Exploring the effects of hormone manipulation on the cold tolerance of cool season turfgrasses and cereals

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ABSTRACT

EXPLORING THE EFFECTS OF HORMONE MANIPULATION ON THE COLD TOLERANCE OF COOL SEASON TURFGRASSES AND CEREALS

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Trinexapac-ethyl (TE), product Primo Maxx, is a gibberellic acid (GA) inhibitor produced by Syngenta. A controlled acclimation study and two field studies were performed, one on AB and CB, and another on cereal crops with known genetic differences in cold tolerance. The results of the field study and the controlled acclimation study on the turfgrass species showed an increased concentration of high molecular weight (HMW) fructan for AB compared to CB. Results from the controlled acclimation study indicated that AB photosynthesis rates were higher than CB 20°C and 10°C. Carbohydrate concentration increased following five weeks of acclimation for both AB and CB in the controlled acclimation study. Both of the field studies had a significant date effect for carbohydrate status, with an increased concentration in HMW fructan in the fall when compared to the spring.

EXECUTIVE SUMMARY

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Cold tolerance is a major issue for turfgrass in parts of the world that experience sub-zero temperatures in the winter months. Identifying management practices that can improve stress tolerance during the acclimation period in the fall for turfgrasses has the potential to improve winter survival. The application of trinexapac-ethyl (TE), a gibberellic acid (GA) inhibitor, is widely used in the turfgrass industry as a management technique to improve playing quality of putting green surfaces. The TE product Primo Maxx produced by Syngenta has previously been identified as a product with the potential to improve stress tolerance of turfgrass. Total non-structural carbohydrates (TNC) plays a central role in stress tolerance and TE has previously been shown to increase TNC in turfgrass species. In a preliminary study, the objective was to assess the physiological changes that occur following TE application. The results indicated an increase in chlorophyll content and photosynthesis following the application of TE when compared to the application of GA_3. Carbohydrate status increases when photosynthesis is greater than respiration. Subsequently, a controlled acclimation study and two field studies were performed, one on AB and CB, and another on cereal crops with known genetic

differences in cold tolerance. The results of the field studies on the turfgrass species AB and CB, showed an increased concentration of high molecular weight (HMW) fructan for AB. Annual bluegrass also had an increased HMW fructan concentration in the controlled environment. For all of the turfgrass studies there was a significant species effect with AB benefitting more from the TE application then the CB when compared to the GA_3 application. The second field study on seven different cereal crops with known genetic differences in cold tolerance gave a significant date effect for HMW fructan. The controlled acclimation study indicated that AB photosynthesis rates were higher at 20°C and 10°C when compared to CB. There was no significant treatment effect for carbohydrate status in the controlled acclimation study or either field study. Carbohydrate concentration increased following five weeks of acclimation in the controlled environment study. Both of the field studies had a significant date effect for carbohydrate status, with an increased concentration in the fall when compared to the spring.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AB	Annual bluegrass
Chl.	Chlorophyll
CB	Creeping bentgrass
DW	Dry weight
EC	Electric conductivity
GA	Gibberellic acid
GA_3	Gibberellic acid 3
GTI	Guelph Turfgrass Institute
HMW	High molecular weight fructan
LMW	Low molecular weight fructan
mg/L	Milligrams per litre
TNC	Total non-structural carbohydrates
TE	Trinexapac-ethyl
µmol	Micromoles
pH	Potential hydrogen

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1.0 INTRODUCTION

1.1 WHY IS COLD TOLERANCE IMPORTANT

Plants that are grown in temperate climates and experience severe cold in the winter require a high level of cold tolerance for longevity and economical stability. Within the turfgrass industry specifically, cold tolerance is important for economic viability. If a golf course loses turfgrass on the greens due to winter kill, reconstruction can cost anywhere from $20,000 to $45,000 per green, depending on the extensiveness of the work. For 18 greens, the total reconstruction costs can reach $810,000. Therefore golf clubs can incur substantial costs at the start of the new golfing season. In terms of winter cereal production, if a grower loses a large percent of their crop it could potentially put the farm at risk. Abiotic stresses can have a profound effect on crop yield. A study in 1982 demonstrated that major U.S. crops, including wheat and barley, were only reaching 20% of their genetic potential due to drought and cold stress (Boyer 1982). Within the grass family there is great diversity in terms of cold tolerance. It is this diversity that continues to push research to try and improve winter hardiness within specific species for the desired purpose of improving cold tolerance and winter survival of turfgrass species and winter cereal crops.

1.2 WHAT IS COLD TOLERANCE

Cold tolerance can be defined as the ability of a plant to change the physiology and biochemistry, in order to survive abiotic stresses and pathogens associated with sub-zero temperatures. Poor cold tolerance has a negative economic impact on any business involved in crops that must survive an overwintering period.

Cold acclimation is a critical phase in developing cold tolerance and is induced by short days or cool yet above freezing temperatures (Thomashow 1999; Hoffman et al. 2010; Landry and Wolyn 2011). Cold acclimation is initiated by physiological changes through a series of biochemical signals leading to a cold hardened plant (Thomashow 1999; Thomashow 2010). The main result of hardening of the plant is that the plant can survive the abiotic stress associated with winter. One of the most important physiological changes is the reduction of intracellular freezing and promotion of extracellular freezing (Pearce 1999a). Intracellular freezing is the formation of ice within the cell that results in the plasma membrane being compromised. Extracellular freezing is ice formation in the areas external to the cells; therefore the plasma membrane is not compromised by expanding water. Subzero tolerant species are believed to prevent cell death by limiting the growth of the ice crystals and keeping them in the extracellular area (Pearce 1999a). However the longer a plant is exposed to freezing temperatures the larger the extracellular ice crystals become and eventually the crystals penetrate the membrane (Pearce 1999a). In addition, as the moisture from within the cell is pulled into the extracellular space by the osmotic potential of ice formation, the plant becomes desiccated; hence cold tolerance and drought responses are linked (Pearce 1999a; Thomashow 2010).

Landry and Wolyn (2011) worked on two cultivars of asparagus (*Asparagus officinalis*), Guelph millennium and Jersey giant that showed differences in fern senescence. Guelph millennium starts senescence (turning yellow) by mid October; in contrast Jersey giant stays green until the first heavy frost (Landry and Wolyn 2011). The study was carried out to investigate the relationship of early fern senescence in cold acclimation capacity and cold

tolerance (Landry and Wolyn 2011). As Guelph millennium consistently out yields and has superior stand longevity compared to Jersey giant in Ontario, it was hypothesised that Guelph millennium has better cold tolerance traits than Jersey giant. The study was carried out from mid August to November, focussing on physiological traits relating to cold acclimation and cold tolerance in the storage root, fern and rhizome (Landry and Wolyn 2011). Results indicated that during the fall storage root LT_{50}, fern chlorophyll concentration, crown water content and rhizome nitrogen concentration all decreased. Guelph millennium rhizomes had a lower water content than Jersey giant indicating greater dehydration however both cultivars' storage root LT_{50} values were -19°C (Landry and Wolyn 2011). With the cultivars showing no differences in LT_{50} values but showing differences in other traits believed to be important for freezing tolerance, it was concluded that timing of fern senescence combined with rhizome characteristics play a critical role in cold acclimation and freezing tolerance of asparagus (Landry and Wolyn 2011).

Landry and Wolyn (2012) established another study with the same two cultivars of asparagus Guelph millennium and Jersey giant that looked at developing a method to assay seedlings for cold tolerance and freezing tolerance grown in a controlled environment (Landry and Wolyn 2012). Trying to assay field grown seedlings for cold tolerance and freezing tolerance is very complex as there are a lot of variables affecting the traits (Landry and Wolyn 2012). Developing an assay method in controlled environments would be beneficial for breeding programs as it would help develop superior genotypes for cold tolerance and freezing tolerance (Landry and Wolyn 2012). The seedlings were exposed to cold acclimation temperatures (10°C day/5°C night) or cold acclimation followed by freezing temperatures (3°C

day/-3°C night) in growth chambers, the freezing temperature was to mimic a thaw-refreeze cycle (Landry and Wolyn 2012). The physiological parameters, crown dry weight, crown nitrogen concentration, total non-structural carbohydrates, fern chlorophyll concentration, LT_{50} and crown water content were analyzed (Landry and Wolyn 2012). Chlorophyll concentration decreased during cold acclimation for both cultivars, however there was a significantly greater decrease for Guelph millennium which was consistent with their previous study. Following cold acclimation LT_{50} values were -8°C for both cultivars, however following thaw-refreeze cycle the LT_{50} values increased to -5°C for Jersey Giant. Therefore the freezing tolerance of Jersey giant decreased following the thaw-refreeze cycle (Landry and Wolyn 2012). It was concluded that the superior freezing tolerance of Guelph millennium in the growth chambers was due to high proline concentration, reducing sugar concentration, stable protein and early senescence (Landry and Wolyn 2012). Futher studies will be required to determine if the differences in freezing tolerance between cultivars can be achieved with field grown asparagus (Landry and Wolyn 2012).

Espevig et al. (2011) conducted a study looking at the effect of different cold acclimation treatments on freezing tolerance and carbohydrate status of two bentgrass species, creeping bentgrass (*Agrostis stolonifera* L.) (CB) and velvet bentgrass (*Agrostis canina* L.). The cold acclimation treatments were 1) non-acclimated, 2) two weeks acclimation at a constant 2°C, 3) four weeks acclimation at a constant 2°C, and 4) four weeks acclimation at a constant 2°C plus two weeks at a constant -2°C for sub zero acclimation (Espevig et al. 2011). Acclimation treatment number 4 produced the lowest LT_{50} value of -14.6°C. Previous studies have also shown the importance of a sub zero acclimation period in freezing tolerance (Gay and Eagles

1991). The LT_{50} values between the two species were not significantly different (Espevig et al. 2011). Sucrose and fructans were significantly higher in the plants that were in the acclimation treatment number 2 when compared to non-acclimated plants in both species. However, only fructans continued to increase in concentration in acclimation treatment number 3 for both species (Espevig et al. 2011).

Dionne et al. (2001) published a cold acclimation study investigating the differential freezing tolerance and carbohydrate status of three annual bluegrass ecotypes; the ecotypes came from Central Quebec (CR), Western Pennsylvania (OK) and Coastal Maryland (CO). Cold acclimation occurred in a controlled environment (heated greenhouse) and a uncontrolled environment (unheated greenhouse) (Dionne et al. 2001). Previous studies had established that the three ecotypes differed significantly in their freezing tolerance. Dionne et al. 2001 concluded the freezing tolerance pattern, based on LT_{50} values; OK < CO < CR, this same pattern of freezing tolerance was exhibited in both the controlled environment (heated greenhouse) and the uncontrolled environment (unheated greenhouse). Concentrations of monosaccharide's, disaccharides and fructans were quantified and there was no significant correlation between the LT_{50} values and carbohydrate status (Dionne et al. 2001).

