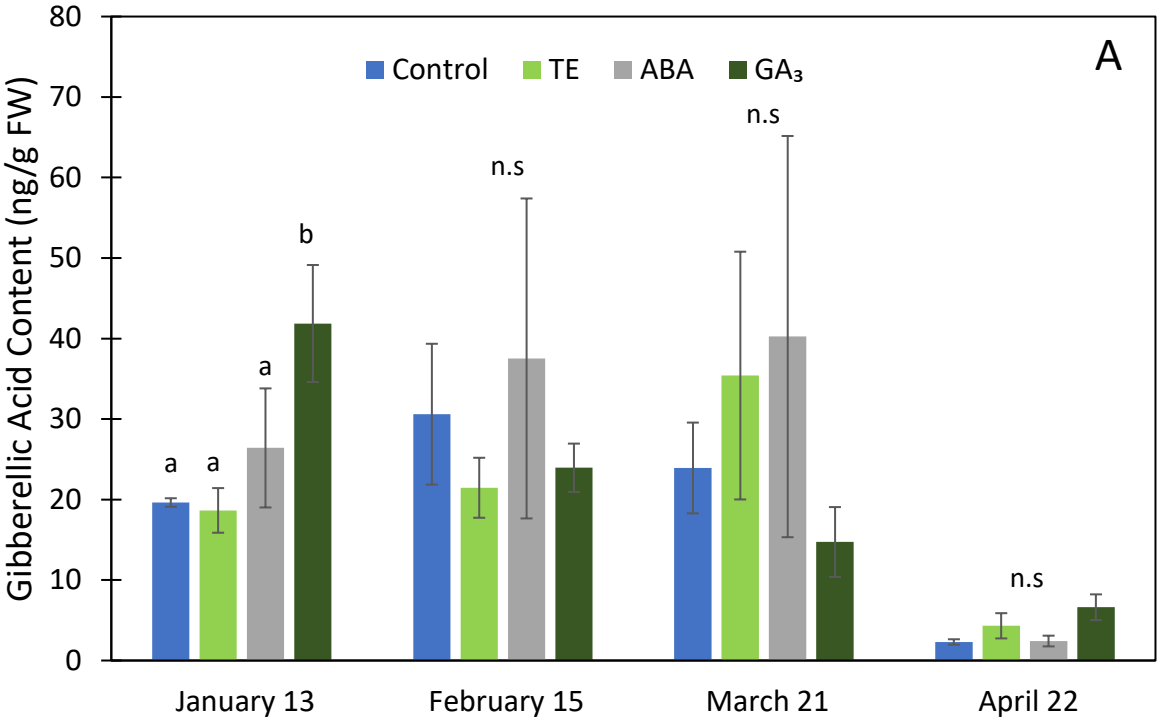

B
Control
TE
ABA
GA3
LT50 (°C)
-30
-25
-20
-15
-10
-5
0
a
a
b
b
January 13
February 15
March 21
April 22

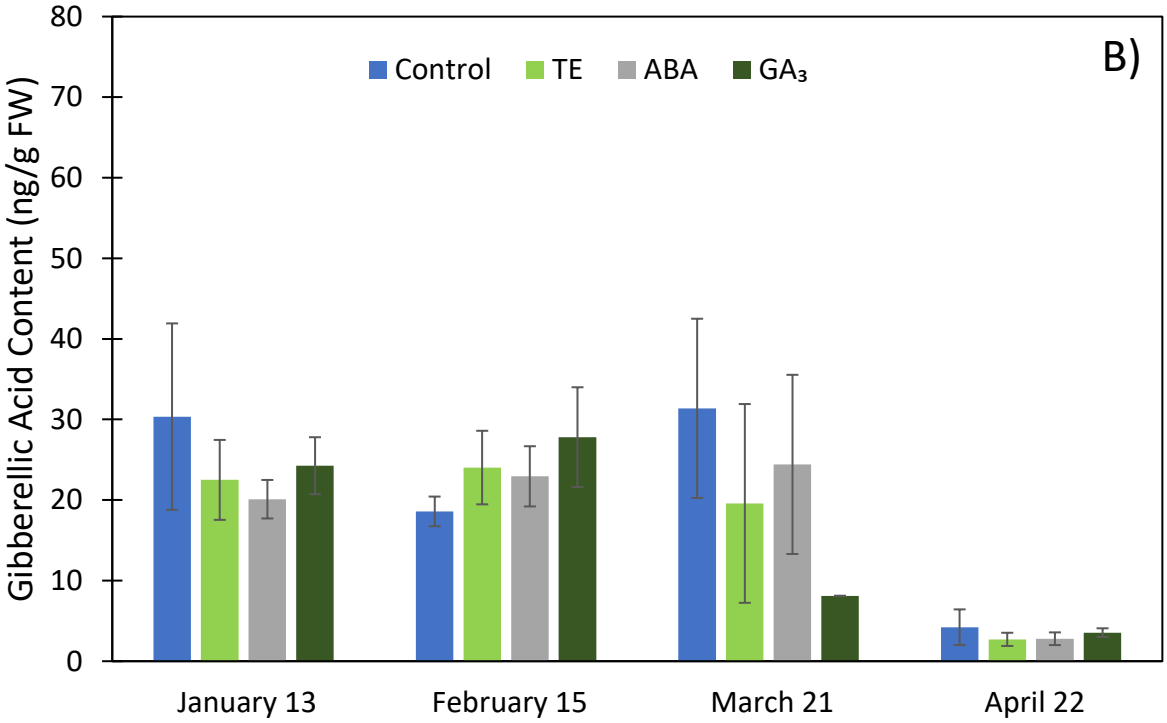
Supplemental Figure B3.1 Assesment of monthly acclimation status by lethal temperature of 50% survival (LT_{50}) for annual bluegrass (A) and creeping bentgrass (B) to a minimum temperature of -24^{o}C throughout the winter after fall application of abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Different lowercase letter indicate significant differences ($P \leq 0.05$) for means ($n = 4$) within individual dates based on a pairwise comparisons with least square means.


A
Control
TE
ABA
GA₃
Absiscic Acid Content (ng/g FW)
40
35
30
25
20
15
10
5
0
b
b
a
b
b
b
a
b
n.s
n.s
January 13
February 15
March 21
April 22

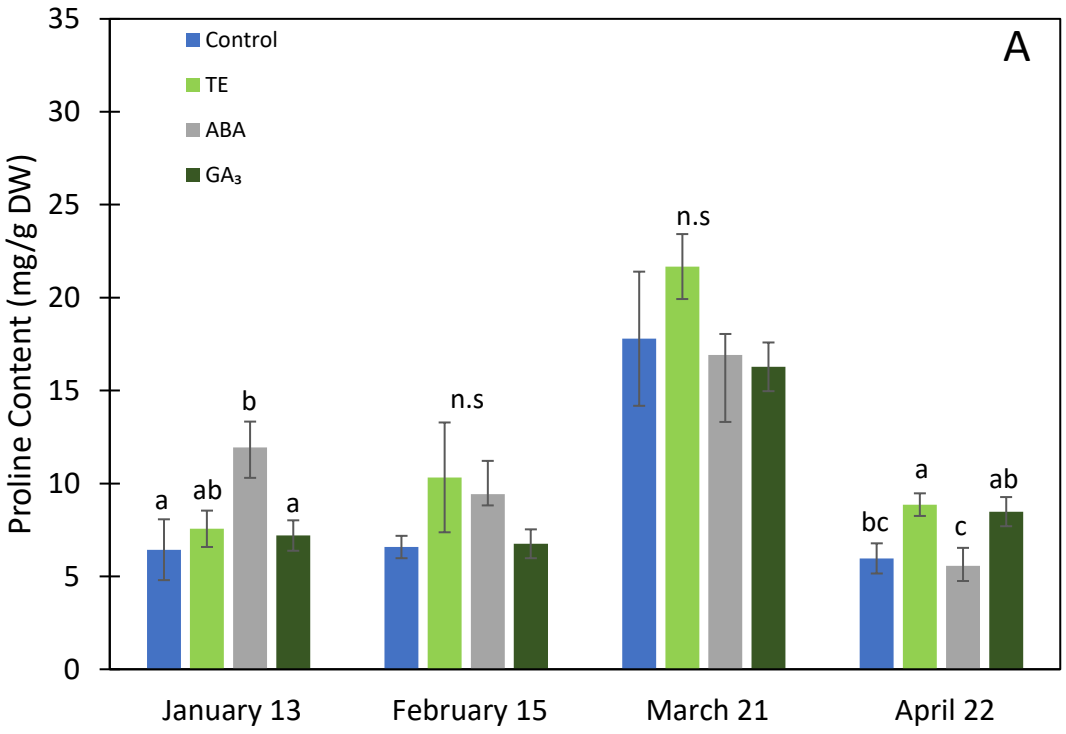

B
Control
TE
ABA
GA₃
Absiscic Acid Content (ng/g FW)
50
45
40
35
30
25
20
15
10
5
0
January 13
February 15
March 21
April 22

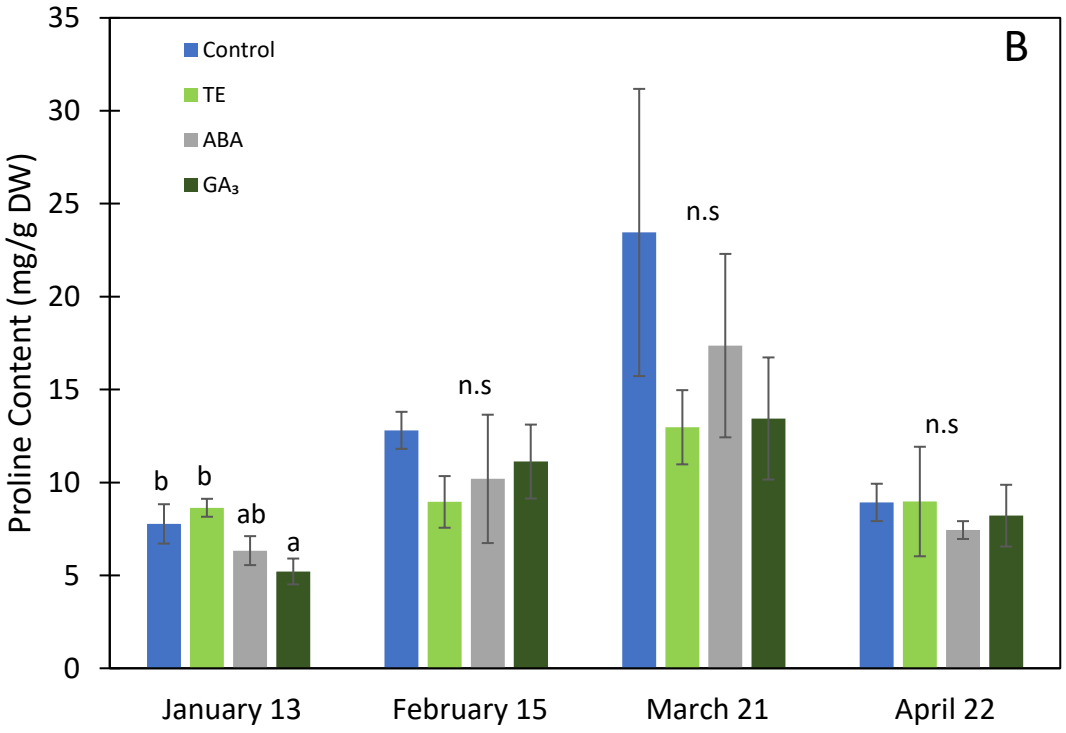
Supplemental Figure B3.2 Monthly assessment of endogenous levels of abscisic acid content (ng/g FW) within the crown tissue of (A) annual bluegrass and (B) creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means (n = 4) within individual dates based on a Tukey's HSD test.


A
Control
TE
ABA
GA₃
Gibberellic Acid Content (ng/g FW)
80
70
60
50
40
30
20
10
0
a
a
a
b
n.s
n.s
n.s
January 13
February 15
March 21
April 22

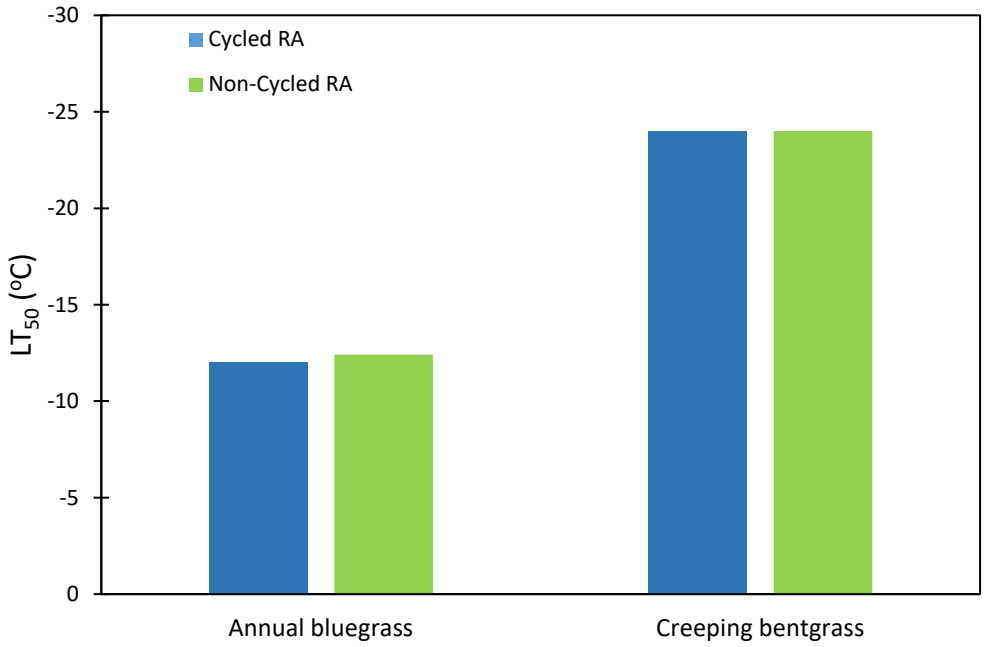

B)
Control
TE
ABA
GA₃
Gibberellic Acid Content (ng/g FW)
80
70
60
50
40
30
20
10
0
January 13
February 15
March 21
April 22

Supplemental Figure B3.3 Monthly assessment of endogenous levels of gibberellic acid content (ng/g FW) within the crown tissue of (A) annual bluegrass and (B) creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means ($n = 4$) within individual dates based on a Tukey's HSD test.