Winter hardiness is a complex trait involving resistance or tolerance to many abiotic stresses including freezing temperatures, ability to withstand thaw-refreeze events, ice encasement, disease, desiccation, frost heaving and flooding (Brown and Blackburn 1987; Pearce 1999b). Brown and Blackburn (1987) concluded freezing tolerance in the spring during

thaw-refreeze events is the most important trait for cereal production in terms of winter survival in Ontario.

Livingston (1996) published a study assessing the relationship between subzero acclimation, freezing tolerance and carbohydrate status of cereal crops. For freezing tolerance the study focused on oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) which were acclimated in a controlled environment at -3°C for a total of seven days (D. Livingston 1996). The oat cultivar Wintok exhibited greater freezing tolerance as shown by an increase in LT_{50} by 7°C after being exposed to -3°C for 7 days. On day zero of the experiment the LT_{50} value for Wintok was -13°C on day 7 the LT_{50} value was -20°C. The author concluded that the increase in freezing tolerance resulted from a higher concentration of carbohydrates (D. Livingston 1996). The study identified a correlation between carbohydrate status and winter survival, the greater the carbohydrate concentration the greater the winter survival (D. Livingston 1996).

1.3 COLD ACCLIMATION

Plants grown in temperate climates need a period of cool weather in order to fully develop cold tolerance; this process is called cold acclimation and involves many physiological traits (Taiz and Zeiger, 1991; Smith et al, 2010). Optimum temperatures for the acclimation of cereal crops are when temperatures fall into the range of 3°C to 10°C. Cold tolerance occurs when the plants are fully acclimated, typically plants reach maximum cold tolerance in the middle of the winter (Ruelland, Vaultier, and Zachowski 2009). The first environmental signal is a decrease in light levels in the late summer/early fall which is followed by the second signal of decreasing temperatures. The plant can sense the day length by the hours of day light and this

signals the plant to produce a photochemical reaction within the plant that results in the biochemical and physiological changes.

It has been well documented in many species that total non-structural carbohydrates (TNC) increase during cold acclimation (Olien and Clark 1993; Dionne et al. 2001; Ruelland et al. 2009; Espevig et al. 2011; Theocharis et al. 2012). The main role of total non-structural carbohydrates in cold tolerance is cryoprotection as they interact with the lipids in the cell membranes and change the cell structure (Livingston and Henson 1998; Vereyken et al. 2003; Livingston et al. 2009). Fructan interacts with the lipid by inserting part of the polysaccharide into the head group of the lipid; this stabilizes the cell during desiccation (Vereyken et al. 2003; D. P. Livingston, Hincha, and Heyer 2009;). Sucrose is also thought to be a signal molecule for genes associated with cold tolerance (D. P. Livingston, Hincha, and Heyer 2009). The accumulation of TNC within the cell lowers the freezing point of the cell and this along with the formation of extracellular ice protects and maintains the cells integrity (D. P. Livingston, Hincha, and Heyer 2009).

For grasses one of the main physiological responses of cold acclimation is dehydration of the crown, the growing point of the plant (Olien and Clark 1993; Landry and Wolyn 2011; Hoffman et al. 2013). The crown consists of the apical meristem where the leaf primordia and axillary buds are produced. The crown is essentially a compressed stem and is the most important part of the grass plant. As ice formation occurs in the extracellular area water is drawn from the intercellular areas. Hoffman et al. (2010) published a cold acclimation study investigating physiological changes of perennial ryegrass (*Lolium perenne*) accessions known to

have genetic-based differences in freezing tolerance. There were four accessions used, two accessions were freezing damage susceptible and two accessions were freezing tolerant (Hoffman et al. 2010). Cold acclimation was conducted in a controlled environment with a constant 2°C for 21 days and crown material was harvested throughout the acclimation period on days 0, 7, 14 and 21 (Hoffman et al. 2010). The harvested crown material was used to determine lipid composition, water soluble carbohydrates and proline (Hoffman et al. 2010). Water soluble carbohydrates concentrations were significantly higher in the freeze tolerant accessions by day 21 of acclimation at a constant 2°C (Hoffman et al. 2010).

1.4 COLD DEHARDENING OR DEACCLIMATION

Cold dehardening is when the plant loses the cold tolerance previously produced by the acclimation period (Kalberer et al. 2006). Cold acclimation can take weeks to months before full cold tolerance is achieved while in comparison, dehardening can occur in hours to days. Cold tolerance can be rapidly lost if temperatures reach 3°C or above (Smith et al, 2010). If the conditions are right, the plant material can re-acclimate following a thaw or de-acclimation period (Kalberer, Wisniewski, and Arora 2006). The most critical time for winter survival is in the spring as temperatures can fluctuate between above and below freezing, known as a thaw-refreeze cycle. Due to the increasing temperatures the plant loses its previously acclimated cold tolerance. The processes involved in dehardening are not fully understood at this point as literature on deacclimation effects on turfgrass species is still very limited.

Hoffman et al. (2014) published a study on cold deacclimation or dehardening, the study assessed the effect of above freezing temperatures immediately following cold acclimation on

freezing tolerance of annual bluegrass (*Poa annua* L.) (AB) and CB. Hoffman et al. (2014) state the objective of the study as "*determining the deacclimation sensitivity of one annual bluegrass ecotype and one creeping bentgrass cultivar to varying temperature increases and durations*." The cold acclimation procedure was as follows: two weeks at 20°C with a 10 hour photoperiod (light intensity of 300 µmol m^{-2} sec^{-1}), then two weeks at 2°C with a 10 hour photoperiod (light intensity of 300 µmol m^{-2} sec^{-1}) followed by sub zero acclimation at -2°C in complete dark (Hoffman, DaCosta, and Ebdon 2013). Deacclimation was carried out at each step of the acclimation procedure, plants were removed from acclimation temperatures and subjected to 4°C, 8°C and 12°C for both one day and five days the plant material was then subjected to freezing tests (Hoffman, DaCosta and Ebdon 2013). Following a four week recovery period in the greenhouse, the LT_{50} was calculated for the various treatments based on percent survival (Hoffman, DaCosta and Ebdon 2013). The study identified a 2.5 fold decrease in AB freezing tolerance when compared to CB following the deacclimation temperatures (Hoffman, DaCosta and Ebdon 2013). In general, AB was more susceptible to freezing temperatures following the deacclimation in all treatments then CB (Hoffman, DaCosta and Ebdon 2013).

Tompkins et al. (2000) conducted a study in the late winter/early spring of 1996 and 1997, to investigate the dehardening of AB and CB as affected by different hydration treatments. Hydration treatments were; 1. Snow cover was maintained for as long as possible (snow cover), 2. Snow was removed in early March (dry treatment), 3. Snow was piled around the plots in early March and allowed to melt onto the plot only (partial wet treatment), 4. Snow was again piled around the plots in early March and allowed to melt onto the plot only, but prior to sampling water was applied to the plot at a rate of 5 L m^{-2} (wet treatment) (Tompkins,

Ross, and Moroz 2000). Plant material was sampled on March 18th, March 25th, April 1st, April 9th, April 15th and April 22nd of each year, respectively (Tompkins, Ross, and Moroz 2000). The samples were used to determine LT_{50} and percent crown moisture content (Tompkins, Ross, and Moroz 2000). The authors concluded that soil temperature was one of the main contributing factors to turfgrass losing its hardiness, as soil temperatures rise above 0°C the turfgrass plants deacclimate (Tompkins, Ross, and Moroz 2000). In 1996, prolonging snow cover increased cold tolerance from 6 days to 9 days but, had no effect in 1997 (Tompkins, Ross, and Moroz 2000).

1.5 CHILLING AND FREEZING TEMPERATURES

Chilling and freezing temperatures are very different in the physiological responses they produce in the plant, thus it is important to understand the differences between them. Chilling temperatures are considered low but above 0°C, while freezing are below 0°C (Ruelland, Vaultier, and Zachowski 2009). The kinetics of the metabolic system are negatively affected in terms of thermodynamics by lowering temperatures; as temperatures decrease essential processes like photosynthesis are negatively affected (Ruelland, Vaultier, and Zachowski 2009). Proteins and protein complexes become unstable, as does metabolic regulation of enzymes. Membranes become rigid, which causes disturbance to all membrane processes (Ruelland, Vaultier, and Zachowski 2009). Chilling tolerant species change their physiology to prevent membranes from becoming rigid. The freezing point of plant tissue is -1.5°C and it is at this point that freezing damage occurs in non-acclimated plants (Graham and Patterson 1982).

1.6 CHILLING DAMAGE

Plants that originate from tropical or sub tropical regions of the world can experience chilling damage. Chilling damage can occur within a wide range of positive temperatures from 0°C to 20°C, apples (*Malus domestica*) are considered a temperate fruit and do not show signs of chilling damage until temperatures are between 0°C and 4°C (Lyons 1973; Graham and Patterson 1982). However bananas (*Musa acuminata*) can show chilling damage at temperatures below 12°C (Lyons 1973; Graham and Patterson 1982). Typically chilling damage occurs on susceptible banana species at around 10°C to 12°C, but there are differences among species, depending on the origin (Lyons 1973; Graham and Patterson 1982). Chilling injury is superficial or it will never cause plant death; a plant will be damaged by chilling injury but will survive. Whereas with freezing damage, often the plant cannot recover and simply dies (Lyons 1973; Graham and Patterson 1982). Chilling damage affects the post harvest storage as the fruit degrades more quickly when stored at cooler temperatures. Therefore, varieties with better cold tolerance are more desirable because fruit could be stored for longer at cool temperatures (Lyons 1973).

Photosynthesis is the main physiological trait affected by the chilling temperatures which reduces the plant's ability to photosynthesize effectively (Ruelland, Vaultier, and Zachowski 2009). More specifically, the lack of growth caused by exposure to the cold temperatures, leads to the energy produced through photosynthesis not being utilized, leading to feedback inhibition of photosynthesis (Ruelland, Vaultier, and Zachowski 2009; Theocharis, Clément, and Barka 2012).

Chilling temperatures also cause an increase in reactive oxygen species (ROS) due to a decrease in scavenging enzymatic activity and the chloroplast electron transfer chain activity being reduced (Ruelland, Vaultier and Zachowski 2009). Reactive oxygen species cause extensive damage to membranes leading to ion leakage (Ruelland, Vaultier, and Zachowski 2009). Although ROS are known to cause damage to the membranes, it has also been reported that ROS are involved in plant stress acclimation (Theocharis, Clément and Barka 2012). Reactive oxygen species are thought to be a signalling molecule for antioxidant enzymes (Theocharis, Clément, and Barka 2012). It is important to understand that chilling tolerance and cold tolerance initiate different physiological and biochemical responses.