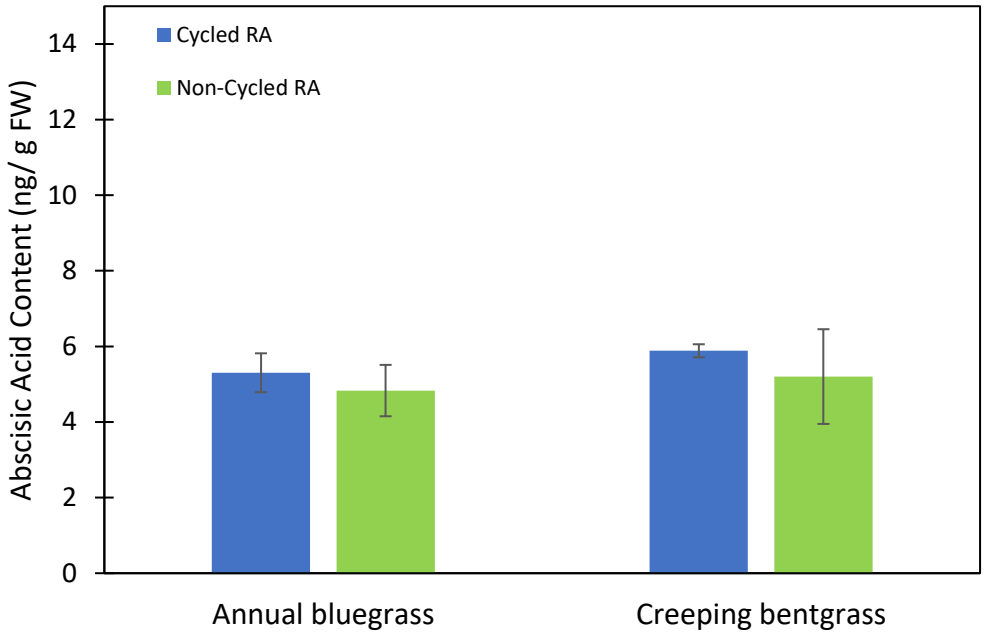

A
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
35
30
25
20
15
10
5
0
a
ab
b
a
n.s
n.s
bc
a
c
ab
January 13
February 15
March 21
April 22


B
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
35
30
25
20
15
10
5
0
b
b
ab
a
n.s
n.s
n.s
January 13
February 15
March 21
April 22

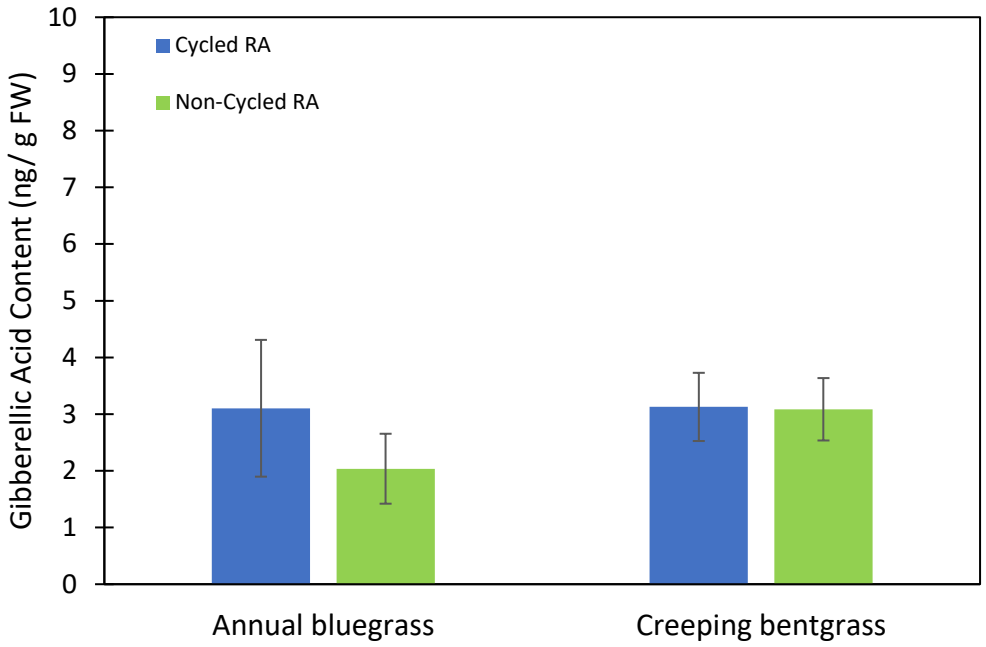
Supplemental Figure B3.4 Monthly assessment of endogenous levels of proline content (mg/g DW) within the crown tissue of (A) annual bluegrass and (B) creeping bentgrass after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Error bars represent the standard error for four replicates within each plant growth regulator treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means (n = 4) within individual dates based on a Tukey's HSD test.



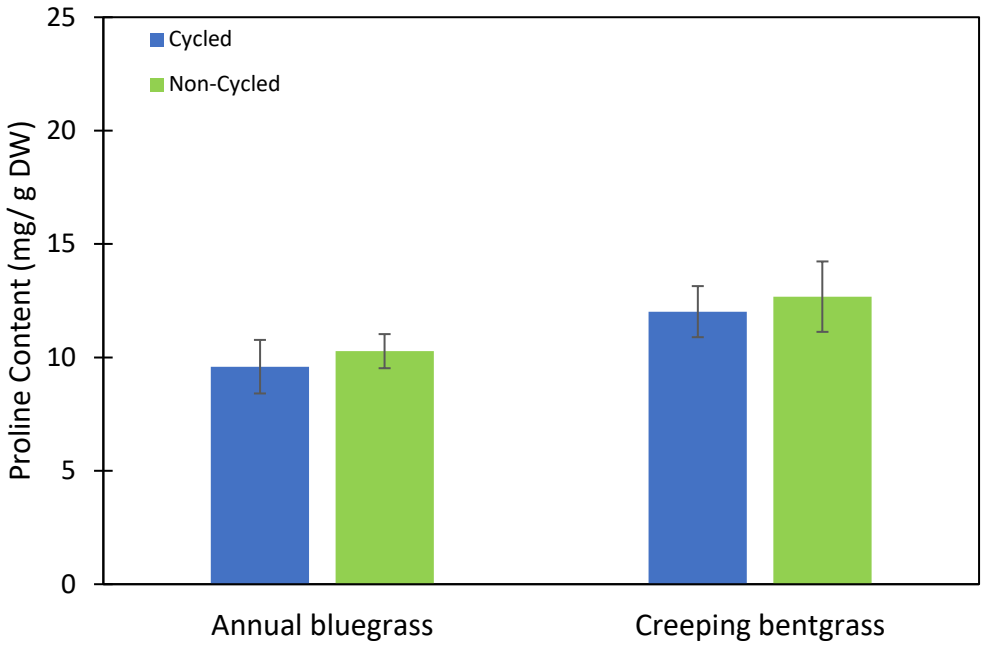
Supplemental Figure B3.5 Assessment of acclimation status lethal temperature of 50% survival (LT50) for field acclimated creeping bentgrass and annual bluegrass bentgrass after fall application of PGR treatment: abscisic acid, trinexapac-ethyl, and gibberellic acid. All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24th and March 8th and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field for one month to be removed on March 31 (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2°C (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31and April 8. No significant differences were found ($P \leq 0.05$) for means ($n = 16$) within de-acclimation treatment based on a Tukey's HSD test.



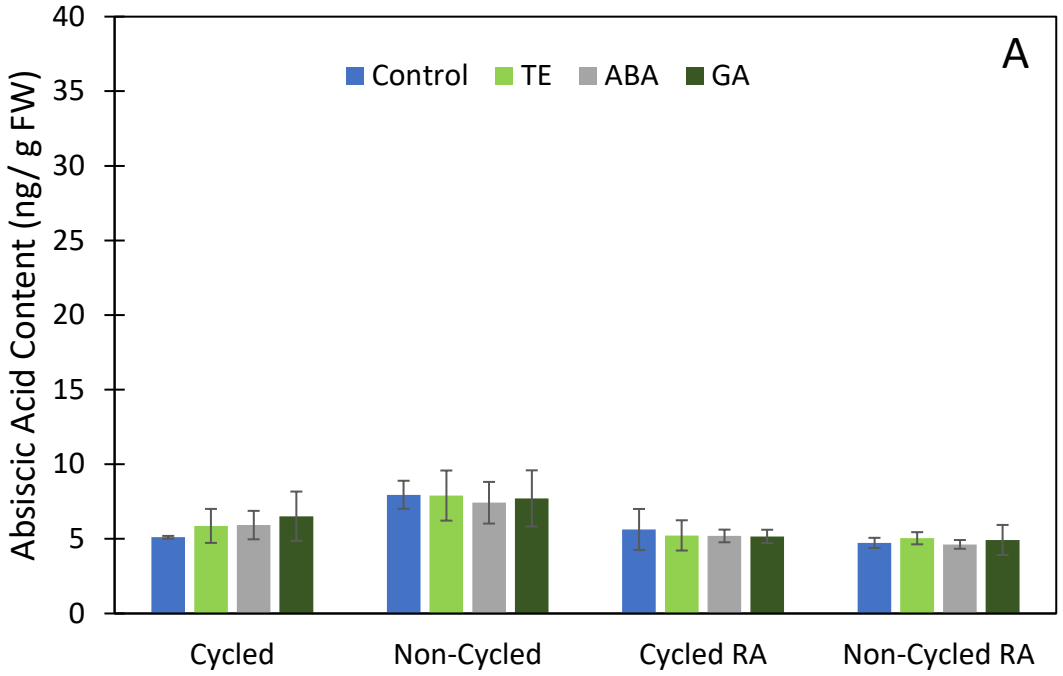
Supplemental Figure B3.6 Abscisic acid content (ng/g FW) within the crown tissue of field acclimated creeping bentgrass and annual bluegrass after fall application of PGR) treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field for one month to be removed on March 31 (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2^{o}C (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31 and April 8. Error bars represent the standard error for means of each de-acclimation treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means (n = 16) within de-acclimation treatment and species based on a Tukey's HSD test.

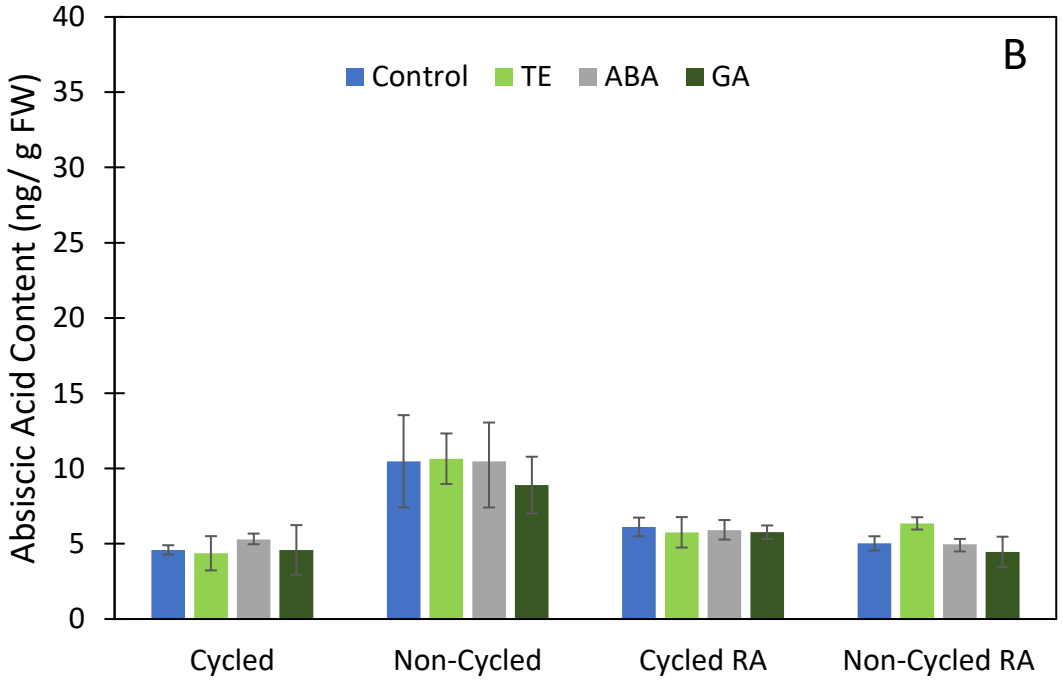


Supplemental Figure B3.7 Gibberellic acid content (ng/g FW) within the crown tissue of field acclimated creeping bentgrass and annual bluegrass after fall application of PGR treatment: abscisic acid, trinexapac-ethyl, and gibberellic acid. All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24 and March 8 and subjected to one of two de-acclimation treatments: five days of simulated warming in a growth chamber and then placed back in the field (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2^{o}C and then placed back into the field (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31 and April 8. Error bars represent the standard error for means of each de-acclimation treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means (n = 16) within de-acclimation treatment and species based on a Tukey's HSD test.



Supplemental Figure B3.8 Proline content (mg/g DW) in the crown tissue of field acclimated creeping bentgrass and annual bluegrass after fall application of PGR treatment: abscisic acid, trinexapac-ethyl, and gibberellic acid. All PGR treatments were pooled for each individual species. Samples were removed from the field on February 24 and March 8 and subjected to one of two de-acclimation treatments: five days of simulated warming in a growth chamber (Cycled), five days of simulated warming in a growth chamber and five days in a freezer at -2°C (Non-Cycled). Error bars represent the standard error for means of each de-acclimation treatment. Different lowercase letter indicates significant differences ($P \leq 0.05$) for means ($n = 16$) within de-acclimation treatment and species based on a Tukey's HSD test.