1.7 FREEZING DAMAGE

Non-acclimated plants exposed to freezing temperatures will experience dramatic physiological damage due to the expansion and shrinkage experienced by the membranes during freeze-thaw cycles. Freezing and desiccation of the cell occur at the same time leading to shrinkage of the cell, in non-acclimated plants this will lead to expansion-induced lysis (Ruelland, Vaultier, and Zachowski 2009). Expansion-induced lysis is associated with species that experience the formation of endocytotic vesicles following cell shrinkage. Vesicles are types of endosomes and are involved in recycling substances from within the cell; they also play a key role in cell to cell signaling (Samaj et al. 2005). Vesicles are produced as the cell shrinks; they are made up of the extra membrane material that is produced during shrinkage.

Dowgert and Steponkus (1984), working on rye (*Secale cereale*) leaves, measured the effect of exposure to freezing temperatures. The study found that the predominate form of

injury following a freeze-thaw cycle for non-acclimated rye leaves was expansion-induced lysis due to the formation of endocytotic vesicles. Acclimated rye leaves produced exocytotic extrusions on the surface of the protoplasts (Dowgert and Steponkus 1984). During a thaw, osmotic expansion occurs and therefore the plasma membrane surface needs to expand to the original size. However, due to the formation of endocytotic vesicles the original material is no longer available for reincorporation to the plasma membrane. Therefore the result is expansion-induced lysis and ultimately cell death (Ruelland, Vaultier and Zachowski 2009). Whereas when exocytotic extrusions are produced in response to freezing temperatures, the plasma membrane can return to the original size and osmotic expansion will not lead to lysis (Ruelland et al. 2009; Dowgert and Steponkus 1984). The exocytotic extrusions are produced by freezing temperatures in cold acclimated plants while endocytotic vesicles are produced in non-acclimated plants (Uemura and Steponkus 1999).

1.8 TOTAL NON-STRUCTURAL CARBOHYDRATES

Total non-structural carbohydrates (TNC) refers to sugars like starch, sucrose, glucose, fructose and fructans and are not involved in the production of structural components of the plant. Fructans are used by the plant as short term storage units and are synthesised when photosynthesis is greater or equal to respiration and during low temperatures (Hendry 1987; Jeong and Housley 1990; Olien and Clark 1993). Jeong and Housley (1990) carried out a study looking at the effect of alternating cold and warm temperatures on fructan metabolism in wheat seedlings (*Triticum aestivum* L. cv Caldwell). Originally the study was set up to develop a method to control the metabolism direction of fructans because in warm temperatures fructans are being used by the plant while in cold temperatures fructans are stored by the

plant. Fructan synthesis was initiated by removing three week old seedlings grown at 25˚C (warm) and transferring them to 10˚C (cold). Following the cold treatment foliage was removed and placed in continuous darkness at 25˚C where fructan degradation was monitored. Due to defoliation the remaining shoot tissue was comprised of 90% leaf sheath. One day after transferring from 25˚C to 10˚C total fructan concentration increased significantly in both the leaf blades and shoot tissue. Higher concentrations of fructans were found to accumulate in the leaf sheath when compared to the leaf blades. However when re-transferred to 25˚C in darkness fructan content in the leaf sheaths declined rapidly and were completely degraded within 48 hours. This study demonstrated the role low temperature plays in fructan synthesis and degradation; it also identified a method for controlling the metabolism direction of fructan.

Twelve to 15% of angiosperms produce fructans and accumulate fructans for winter hardening (Hendry 1987; D. P. Livingston, Hincha and Heyer 2009). Grasses that originate in temperate zones accumulate fructans for over-wintering and utilize them as the main source of sugar during spring re-growth (Suzuki 1988). Plants in parts of the world that experience drought and freezing temperatures have evolved to produce fructans in the highest quantities (Livingston III 1991). Changes in carbohydrates composition from fructans to sucrose, glucose and fructose in wheat, barley and rye during the winter have been documented (Olien and Clark 1993). Olien and Clark (1993) found that the plant depolymerises or breaks the fructans down into sugars that the plant utilizes throughout the late winter and spring. This has also been investigated by Dionne et al. (2001) who found fructans and sucrose to be the most accumulated carbohydrates in the crowns of cold-hardened AB. Smith et al. (2010) states that high levels of sucrose cause the expression of genes that synthesize fructan and this would be

in agreement with the work from Dionne et al. (2001). Fructans have a cryoprotective quality, meaning they interact with the membranes to prevent the cells from becoming unstable during the shrinking and dehydration of acclimation (Olien and Lester 1985; Suzuki 1988; Livingston III 1991; Olien and Clark 1993; Dionne et al. 2001). This work shows the importance of TNC for winter survival and also highlights the importance of accumulating a high quantity during acclimation.

1.9 ABSCISIC ACID

Abscisic acid (ABA) was first thought to be the only hormone involved in the abscission process. However, further work found that ABA and ethylene are involved in organ abscission (Taiz and Zeiger, 1991). It has also been suggested that ABA promotes leaf senescence by stimulating the formation of ethylene, therefore promoting the abscission process (Taiz and Zeiger, 1991). There are two forms of the isomer ABA: cis and trans but in nature it is nearly always found in the cis form. Abscisic acid is a known plant hormone which acts as an inhibitor of growth, but it also has a role in stress conditioning (Zeevaart and Creelman 1988). The level of stress conditioning is dependent upon the endogenous concentration of ABA, which is a result of the environmental conditions and plant growth. There is evidence to support a correlation between the amount of endogenous ABA and the level of cold tolerance (Taylor et al. 1990; Zhang et al. 2011). A study conducted by Zhang et al. (2011) shows increased levels of ABA during and at the end of cold acclimation of cold-tolerant varieties of bermudagrass in comparison to cold-sensitive varieties. Taylor et al. (1990) studied the level of endogenous ABA in winter wheat during cold acclimation, both throughout the winter and into the spring months. The study showed ABA endogenous levels increased from October to December and

then declined from January to March. Cold tolerance improved when ABA levels were high as indicated by lower LT_{50} values (Taylor et al. 1990). Studies have shown that the application of exogenous ABA can increase the amount of sugars in barley during cold acclimation (Bravo et al. 1998). However, there were variable results in terms of increasing the freezing tolerance, depending on the original endogenous content of ABA (Bravo et al. 1998). Studies have shown that there is a relationship between ABA content and the level of cold tolerance by the use of ABA-deficient mutants and exogenous ABA (Gilmour and Thomashow 1991; Heino et al. 1990). Exogenous ABA has shown to produce a similar level of cold tolerance in non-acclimated plants (Gilmour and Thomashow 1991; Heino et al. 1990; Lång et al. 1989). Heino et al. 1990 working with an ABA-deficient mutant of *Arabidopsis thaliana* (L.) Heyn, found that when the mutant was subjected to low-temperature treatment cold tolerance did not increase and was the same as the non acclimated wild type. However, when the mutant was treated with exogenous ABA through the growth medium the cold tolerance returned to a similar level of the wild type. Gilmour and Thomashow (1991) determined ABA-deficient mutants had lower cold tolerance as measured by higher ion leakage when compared to ABA insensitive mutants and wild type Arabidopsis thaliana (L.) Heynh. Lång et al. (1989) treated arabidopsis plantlets with exogenous ABA (15mg/1) at a non-acclimating temperature (20°C) and found that cold tolerance increased more rapidly when compared to a low temperature treatment (4°C). These three studies demonstrate the importance of the ABA role in cold acclimation and cold tolerance. It's thought that ABA could signal other responses which relate to cold acclimation. There is still a lot of work that can be done to investigate the role ABA plays in cold tolerance.

1.10 GIBBERELLIC ACID

Japanese rice farmers in the 1930's had to deal with a fungal disease known as *bakanae* or "foolish seedling" (Eckardt 2002; Taiz and Zeiger 2010). The fungal disease caused the rice plants to grow too tall and not produce any seed, investigations by plant pathologists at the time lead to the discovery of the pathogenic fungus *Gibberella fujikuroi* (Brian et al. 1954). Impure crystals which had plant growth promoting properties were discovered in the fungus culture filtrate (Brian et al. 1954; Taiz and Zeiger 2010). The Japanese scientists named the compounds gibberellin A. (Taiz and Zeiger 2010). During the 1950's two research groups, one in the United States and one in Britain, were working on identifying the structure of one of the compounds purified from the *Gibberella* cultures (Brain et al. 1954). During the same period Japanese scientists were working on separating and identifying different compounds from the original gibberellin A. sample (Taiz and Zeiger 2010). This lead to the identification of three gibberellin compounds; gibberellin A_1 (GA_1), gibberellin A_2 (GA_2) and gibberellin A_3 (GA_3). It was later discovered that the GA_3 compound discovered by the Japanese was identical to the "gibberellic acid" compound discovered in the United States and Britain (Taiz and Zeiger 2010). Thus the term "gibberellic acid" relates solely to GA_3 whereas the term "gibberellin" can relate to the entire family of hormones (Taiz and Zeiger 2010). Since the initial work performed on the original gibberellin compounds, many more have been discovered. To date there are 126 naturally occurring gibberellins found in plants, bacteria and fungi (MacMillan 2001). Gibberellins play a key role in a variety of plant growth and development phenomena, such as stem elongation (Kende, Knaap, and Cho 1998), flower initiation (Colasanti and Coneva 2009), fruit development (Groot, Bniinsma, and Karssen 1987), seed germination (Plummer and Bell

1995) and pollen development (Hansen, Bellman, and Sacher 1976). Gibberellins have also been shown to play critical roles in stress tolerance (Vettakkorumakankav et al. 1999). Vettakkorumakankav et al. (1999) studied the effect of the plant growth regulator paclobutrazol on stress tolerance. The hypothesis was tested using near-isogenic lines of normal and GA responsive dwarf mutant of barley (Vettakkorumakankav et al. 1999). Paclobutrazol, a triazole, works by inhibiting cytochrome P450 monooxygenase reactions during gibberellin and ergosterol biosynthesis (Vettakkorumakankav et al. 1999). The cytochrome P450 also plays a role in the degradation of ABA (Korol and Klein 2002). It was hypothesized that by reducing the endogenous levels of GA via exogenous paclobutrazol the stress tolerance would increase. Stress tolerance was improved by a number of physiological phenomena triggered by reduced endogenous GA levels (Kraus and Fletcher 1994). Included in the effects of reduced endogenous GA are increased antioxidant enzymes to scavenge ROS produced during abiotic stress, increased biosynthesis of photosynthetic pigments and altered root to shoot ratio (Kraus and Fletcher 1994; Vettakkorumakankav et al. 1999). Vettakkorumakankav et al. 1999 concluded stress tolerance was increased by using GA inhibitors.