A
Control
TE
ABA
GA
Absiscic Acid Content (ng/ g FW)
40
35
30
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA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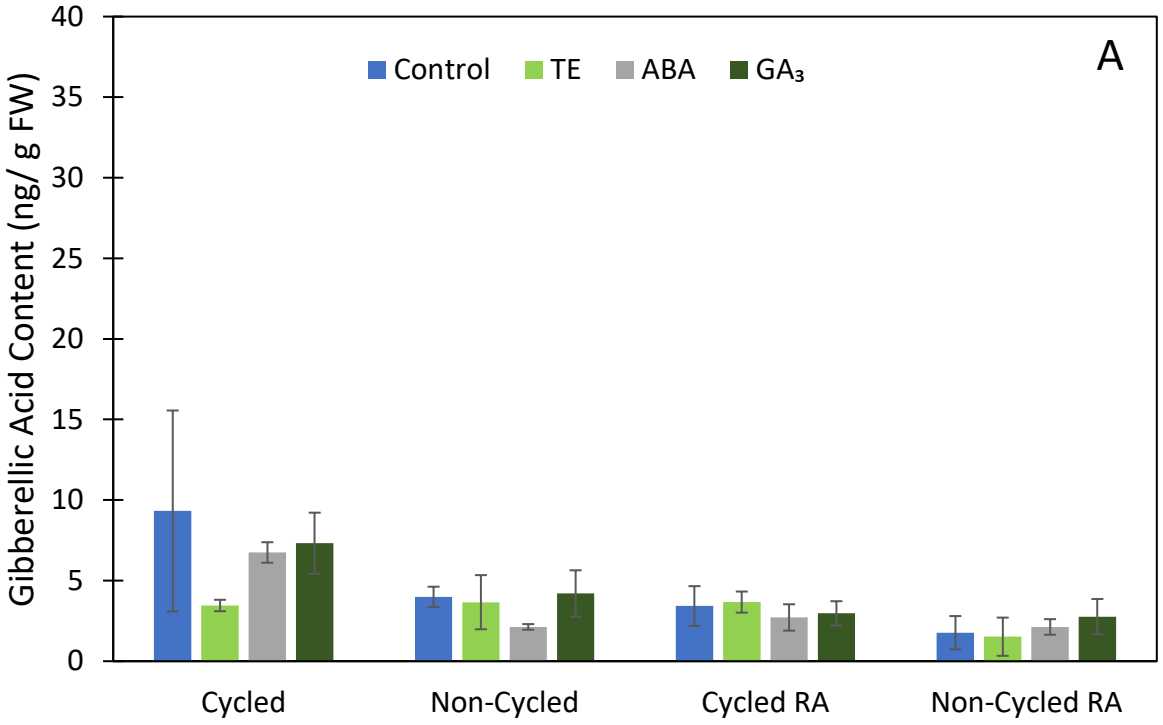
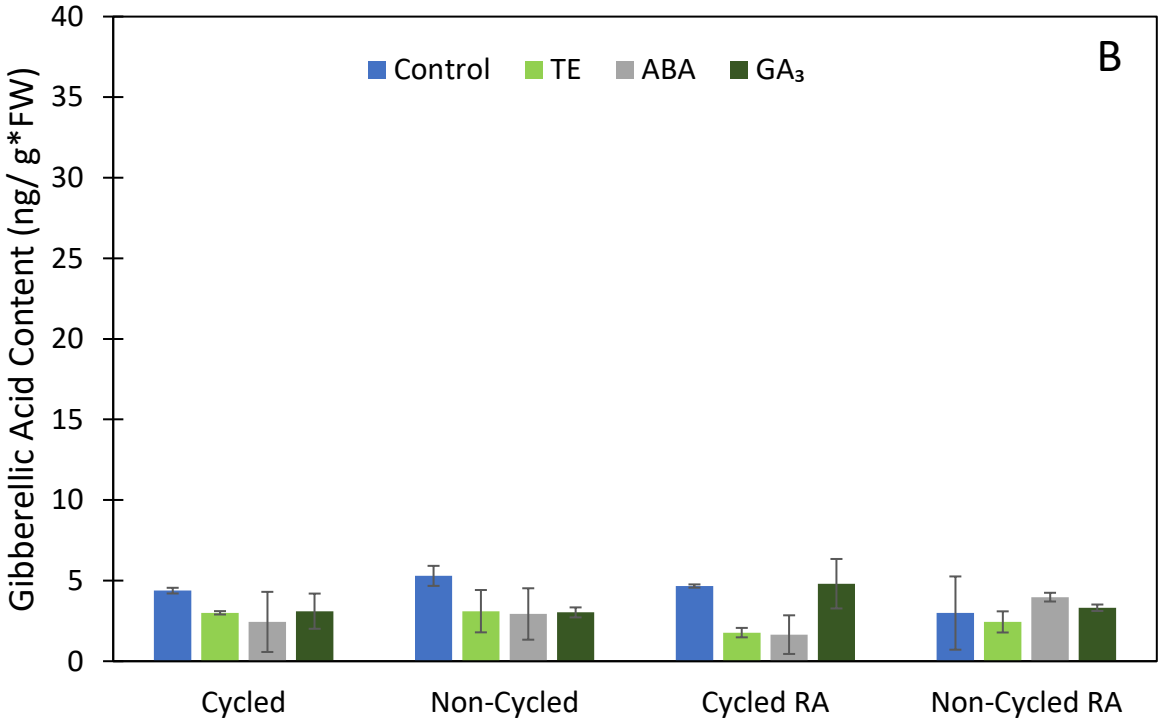
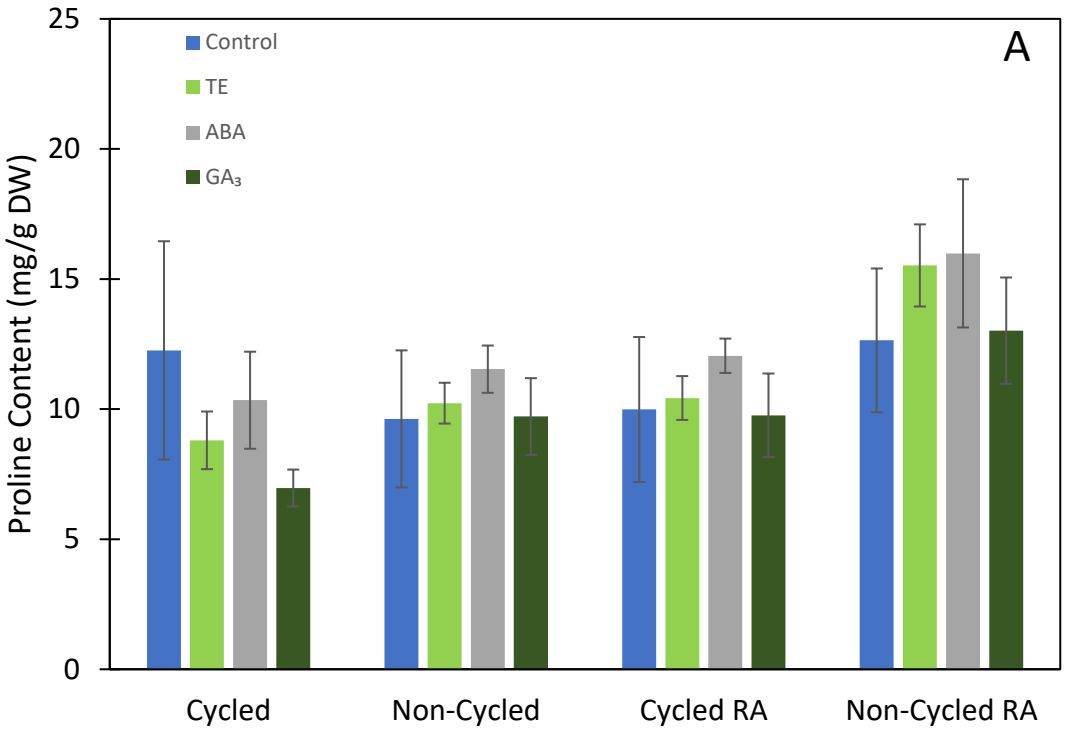
TE
ABA
GA
Absiscic Acid Content (ng/ g FW)
40
35
30
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA

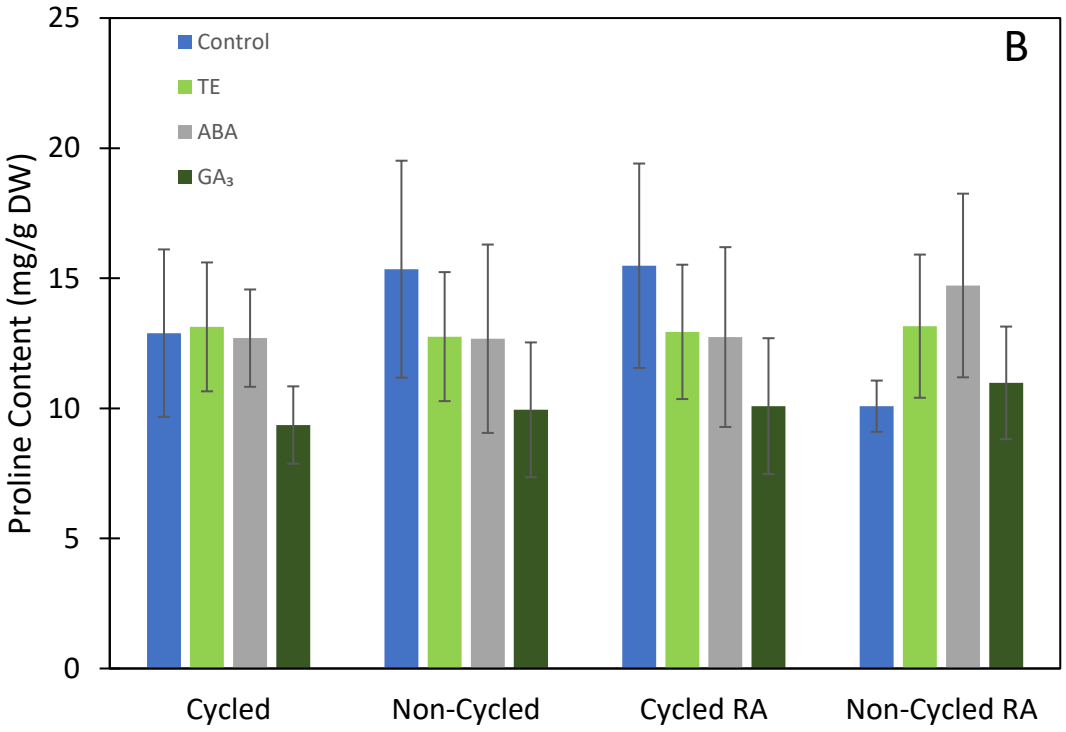
Supplemental Figure B3.9 Abscisic acid content (ng/g FW) within the crown tissue of field acclimated annual bluegrass (A) and creeping bentgrass (B) after fall application of PGR treatment: abscisic acid (ABA), trinexapac-ethyl (TE), and gibberellic acid (GA_3). Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2°C and then placed back into the field (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31 and April h. Error bars represent the standard error for means (n = 4) of each PGR treatment. No significant differences were found.


A
Control
TE
ABA
GA₃
Gibberellic Acid Content (ng/ g FW)
40
35
30
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA

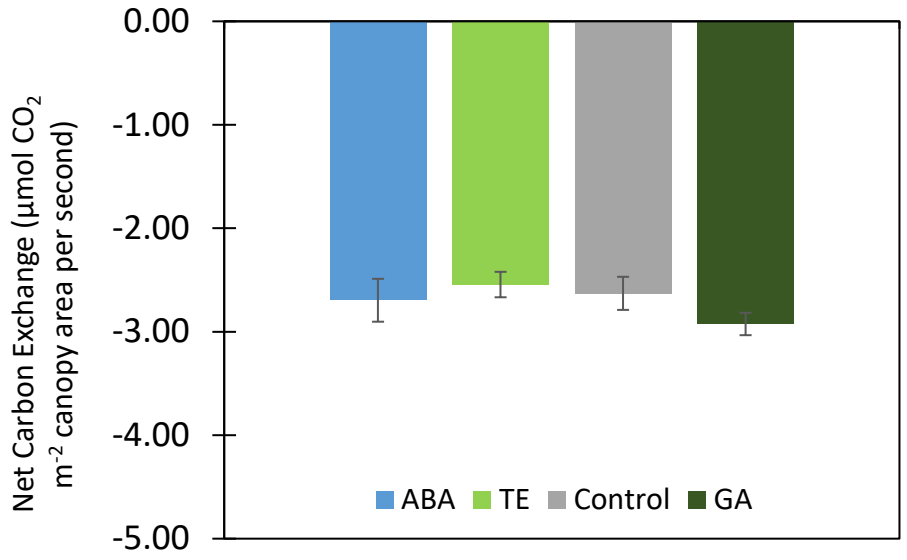

B
Control
TE
ABA
GA₃
Gibberellic Acid Content (ng/ g*FW)
40
35
30
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA

Supplemental Figure B3.10 Gibberellic acid content (ng/g FW) within the crown tissue of field acclimated annual bluegrass (A) and creeping bentgrass (B) after fall application of PGR treatment: abscisic acid (ABA), Trinexapac-ethyl (TE), and gibberellic acid (GA_3). Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2°C and then placed back into the field (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31 and April 8. Error bars represent the standard error for means (n = 4) of each PGR treatment. No significant differences were found.