Bingham and McCabe (2006) studied the effect of Moddus (trinexapac-ethyl) and Route (Zinc ammonium acetate) an auxin promoter on root growth, drought response and soil water extraction in spring barley (*Horduem vulgare L*.). The study was carried out under a rain-proof shelter and irrgation was withheld from the drought-induced plants. The results indicated that both Route and Moddus significantly increased ABA accumulation in the stem base of the drought-induced plants (Bingham and McCabe 2006).

1.11 EFFECTS OF TRINEXAPAC-ETHYL

The company Syngenta which manufactures the trinexapac-ethyl (TE) product Primo Maxx, suggests it improves stress tolerance. McCann and Huang (2007) investigated the effect of TE application and combined heat and drought stress of creeping bentgrass. The TE application in the study resulted in a constant photosynthesis rate, increased chlorophyll content, higher turf quality and a higher rate of growth during the stress period (McCann and Huang 2007). Total non-structural carbohydrate concentration was lower in TE treated plants during the stress period (McCann and Huang 2007).

McCann and Huang (2008) also studied drought tolerance and the effect of applying TE and ABA on cool season turfgrasses kentucky bluegrass and creeping bentgrass. The application of ABA and TE resulted in the same physiological response in both turfgrass species. The researchers concluded that CB and kentucky bluegrass responses to drought were improved by both TE and ABA applications when compared to the untreated control (McCann and Huang 2008).

Bian et al. (2009) studied the effect of TE application on creeping bentgrass that either experienced drought or well watered for 28 days. Their study focused on solute concentrations known to be involved in osmotic adjustment. Trinexapac-ethyl treated plants had higher concentrations of soluble sugars and had greater water use efficiency for the 28-day period of drought when compared to the untreated control (Bian, Merewitz and Huang 2009). Inorganic ions (Ca and K) increased in the TE-treated plants when compared to the untreated control (Bian, Merewitz and Huang 2009). The authors concluded that TE benefits turfgrass during

drought by promoting lower water use and by increasing soluble sugar concentrations (Bian, Merewitz and Huang 2009).

Ervin & Koski (2001) conducted a greenhouse study to investigate the physiological effects of TE on Kentucky bluegrass. The study focused on chlorophyll concentration, leaf blade cell density and structural carbohydrates (Ervin and Koski 2001). There was only one application of TE made and sampling occurred 2 and 4 weeks after the application date (Ervin and Koski 2001). The authors found that after one application of TE there is no significant effect on structural carbohydrates; however, chlorophyll-b concentration and cell density were both increased (Ervin and Koski 2001).

1.12 TRINEXAPAC-ETHYL AND TOTAL NON-STRUCTURAL CARBOHYDRATES

To produce a better-performing putting surface golf course managers regularly use TE on putting greens. Trinexapac-ethyl works by interfering with the biosynthetic pathway of GA; more specifically, it prevents the conversion of GA_{20} to GA_1, reducing cell elongation and producing a tighter, denser turfgrass sward. On golf courses, putting greens are mowed on a daily basis to ensure high performance and conditioning, and by reducing cell elongation putting green performance is increased.

Previous studies have looked at the effect TE has on the physiological aspects of plant growth and stress tolerance. Steinke and Stier (2004) studied the effect of TE on cold tolerance of *Poa supina* grown in shaded conditions. The study calculated freezing tolerance as determined by LT_{50} and analyzed the concentrations of the carbohydrates glucose, fructose and sucrose. The study found that sequential applications of TE significantly increased freezing

tolerance in the second year of the study, but not significantly in the first year. Freezing tolerance was measured by removing samples (prepared in October) from the field in January, February and March of both 2001 and 2002 (Steinke and Stier 2004). Steinke and Stier (2004) determined LT_{50} with a glycol bath and lowering the temperature until target temperatures were reached. A total of 7 target temperatures were used: 0, -4, -8, -12, -16, -20 and -24°C, at which point plant material was removed. The plant material was then kept at 3°C for six days and once thawed, the plant material was placed in a greenhouse for re-growth (Steinke and Stier 2004). The carbohydrate concentrations were measured on samples (prepared in October) that were removed from the field in January, February and March in 2001 and 2002 (Steinke and Stier 2004). Steinke and Stier (2004) found TE did not significantly affect the concentrations of glucose, sucrose or fructose from January through March of either 2001 or 2002. Although TE application didn't increase the carbohydrate accumulation, TE application resulted in a slower loss of carbohydrates from January through March in both years (Steinke and Stier 2004).

Arghavani et al. (2012) improved the salt tolerance of Kentucky bluegrass (*Poa pratensis* L.) with the application of TE. The study found that by applying TE at 1g/100m^2 twice with a 4-week interval TNC increased. TNC was calculated by measuring the concentrations of glucose, fructose and sucrose (Arghavani, Kafi, and Babalar 2012). Interestingly the study found that applying TE at 1.7g/100m^2 twice with a 4 week interval decreased the salt stress (Arghavani, Kafi, and Babalar 2012). This evidence suggests that the relationship between TE and TNC concentrations is complex. Arghavani et al. (2012) suggests it is TE's ability to maintain antioxidant activity that leads to better root growth and increases in TNC.

1.13 PROJECT RATIONALE

The GA inhibitor TE is widely used in the turfgrass industry as a management technique for improving the putting quality of putting greens. Improved putting quality is achieved by altering growth of grasses to be more compact through reduced leaf elongation. This is important in the turf industry as putting greens are mowed daily throughout the growing season. So by utilizing plant growth regulators growth is being reduced on the putting surface which results in a more consistent surface and reduces potential labour costs if mowing cannot be accomplished daily.

The manufacturer of the TE plant growth retardant product Primo Maxx suggests it also improves stress tolerance. It has been suggested the product does this by increasing TNC. Total non-structural carbohydrates increase when the rate of photosynthesis is greater than the rate of respiration and plant growth.

Cold tolerance of cereal crops has not significantly improved in the last 20 - 30 years. The vast majority of research efforts have been on improving the genetic background of cereal crops through breeding. Both classical and molecular breeding methods have been utilized to improve cold tolerance, although molecular breeding has been the main focus for approximately 15 years. Little research has focused on manipulating the physiology of cereals in the fall to improved cold tolerance.

The following experiments investigate the physiological effects of TE in a non-stressed environment (greenhouse) versus a stressed environment (cold acclimation) in two field experiments, one on turfgrass species and a second on cereal crops.

1.14 Hypothesis

Manipulating GA will affect winter acclimation, cold tolerance and will be inversely related to carbohydrate status in both species. Application of the GA inhibiter TE will increase carbohydrate concentration, cold acclimation and cold tolerance. The application of GA_3 will decrease carbohydrate content, cold acclimation and cold tolerance. Annual bluegrass and CB will differ in cold tolerance as exhibited by $LT_{50.}$

1.15 Objectives

1. To determine if there are different physiological responses (photosynthesis and chlorophyll content) in an unstressed environment between AB and CB.
2. To determine if the application of GA inhibitors and/or exogenous GA are related to carbohydrate status and LT_{50}.
3. To determine if there are different physiological responses (photosynthesis and respiration rates) at different acclimation temperatures between the two species of turfgrass tested.
4. To determine if these two cools season turfgrass species can be used as a model system for similar physiological responses in cereal C_3 crops.
5. To determine if the application of GA inhibitors and/or exogenous GA correlate to carbohydrate status in different cereal crops with different genetic tolerances to cold tolerance.

2.0 MATERIALS AND METHODS

2.1 ASSESING THE EFFECT OF TRINEXAPAC-ETHYL AND GA_3 ON ANNUAL BLUEGRASS AND CREEPING BENTGRASS IN GREENHOUSE CONDITION

The objective of this study was to determine if photosynthesis rates increase after two applications of the TE product Primo Maxx in an unstressed environment on creeping bentgrass (CB) and annual bluegrass (AB).

Creeping bentgrass and AB plugs (from a sand-based, research green) 3.7cm in depth were taken from the Guelph Turfgrass Institute (GTI) and a total of 72 conetainers were established on April 18th 2012. The conetainers were filled with pea gravel (1.27cm) at the bottom of the conetainer and topped with USGA specification rootzone (Green Section Staff, 1993) to simulate a USGA constructed green. The conetainers have a diameter of 13cm^2 and are 15cm long. Three treatments TE, Control (water) and GA_3 with four subsamples resulting in twelve conetainers (Stuewe and Sons Inc., Corvallis, OR) of each species were used for each replicate and the study was replicated three times over time.

Fertilisation was carried out bi-weekly with a 20-8-20 water-soluble fertiliser (Plant products, Guelph, Ontario) in a 250 ppm N solution with an EC of 2.54 and pH of 6.1 (Ron Dutton, Bovey Greenhouse, University of Guelph). Fungicides were applied to the plant material twice using Banner Maxx (Syngenta, Guelph, Ontario) at 75ml/100m^2 for control of Fusarium with a 30 day interval. Mowing occurred twice per week with hand scissors and plants were kept to a height of ~3mm. Irrigation was applied every 2 days until the plant material and soil profile of the conetainer were saturated.

Treatments were applied using a hand-held sprayer from a height of 30cm above the turfgrass and crop surface and this application method was used for all of the studies. Treatments were GA_3 (60 mg/100m^2 of GA_3 in 6L/100m^2), control (6L/100m^2 of water) and TE (3.8ml/100m^2 in 6L/100m^2). Applications were made on day 1 and day 11 of the experiment.

Chlorophyll content of each conetainer was quantified by the collection of clippings on the third replicate the day before photosynthesis and respiration rates were recorded. The method used for extraction and quantification of leaf chlorophyll content was as described by Richardson et al. (2002). Briefly, leaf tissue was added to a 15 ml polypropylene centrifuge tube containing 7 ml of preheated (65°C) Dimethyl sulfoxide (DMSO). All the available leaf tissue was used for the sample and standardized for the calculation on chlorophyll content. The centrifuge tubes were then placed in a water bath for 30 minutes at 65°C for extraction. Following the 30 minute incubation, the centrifuge tubes were removed from the water bath and topped up to 10 ml with DMSO. For quantification, 3 ml of the DMSO extract was removed from each polypropylene centrifuge tube and placed into disposable polystyrene cuvettes. Pure DMSO was used as the blank and absorbance was recorded at 645 and 663 nm for each of the samples using a spectrophotometer (Thermo Scientific, Evolution 60). The following equations were used to calculate total chlorophyll (Chl), chlorophyll a (Chl. A) and chlorophyll b (Chl. B).