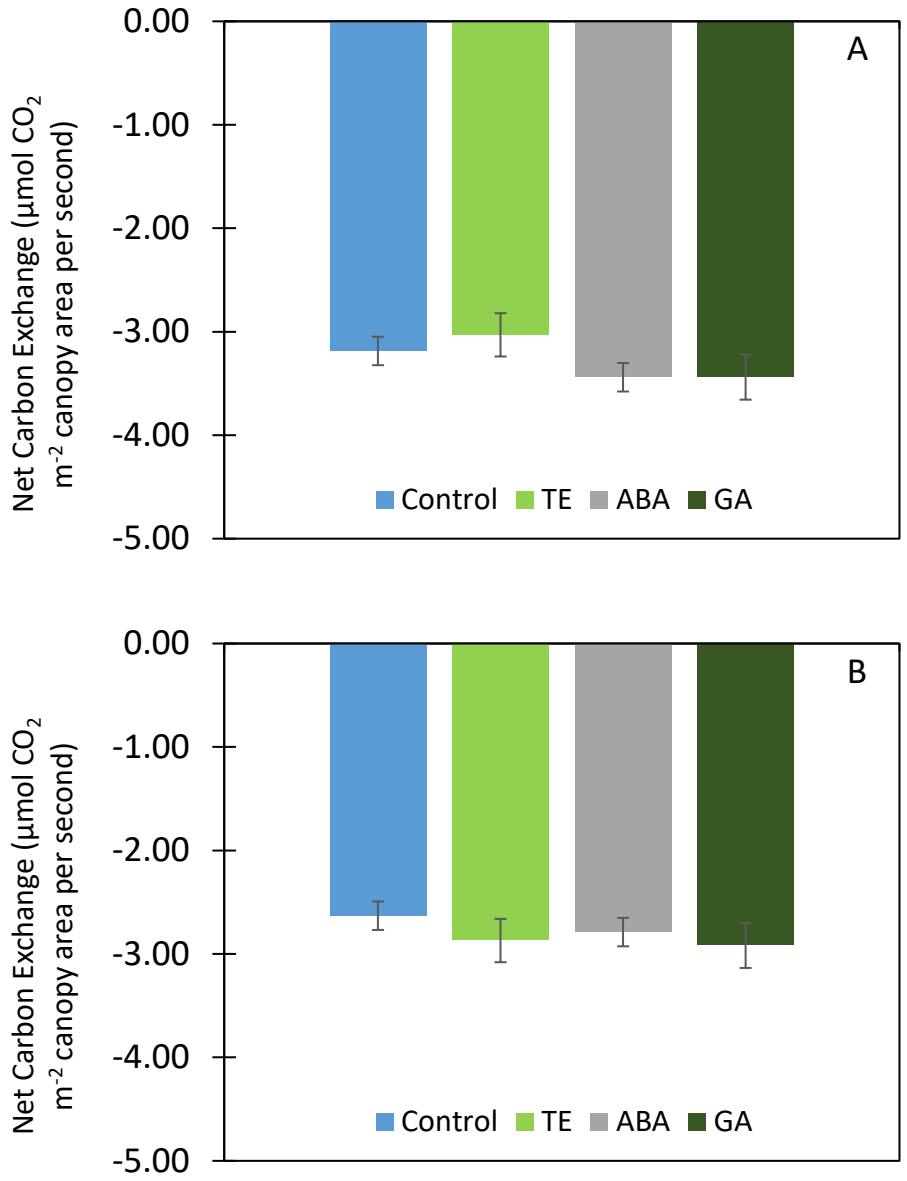

A
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA


B
Control
TE
ABA
GA₃
Proline Content (mg/g DW)
25
20
15
10
5
0
Cycled
Non-Cycled
Cycled RA
Non-Cycled RA

Supplemental Figure B3.11 Proline content (mg/g DW) within the crown tissue of field acclimated annual bluegrass (A) and creeping bentgrass (B) after fall application of PGR treatment: abscisic acid (ABA), Trinexapac-ethyl (TE), and gibberellic acid (GA_3). Samples were removed from the field on February 24 and March 8 and subjected to one of two "de-acclimation" treatments: five days of simulated warming in a growth chamber and then placed back in the field (Cycled RA), five days of simulated warming in a growth chamber and five days in a freezer at -2^{o}C and then placed back into the field (Non-Cycled RA). Samples from Cycled RA and Non-Cycled RA were removed from the field on March 31 and April 8. Error bars represent the standard error for means (n = 4) of each PGR treatment. No significant differences were found.



Supplemental Figure B3.12 Assessment of net carbon exchange in the absence of light (dark respiration) after two days in a growth chamber with 10°C day and 4°C night for creeping bentgrass treated with the following plant growth regulators: abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3). Error bars represent the standard error for four replicates with three subsamples for each PGR treatment.



Supplemental Figure B3.13 Assessment of net carbon exchange in the absence of light (dark respiration) after four days in a growth chamber with 10°C day and 4°C night for annual bluegrass (A) and creeping bentgrass (B) treated with the following plant growth regulators: abscisic acid (ABA), trinexapac-ethyl (TE) and gibberellic acid (GA_3). Error bars represent the standard error for four replicates with three subsamples for each PGR treatment.

Supplemental Table B3.1 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated annual bluegrass and creeping bentgrass to a minimum temperature of -24^{o}C after fall application PGR treatments: abscisic acid, trinexapac-ethyl, and gibberellic acid with all PGR treatments pooled and LT_{50} assessed in January, February, March and April 2018.

Species	Janauary	February	March	April
Annual bluegrass	-18.8c	-16.4d	-19.9b	-4.0f
Creeping bentgrass	-24.0a	-24.0a	-24.0a	-8.3e
p-value	< 0.0001			

[1]Different lowercase letter indicates significant differences ($P \leq 0.05$) for means (n = 16) pairwise comparisons with least square means

Supplemental Table B3.2 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated annual bluegrass to a minimum temperature of -24°C after fall application PGR treatments: abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018.

PGR Treatment	Janauary	February	March	April
Control	-18.8	-16.4	-19.9	-4.0 a[1]
Trinexapac-ethyl	-18.8	-15.9	-20.4	-0.6 b
Abscisic Acid	-17.9	-16.3	-21.0	-1.9 b
Gibberellic Acid	-18.3	-16.2	-21.3	-1.8 b
p-value	n.s[2]	n.s	n.s	0.0016

[1] Different lowercase letter indicates significant differences (P ≤ 0.05) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.3 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated creeping bentgrass to a minimum temperature of -24°C after fall application PGR treatments: abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018.

PGR Treatment	Janauary	February	March	April
Control	-24.0	-24.0	-24.0	-8.2 b[1]
Trinexapac-ethyl	-24.0	-24.0	-24.0	-8.5 b
Abscisic Acid	-24.0	-24.0	-24.0	-10.8 a
Gibberellic Acid	-24.0	-24.0	-24.0	-11.3 a
p-value	n.s[2]	n.s	n.s	< 0.0001

[1] Different lowercase letter indicates significant differences (P ≤ 0.05) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.4 Analysis of variance for abscisic acid (ABA) content for field acclimated annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	0.3620	1.1483		
Residual	16.3126	3.4390		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	45	0.58	0.6323
Date	3	45	63.19	< 0.0001
Treatment*Date	9	45	0.44	0.9054

Supplemental Table B3.5 Analysis of variance for abscisic acid (ABA) content for field acclimated creeping bentgrass after fall application (treatment) of ABA, Trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	0	-		
Residual	10.8641	2.2176		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	45	2.32	0.0885
Date	3	45	201.33	< 0.001
Treatment*Date	9	45	2.54	0.0189

Supplemental Table B3.6 Analysis of variance for gibberellic acid content for field acclimated annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	3.0866	26.2043		
Residual	418.45	94.3859		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	39	0.39	0.7590
Date	3	39	4.73	0.0065
Treatment*Date	9	39	0.73	0.6797

Supplemental Table B3.7 Analysis of variance for gibberellic acid content for field acclimated creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	20.8279	25.3006		
Residual	141.64	32.0667		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	39	0.37	0.7786
Date	3	39	10.93	< 0.001
Treatment*Date	9	39	0.52	0.8512

Supplemental Table B3.8 Analysis of variance for proline content for field acclimated annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	1.6539	1.7956		
Residual	8.6524	1.8241		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	45	3.19	0.0326
Date	3	45	48.94	< 0.0001
Treatment*Date	9	45	1.78	0.09888

Supplemental Table B3.9 Analysis of variance for proline content acclimated for the species creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid assessed in January, February, March and April 2018 (date).

Covariance Parameters	Estimate	Standard Error		
Block	5.4604	5.8851		
Residual	27.7335	5.8467		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	45	1.67	0.1870
Date	3	45	10.85	< 0.0001
Treatment*Date	9	45	0.84	0.5873

Supplemental Table B3.10 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated annual bluegrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24th and March 8th, 2018 subjected to a simulated thawing event in a growth chamber for 5 days (Cycled), and placed in a freezer at -2°C for five days (Non-Cycled).

PGR Treatment	Cycled	Non-Cycled
Control	-9.3	-19.6 ab[1]
Trinexapac-ethyl	-9.0	-20.1 a
Abscisic Acid	-8.1	-18.2 b
Gibberellic Acid	-9.2	-18.5 ab
p-value	n.s[2]	< 0.0001

[1] Different lowercase letter indicates significant differences (P ≤ 0.05) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.11 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated creeping bentgrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24and March 8, 2018 subjected to a simulated thawing event in a growth chamber for 5 days (Cycled) and placed in a freezer at -2°C for five days (Non-Cycled).

PGR Treatment	Cycled	Non-Cycled
Control	-15.8 b	-23.0 ab[1]
Trinexapac-ethyl	-17.9 a	-22.3 ab
Abscisic Acid	-16.3 b	-23.9 a
Gibberellic Acid	-17.6 a	-21.9 b
p-value	< 0.0001	n.s[2]

[1] Different lowercase letter indicates significant differences (P ≤ 0.05) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.12 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated annual bentgrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, Trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 subjected to a simulated thawing event in a growth chamber for 5 days and placed in the field for one month (Cycled RA), and placed in a freezer at -2°C for five days and placed in the field for one month (Non-Cycled RA).

PGR Treatment	Cycled RA	Non-Cycled RA
Control	-13.6 a[1]	-13.0
Trinexapac-ethyl	-9.5 b	-12.1
Abscisic Acid	-13.3 a	-13.2
Gibberellic Acid	-11.3 b	-11.6
p-value	0.0001	n.s[2]

[1] Different lowercase letter indicates significant differences ($P \leq 0.05$) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.13 Lethal temperature of 50% survival (LT_{50}) estimates for field acclimated creeping bentgrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, Trinexapac-ethyl, and gibberellic acid removed from the field on February 24th and March 8th, 2018 subjected to a simulated thawing event in a growth chamber for 5 days and placed in the field for one month (Cycled RA), and placed in a freezer at -2°C for five days and placed in the field for one month (Non-Cycled RA).

PGR Treatment	Cycled RA	Non-Cycled RA
Control	-23.0 ab[1]	-24.0
Trinexapac-ethyl	-22.3 ab	-24.0
Abscisic Acid	-23.9 a	-24.0
Gibberellic Acid	-21.9 b	-24.0
p-value	< 0.0001	n.s[2]

[1] Different lowercase letter indicates significant differences ($P \leq 0.05$) for means of treatments (n = 4) within individual dates based on pairwise comparisons with least square means
[2] denotes no significant differences among PGR Treatments

Supplemental Table B3.14 Analysis of variance for abscisic acid content for annual bluegrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, trinexapac-ethyl (TE), and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	71.4529	61.3887		
Residual	29.7554	9.1827		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	0.25	0.8609
Cycle	1	21	13.80	0.0013
Treatment*Cycle	3	21	0.51	0.6825

Supplemental Table B3.15 Analysis of variance for abscisic acid content for creeping bentgrass to a minimum temperature of -24°C after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	57.1357	63.9043		
Residual	165.90	51.1975		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	0.16	0.9232
Cycle	1	21	20.27	0.0002
Treatment*Cycle	3	21	0.13	0.9421

Supplemental Table B3.16 Analysis of variance for abscisic acid content for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid, removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	0	-		
Residual	26.9694	7.7854		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	0.06	0.9806
Cycle	1	21	0.95	0.3420
Treatment*Cycle	3	21	0.12	0.9500

Supplemental Table B3.17 Analysis of variance for abscisic acid content for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid, removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	0.8359	2.4388		
Residual	16.3912	5.0584		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	1.07	0.3817
Cycle	1	21	3.26	0.0855
Treatment*Cycle	3	21	1.31	0.2987

Supplemental Table B3.18 Analysis of variance for gibberellic acid content for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	3.5401	22.8102		
Residual	133.92	47.8016		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	16	0.49	0.6933
Cycle	1	16	4.38	0.0526
Treatment*Cycle	3	16	0.48	0.6983

Supplemental Table B3.19 Analysis of variance for gibberellic acid content for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	0	-		
Residual	42.1599	13.0108		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	18	1.42	0.2706
Cycle	1	18	0.18	0.6755
Treatment*Cycle	3	18	0.07	0.9744

Supplemental Table B3.20 Analysis of variance for gibberellic acid content for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	96.3588	108.49		
Residual	98.5635	33.3375		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	14	1.25	0.3304
Cycle	1	14	1.37	0.2621
Treatment*Cycle	3	14	1.97	0.1646

Supplemental Table B3.21 Analysis of variance for gibberellic acid content for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	0	-		
Residual	45.7306	14.1128		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	18	1.01	0.4104
Cycle	1	18	1.73	0.2048
Treatment*Cycle	3	18	1.12	0.3670

Supplemental Table B3.22 Analysis of variance for proline content for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	4.6696	5.0592		
Residual	12.0050	3.7048		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	1.05	0.3904
Cycle	1	21	0.31	0.5821
Treatment*Cycle	3	21	0.89	0.4615

Supplemental Table B3.23 Analysis of variance for proline content for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl (TE), and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	18.1305	16.3079		
Residual	14.6438	4.5192		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	1.98	0.1475
Cycle	1	21	0.24	0.6300
Treatment*Cycle	3	21	0.22	0.8832

Supplemental Table B3.24 Analysis of variance for proline content for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	0	-		
Residual	16.9515	4.8935		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	6.59	0.0180
Cycle	1	21	0.81	0.5026
Treatment*Cycle	3	21	0.9416	0.9416

Supplemental Table B3.25 Analysis of variance for proline content for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24, 2018 and March 8, 2018 and then subjected to a simulated thawing event in a growth chamber, returned to the field, and harvested a month later (cycle).

Covariance Parameters	Estimate	Standard Error		
Block	9.5115	10.2335		
Residual	23.7719	7.3362		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	21	0.65	0.5934
Cycle	1	21	0.11	0.7420
Treatment*Cycle	3	21	0.91	0.4513

Supplemental Table B3.26 Analysis of variance for net carbon exchange rate in the presence of light for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle). Net carbon exchange rate was assessed after two and four days in the growth chamber.

Covariance Parameters	Estimate	Standard Error		
Block	0.09704	0.09768		
Residual	0.5404	0.08289		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	85	22.49	<0.0001
Cycle	1	85	62.63	<0.0001
Treatment*Cycle	3	85	0.13	0.9411

Supplemental Table B3.27 Analysis of variance for net carbon exchange rate in the presence of light for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle). Net carbon exchange rate was assessed after two and four days in the growth chamber.

Covariance Parameters	Estimate	Standard Error		
Block	0.03271	0.03637		
Residual	0.2826	0.04335		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	85	6.48	0.0005
Cycle	1	85	101.36	<0.0001
Treatment*Cycle	3	85	0.33	0.8012

Supplemental Table B3.28 Analysis of variance for net carbon exchange rate in the absence of light for annual bluegrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle). Net carbon exchange rate was assessed after two and four days in the growth chamber.

Covariance Parameters	Estimate	Standard Error		
Block	0.02313	0.03086		
Residual	0.3498	0.05365		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	85	3.07	0.0321
Cycle	1	85	14.74	0.0002
Treatment*Cycle	3	85	0.18	0.9088

Supplemental Table B3.29 Analysis of variance for net carbon exchange rate in the absence of light for creeping bentgrass after fall application (treatment) of abscisic acid, trinexapac-ethyl, and gibberellic acid removed from the field on February 24 and March 8, 2018 and subjected to a simulated thawing event in a growth chamber (cycle). Net carbon exchange rate was assessed after two and four days in the growth chamber.

Covariance Parameters	Estimate	Standard Error		
Block	0.05328	0.05078		
Residual	0.2132	0.03271		
Fixed Effects	Numerator df	Denominator df	F Value	Pr > F
Treatment	3	85	1.71	0.1700
Cycle	1	85	1.20	0.2756
Treatment*Cycle	3	85	0.68	0.5654