- Total Chl (g L^{-1}) = (0.0202) x (A_{645}) + (0.00802) x (A_{663})
- Chl a (g L^{-1}) = (0.0127) x (A_{663}) - (0.002369) x (A_{645})
- Chl b (g L^{-1}) = (0.0229) x (A_{645}) - (0.00468) x (A_{663})

Photosynthesis rates were recorded using a LICOR® LI-6400 (LI-6400, Li-Cor Inc., Lincoln NE, USA) with a modified *Arabidopsis* whole plant chamber. The LICOR® LI6400 settings were: CO_2 400 µmol $CO_2 mol^{-1}$air, flow rate 400 and photosynthetically active radiation (PAR) 500. Photosynthesis rates were taken prior to the first application of the treatments on day 1 of the experiment. On day 8, the second photosynthesis rates were taken and the second application made 3 days later, on day 11 of the experiment, seven days later, on day 18 of the experiment, the final photosynthesis rates were recorded. After the final photosynthesis rates were recorded, the plant material was harvested by removing the crown material, approximately 0.5cm in depth from the conetainer. The washed samples were wrapped in aluminum foil and flash-frozen with liquid nitrogen, and then stored at -80°C for carbohydrate analysis.

STATISTICAL ANALYSIS

The statistical package JMP version 10 of SAS (SAS Inc. Carey, NC, USA) was used for statistical computations, for both the chlorophyll content and photosynthesis rate, analysis of variance was analyzed using the mixed effect model (REML). A type 1 error rate of 0.05 was set for statistical analysis. Sources of variance were block (random), species AB and CB (fixed), treatment TE, control and GA_3 (fixed) and application 1 and 2 (fixed). Means comparisons were carried out using Tukey's HSD.

2.2 ASSESSING FREEZING TOLERANCE FOLLOWING COLD ACCLIMATION IN A CONTROLLED ENVIRONMENT

The objective of the study was to assess the physiological effects of TE and GA_3 on CB and AB during cold acclimation and to investigate physiological changes related to survival or changes in carbohydrate status.

Creeping bentgrass and AB plugs were established and maintained the same as in the greenhouse study (section 2.1). After a period of approximately 60 days, the turf conetainers were moved to the growth chamber for cold acclimation (Table 1). The conetainers were held in a rack of 92, and each rack was considered an experimental unit. A total of 12 racks consisting of 2 species, AB and CB, and 3 treatments (TE, GA_3 and control) were organized in a randomized complete block design (RCBD), and replicated twice in time. The treatment application method and rates were the same as in the greenhouse study (section 2.1).

Acclimation occurred in a growth chamber in the Bovey building, University of Guelph. Cold acclimation was achieved with four different temperature regimes and two photoperiods. The first treatments were applied three weeks prior to the start of the ambient conditions and the second treatments were applied 14 days later. For two weeks, the plant material was kept at ambient conditions: 20°C in the day, 10°C at night and 12 hours of light at 800 µmol m^{-2} sec^{-1} in the growth chamber. Following the two weeks of acclimation in the growth chamber at ambient conditions, the conditions were changed to 10°C in the day, 4°C at night with 12 hours of light at 800 µmol m^{-2} sec^{-1} for a period of one week. The third stage of cold acclimation involved a period of two weeks at a constant 2°C with a photoperiod of 8 hours at 300 µmol m^{-2}

sec^{-1}. The final stage of cold acclimation involves a period of 2 weeks at a constant -2°C with a photoperiod of 8 hours at 300 µmol m^{-2} sec^{-1}.

Table 1. Summary of the acclimation protocol used in the controlled environment study, all of the treatments Trinexapac-ethyl (TE), control (water) and GA_3 and both species annual bluegrass (AB) and creeping bentgrass (CB) were kept in the following temperature and photoperiod conditions.

ACCLIMATION PROCESS				
	Ambient conditions	Two weeks	Two weeks	Two weeks
Photoperiod	12 hours at 800 µmol m^{-2} sec^{-1}	12 hours at 800 µmol m^{-2} sec^{-1}	8 hours at 300 µmol m^{-2} sec^{-1}	8 hours at 300 µmol m^{-2} sec^{-1}
Night temp.	10˚C	4˚C	2˚C	-2˚C
Day temp.	20˚C	10˚C	2˚C	-2˚C

Once acclimated, the plant material was subjected to freezing temperatures to determine the lethal temperature at which 50% of the plants die, or the LT_{50}. The target temperatures were achieved by dropping temperatures at a maximum rate of 2°C per hour, for a period 8 hours. The -16°C target temperature was used as a guide for the other target temperatures. All of the target temperatures were achieved in the same time it took to get to -16°C, this way all the plant material was at the target temperature for the same period of time. Plant material spent a total of 22 hours in the freezers and was held at the target temperatures for 8 hours. One deep chest freezer of four total was dropped to each of the target temperatures of -4°C, -8°C, -12°C and -16°C, respectively. Each freezer contained all treatments and the LT_{50} measurements were replicated four times in time. Twelve experimental units consisting of 2 replications were used at time 1 and time 2 to determine LT_{50}, a total of 24 experimental units were used for the experiment. From each experimental unit in the growth chamber, 4 conetainers were removed for each temperature in the chest freezer, each day for a total of 4 days for LT_{50} determinations. In a single day, 48 conetainers consisting of 4 conetainers from 12 experimental units, was placed in 4 freezers. A total of 192 conetainers were used on each day (Table 2). The target temperatures assigned to a given freezer were changed each of the 4 days to avoid freezer effect. Once the plants were exposed to freezing temperatures, they were put back into the greenhouse for re-growth. The LT_{50} values were calculated by re-growth based on images taken 3 weeks after exposure to the freezing temperatures. The images were analyzed using Sigma Scan software.

Table 2. Summary of the freezer rotations used for the determination of the LT_{50} values, all of the treatments; Trinexapac-ethyl (TE), control (water) and GA_3 and both species annual bluegrass (AB) and creeping bentgrass (CB) experienced the temperatures in each freezer and on each date.

	Freezer 1	**Freezer 2**	**Freezer 3**	**Freezer 4**
Date 1	-4°C	-8°C	-12°C	-16°C
Date 2	-16°C	-4°C	-8°C	-12°C
Date 3	-12°C	-16°C	-4°C	-8°C
Date 4	-8°C	-12°C	-16°C	-4°C

PHOTOSYNTHESIS AND RESPIRATION RATES

During cold acclimation, photosynthesis and respiration rates were recorded using the LICOR® LI6400 with a modified *Arabidopsis* whole plant chamber. To ensure accurate measurements the LICOR® LI6400 was put into the growth chamber a minimum of 3 hours before the first reading. Photosynthesis and respiration rates were taken at each of the temperatures 20°C, 10°C, 2°C and -2°C. Respiration rates were taken directly after the photosynthesis rate by turning the light off on the whole plant chamber.

SAMPLING FOR CARBOHYDRATE ANALYSIS

Samples were collected before the start of the acclimation process and at the end of acclimation. The conetainers were removed from the growth chamber and kept in a refrigerator at 4°C for a period of 24 hours until all the harvesting was complete. The crown tissue of the samples were removed using a knife. The samples were then washed to remove all sand and debris. Following washing, the samples were wrapped in aluminium foil and flash frozen in liquid nitrogen, then stored at -80°C. The samples were then freeze-dried at a later date at the Laboratory Services, University of Guelph. Once freeze-dried, the samples were returned to -80°C for storage. The plant material was ground into a fine powder using a food blender with small cups in preparation for the carbohydrate assay kits.

CARBOHYDRATE ANALYSIS

Carbohydrate analysis for HMW and LMW fructan was performed using the Megazyme Fructan Assay Procedure (K-FRUC 03/13). McCleary & Rossiter (2004) developed the current assay procedure, contributions to the method were made by Pontis 1966; Lewis 1993;

Quemener et al. 1994; McCleary et al. 2000 for a detailed protocol of the procedure used see appendix. The following modifications were used to quantify HMW and LMW fructan, 100mg of the freeze dried plant material was used for the following extractions. Low molecular weight fructan was extracted twice in 5ml of 90% ETOH at 70°C in a water bath for 20 minutes, the sample was centrifuged at 4000 rpm for 5 minutes after each of the extractions. The resulting supernatant was collected from both extractions and the ETOH was evaporated off. High molecular weight fructan was extracted twice in 5ml of deionised water (dH_2O) at 80°C in a water bath for 20 minutes, the sample was centrifuged at 4000 rpm for 5 minutes after each of the extractions. The resulting supernatant collected from both extractions and used for the determination of HMW fructan concentration.

STATISTICAL ANALYSIS

The statistical package JMP version 10 of SAS (SAS Inc. Carey, NC, USA) was used for statistical computations, for both the photosynthesis rate and carbohydrate status, analysis of variances was analyzed using the mixed effect model (REML). A type 1 error rate of 0.05 was set for statistical analysis. Sources of variance were block (random), species AB and CB (fixed), treatment TE, control and GA_3 (fixed) and application 1 and 2 (fixed). Means comparisons were carried out using Tukey's HSD.

2.3 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS IN THE FIELD

The objective of the study was to assess the physiological effects of TE and GA_3 on CB and AB in the field in the fall and winter and to investigate the effect of hormone treatments in the fall on carbohydrate status in the fall and spring.

Creeping bentgrass and AB plots were set up using a pot-in-pot system. The experimental design was a randomized complete block with treatment rates and method of application being the same as in section 2.1. Twenty-four experimental units were set up on a USGA sand-based green; 12 of the experimental units were in an area established to CB, and the other 12 were in an area that was primarily AB. Within each experimental unit, 24 plugs of turfgrass were cut and placed into a 50mm plant pot, the plant pots were then put into a second 50mm plant pot; then placed back into the ground. The pot-in-pot system was set up to allow sampling over the winter months.

For carbohydrate analysis, one plug from each experimental unit was sampled every month from November 2012 through May 2013, however due to time constraints and sample numbers only the November 2012 and May 2013 sample date were analyzed for carbohydrate status. Once collected, samples were then taken to the Bovey Building, University of Guelph and placed in a refrigerator at 4°C for 24 hours to allow the samples to thaw out. Then the crown material at the top of the plug was cut off and washed. Each sample was wrapped in aluminium foil and flash frozen in liquid nitrogen. The samples were then stored at -80°C and were freeze-dried at a later date. The plant materiel was freeze-dried and ground using the

same method as described in section 2.2. Carbohydrate analysis for HMW and LMW fructan was performed using the Megazyme Fructan Assay Procedure (K-FRUC 03/13). McCleary & Rossiter (2004) developed the current assay procedure, contributions to the method were made by Pontis 1966; Lewis 1993; Quemener et al. 1994; McCleary et al. 2000 for a detailed protocol of the procedure used see appendix. The modifications mentioned in section 2.2 were also used in this section for the determination of HMW and LMW fructan concentrations.

The statistical package JMP version 10 of SAS (SAS Inc. Carey, NC, USA) was used for statistical computations, for both the photosynthesis rate and carbohydrate status, analysis of variances was analyzed using the mixed effect model (REML). A type 1 error rate of 0.05 was set for statistical analysis. Sources of variance were block (random), species AB and CB (fixed), treatment TE, control and GA_3 (fixed), application 1 and 2 (fixed), date before and after winter (fixed). Means comparisons were carried out using Tukey's HSD.

2.4 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS OF SEVEN CEREALS WITH VARYING WINTER HARDINESS IN THE FIELD

The objective of this study was to assess the physiological effects of TE and GA_3 on cereal crops in the field over the winter months and to investigate if hormone manipulation in the fall would relate to changes in carbohydrate status following treatments and/or in the spring.

In September 2012 a study was set up to investigate the effect of hormone manipulation in the fall on carbohydrate status of seven different types of cereal crops during the fall and spring. The experiment was set up as a split plot design with the main plot being hormone manipulation treatment and the sub plot being cereal crop species. Treatment rates and application method was the same as described in section 2.1, and the hand held sprayer was adjusted to ensure the sprayer was 30cm above crop height.

The cereals are all known to have different levels of cold tolerance and included :(1) Fall rye, (2) Froid FW, (3) WB158-25 WB, (4) 2R Sun#1 Fac.Barley , (5) Sable HRS, (6) HY101-6R 6R SB and (7) Newman Oat. In preparation for seeding the ground was tilled and leveled out. The cereals were seeded using a single row seeder with a one-meter gap between plots. The study was done as preliminary work so as such, and due to space restrictions, there was no replication.

For carbohydrate analysis, sampling was carried out every month from November 2012 through May 2013; however, only the November and May dates were analysed for carbohydrate status due to time restrictions. When necessary, snow and ice were removed on

sampling dates. Sampling was carried out by digging up five of the cereal plants from the designated plot. Samples were stored for 24 hours in a refrigerator at 4°C to thaw out in the Bovey Building, University of Guelph. The following day, all soil and debris were washed off the samples and the samples were then wrapped in aluminium foil and flash-frozen in liquid nitrogen and then stored at -80°C. The samples were then freeze dried at Laboratory Services, University of Guelph and then returned to -80°C for carbohydrate analysis. The plant material was ground into a fine powder with a food blender as described in section 2.2. Carbohydrate analysis for HMW and LMW fructan was performed using the Megazyme Fructan Assay Procedure (K-FRUC 03/13). McCleary & Rossiter (2004) developed the current assay procedure, contributions to the method were made by Pontis 1966; Lewis 1993; Quemener et al. 1994; McCleary et al. 2000 for a detailed protocol of the procedure used see appendix. The modifications mentioned in section 2.2 were also used in this section for the determination of HMW and LMW fructan concentrations.

The statistical package JMP version 10 of SAS (SAS Inc. Carey, NC, USA) was used for statistical computations, for both the photosynthesis rate and carbohydrate status, analysis of variances was analyzed using the mixed effect model (REML). A type 1 error rate of 0.05 was set for statistical analysis. Sources of variance were block (random), cereal species (fixed), treatment TE, control and GA_3 (fixed) and application 1 and 2 (fixed), date before and after winter (fixed). Means comparisons were carried out using Tukey's HSD.

3.0 RESULTS

3.1 ASSESSING THE EFFECT OF TRINEXAPAC-ETHYL AND GA_3 ON ANNUAL BLUEGRASS AND CREEPING BENTGRASS IN GREENHOUSE CONDITION

CHLOROPHYLL CONTENT

The following effects were significant for total chlorophyll and chlorophyll A content: treatment (hormone manipulation), species, application and application by treatment interaction (Table 3). Only treatment (hormone manipulation) was significant for Chlorophyll B content (Table 3, Figure 4). Following the first application plants treated with TE had increased concentrations of Chl A (Figure 1 & 2) and total chlorophyll (Figure 1 & 2). Following the second application of treatments (hormone manipulation) TE treated plants had increased concentrations of Chl A and total chlorophyll (Figure 2). Plants treated with TE had increased concentrations of Chl A (Figure 3) and total chlorophyll (Figure 5) when compared to the control and GA_3 treatments. Gibberellic acid decreased the concentrations of Chl A (Figure 3) and total chlorophyll (Figure 5) when compared to the control and TE treatments. When all hormone manipulation treatments were combined AB had more total chlorophyll than CB (Figure 7). Chlorophyll B concentration had no treatment by application interaction (Table 3). Creeping bentgrass had a lower Chl. A (Figure 6) and total chlorophyll (Figure 7) concentration when compared to AB for all treatments with applications combined.

Table 3. Summary of analysis of variance (ANOVA) for total chlorophyll (total Chl.), chlorophyll A (Chl. A) and chlorophyll B (Chl. B), for a study assessing the effect of trinexapac-ethyl and GA_3 (treatment) on annual bluegrass and creeping bentgrass (species) under greenhouse conditions with 2 application events (application).

Source	Total Chl. Pr>F	Chl. A Pr>F	Chl. B Pr>F
Species	0.0003	<.0001	0.5386
Treatment	<.0001	<.0001	0.0027
Application	0.001	<.0001	0.3019
Species*Treatment	0.7682	0.7419	0.8819
Species*Treatment *Application	0.4337	0.3532	0.9348
Application *Treatment	0.0028	0.0018	0.2616

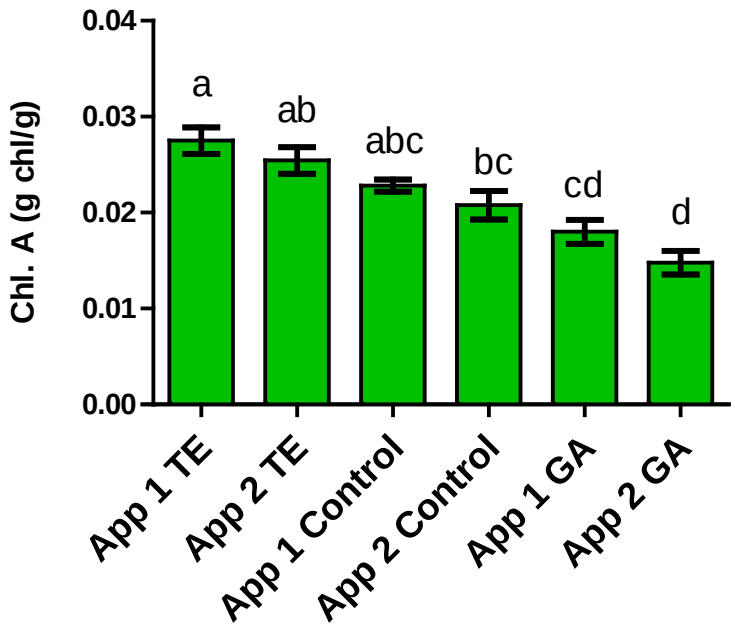

Figure 1. Mean chlorophyll A content (g Chl./g DM) of creeping bentgrass and annual bluegrass (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) once (app 1) and twice (app 2) in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=8.

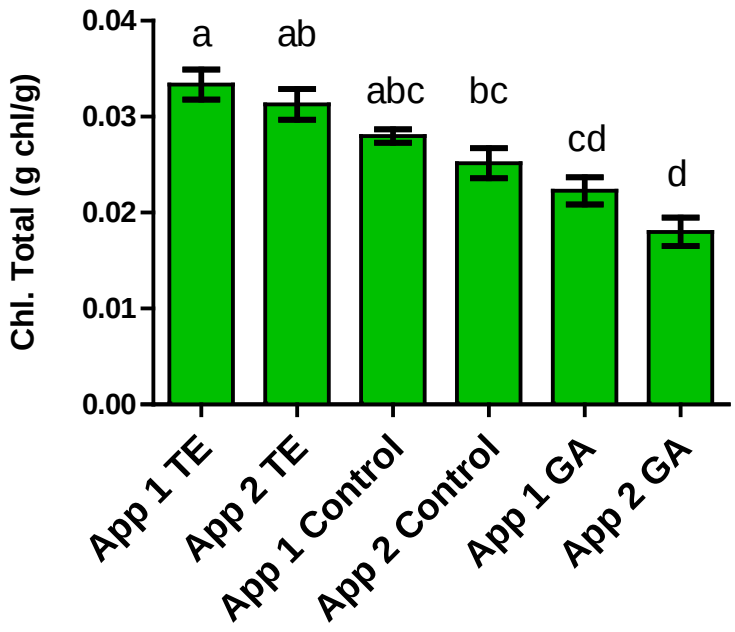

Figure 2. Total chlorophyll content (g Chl./g dried weight of plant material) of creeping bentgrass and annual bluegrass (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) once (app 1) and twice (app 2) in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=8.

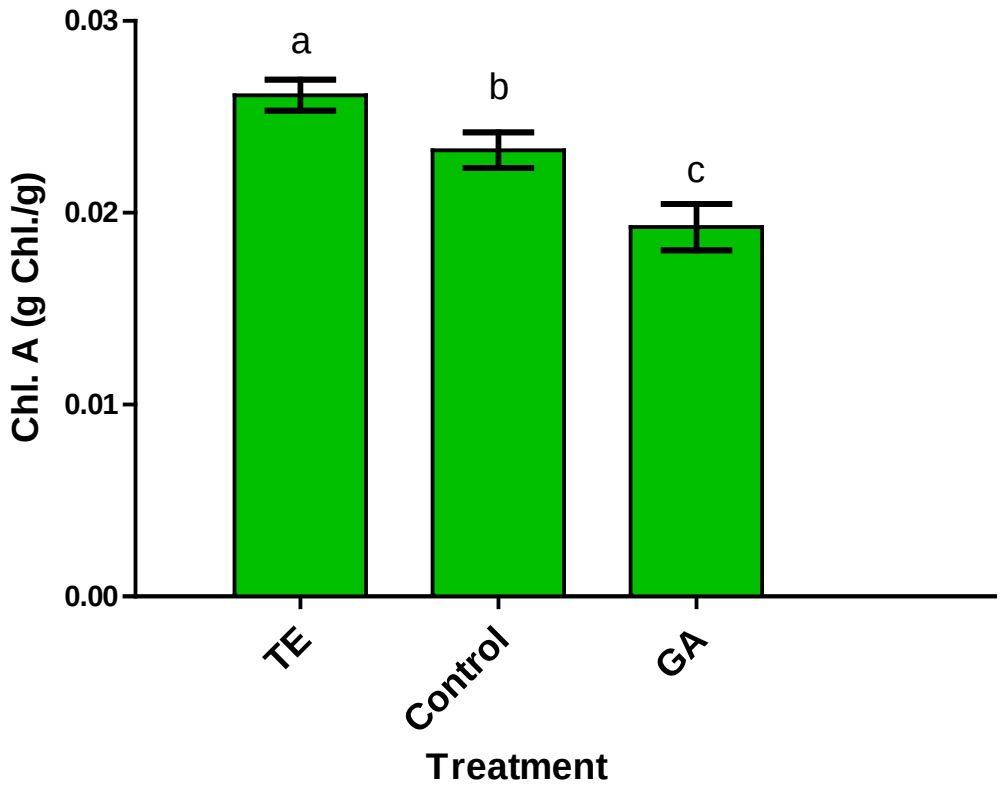

Figure 3. Chlorophyll A content (g Chl./g dried weight of plant material) of creeping bentgrass and annual bluegrass (pooled) after two applications (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=24.

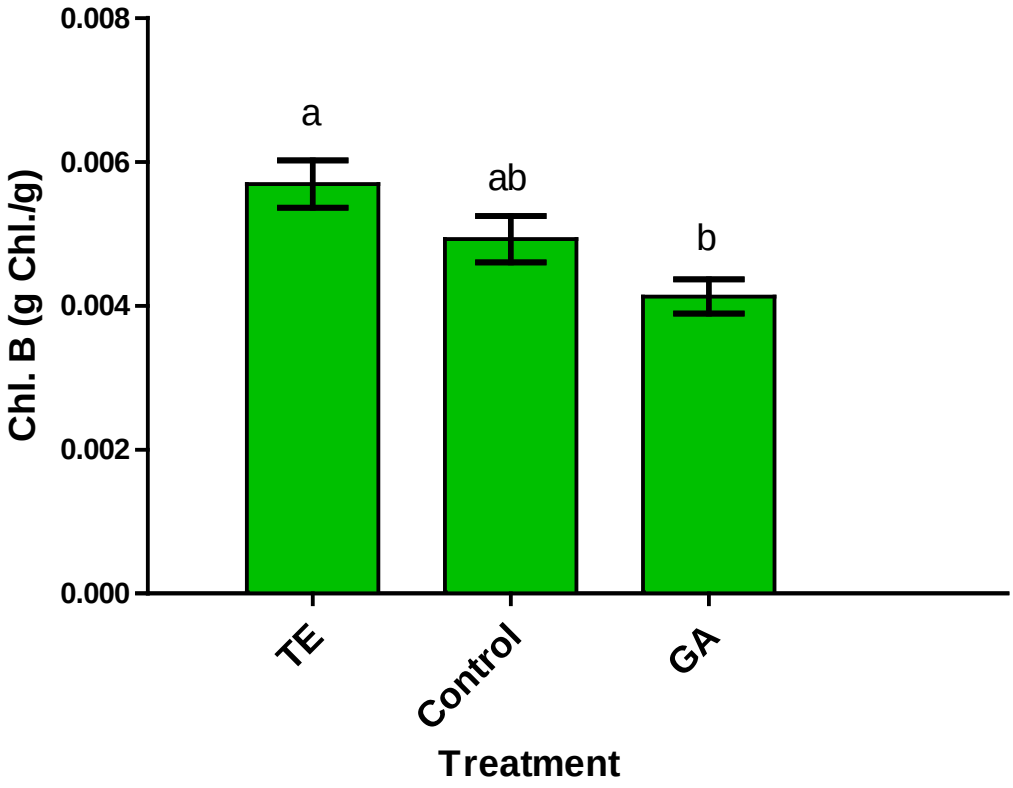

Figure 4. Chlorophyll B content (g Chl./g dried weight of plant material) of creeping bentgrass and annual bluegrass (pooled) after two applications (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=24.

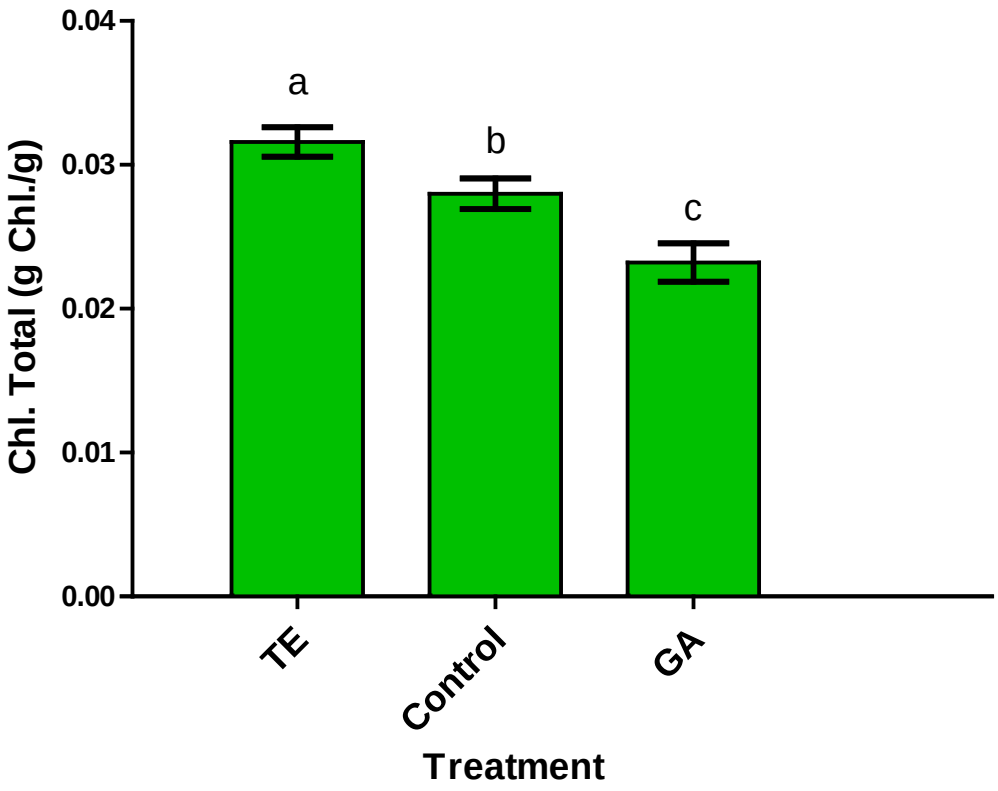

Figure 5. Total chlorophyll content (g Chl./g dried weight of plant material) of creeping bentgrass and annual bluegrass (pooled) after two applications (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=24.

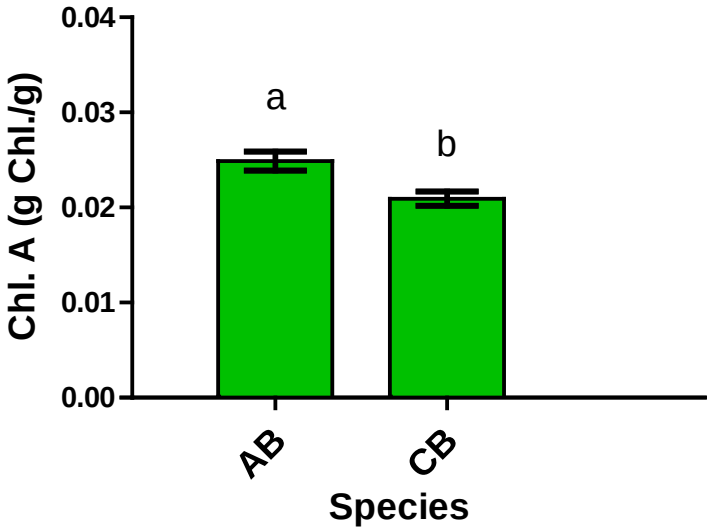

Figure 6. Chlorophyll A content (g Chl./g dried weight of plant material) for annual bluegrass (AB) and creeping bentgrass (CB) with treatments and applications combined in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=36.

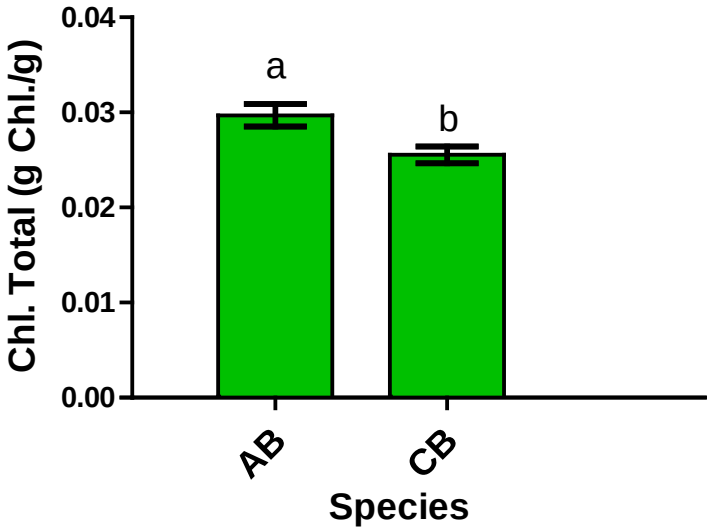

Figure 7. Total chlorophyll content (g Chl./g dried weight of plant material) for annual bluegrass (AB) and creeping bentgrass (CB) with treatments and applications combined in replicate three. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error of chlorophyll content, n=36.

PHOTOSYNTHESIS

A summary of the results for the ANOVA for photosynthesis rates can be found on Table 4. After the first application of the treatments, there was a significant treatment effect, after the second application of treatments, species and treatment were significant. With the first and second applications combined there was a species, treatment and application effect and no significant interactions were found. The first application of TE increased photosynthesis rates when compared to GA_3 with species combined (Figure 8). The second application of TE increased photosynthesis rates when compared to GA_3 with species combined (Figure 9). Trinexapac-ethyl effects were not significant when compared to the control; however GA_3 effects were significant when compared to the control (Figure 9). When the first and second application was combined over species, TE increased photosynthesis rates when compared to GA_3 treatment (Figure 10). Trinexapac-ethyl treatment was not significant when compared to the control; however GA_3 treatment was significant (Figure 10).

Photosynthesis rates were significantly higher for AB after the second application with all the treatments combined (Figure 11). Photosynthesis rates combined for after the first and second application of treatments were significantly higher for AB than CB (Figure 12).

For the application effect with all the treatments and species combined the first application produced a significantly higher photosynthesis rate than the second (Figure 13).

Table 4. Summary of analysis of variance (ANOVA) for photosynthesis rates following each treatment application timings, and both treatment application timings (pooled) for the study assessing the effect of trinexapac-ethyl, water and GA_3 (treatment) on annual bluegrass and creeping bentgrass (species) under greenhouse conditions

	1st Application Pr>F	**2nd Application Pr>F**	**1st and 2nd Application combined Pr>F**
species	0.7603	0.0238	0.0461
treatment	0.0055	<.0001	<.0001
Application	N/A	N/A	0.0013
species*treatment	0.7979	0.4101	0.3441
species*treatment*Application	N/A	N/A	0.7991
Application*treatment	N/A	N/A	0.0742
species*Application	N/A	N/A	0.1044

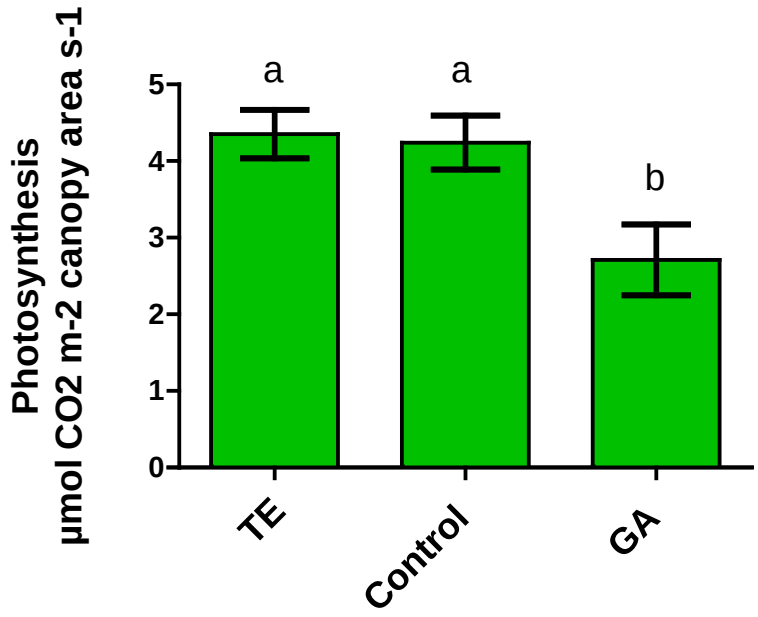

Figure 8. Photosynthesis rates of annual bluegrass and creeping bentgrass (pooled) for the treatment Trinexapac-ethyl (TE), control (water) and GA (GA_3) effect after the 1st application of treatments. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=24.

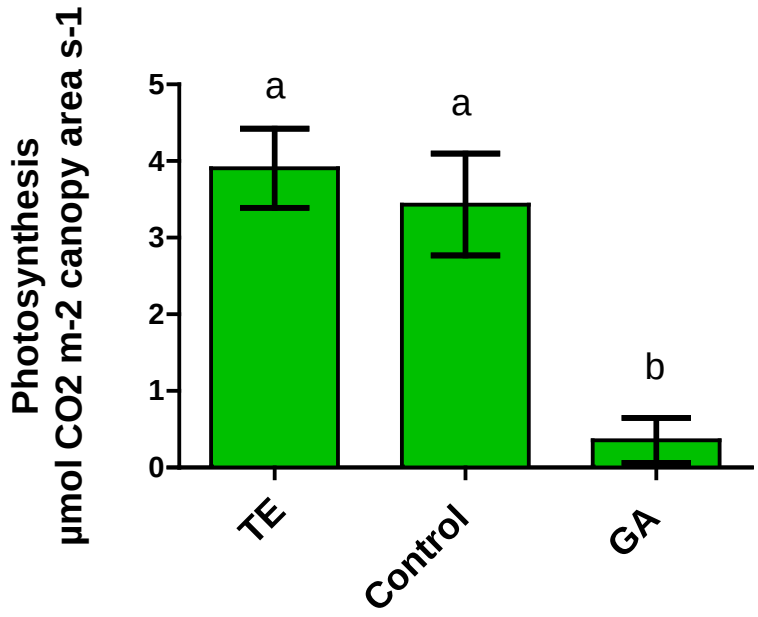

Figure 9. Photosynthesis rates of annual bluegrass and creeping bentgrass (pooled) for the treatment Trinexapac-ethyl (TE), control (water) and GA (GA_3) effect after the 2^{nd} application of treatments. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=24.

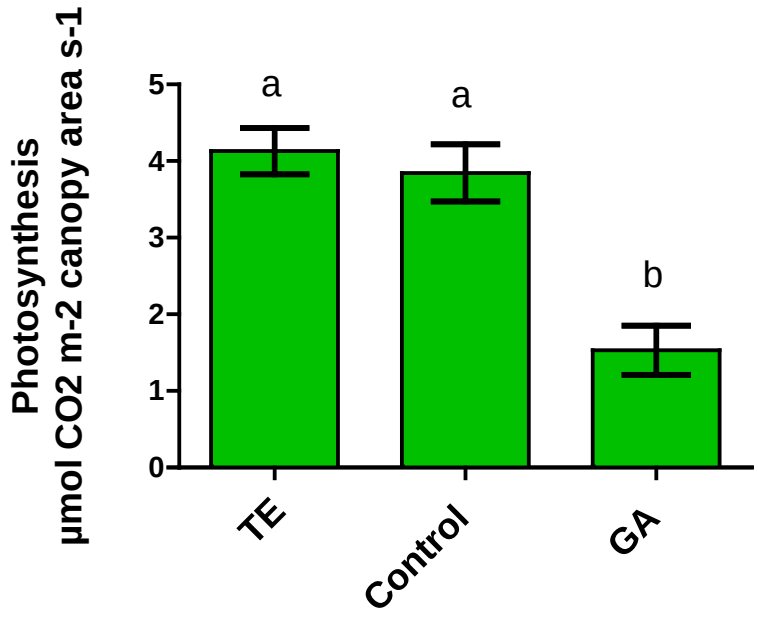

Figure 10. Photosynthesis rates of annual bluegrass and creeping bentgrass (pooled) for the treatment Trinexapac-ethyl (TE), control (water) and GA (GA_3) effect after the 1^{st} and 2^{nd} application of treatments. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=48.

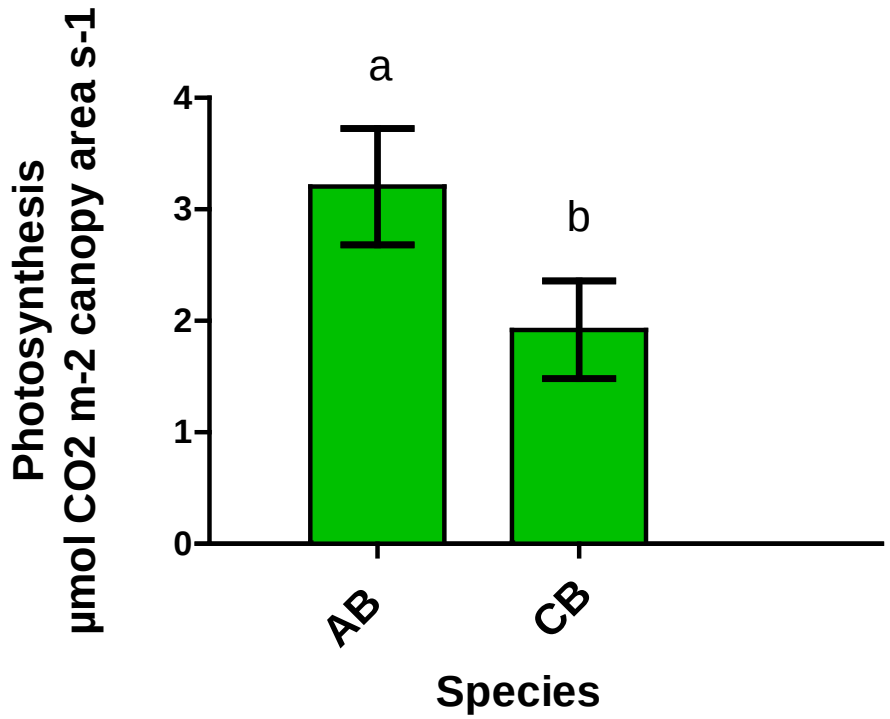

Figure 11. Photosynthesis rates of annual bluegrass (AB) and creeping bentgrass (CB) for the species effect with all treatments; Trinexapac-ethyl (TE), control (water) and GA (GA_3) (pooled) after 2nd application of treatments. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=36.

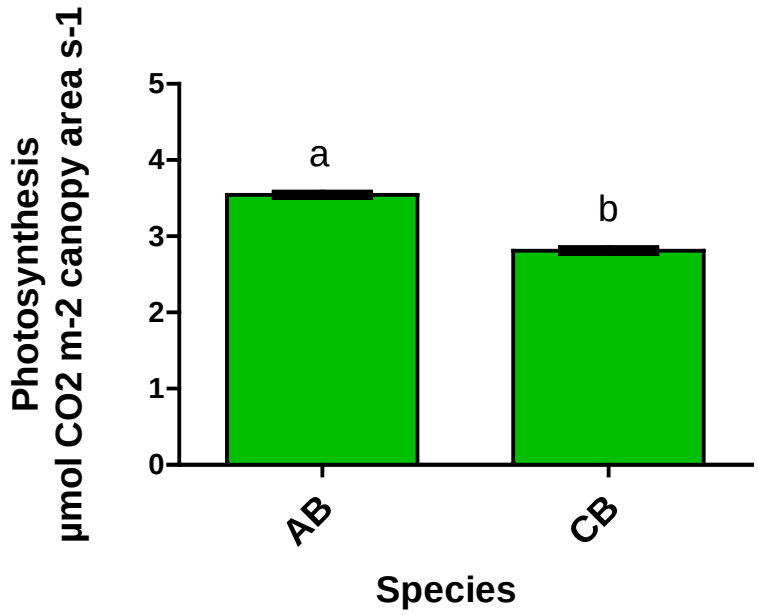

Figure 12. Photosynthesis rates of annual bluegrass (AB) and creeping bentgrass (CB) for the species effect with all treatments; Trinexapac-ethyl (TE), control (water) and GA (GA_3) (pooled) after the 1st and 2nd application of treatments (pooled). Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=72.

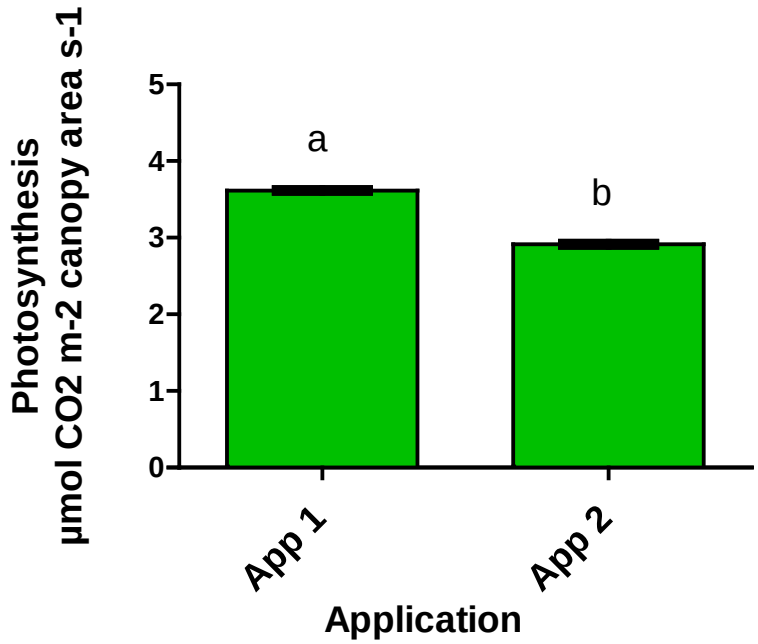

Figure 13. Photosynthesis rates of creeping bentgrass and annual bluegrass (pooled) treated with Trinexapac-ethyl (TE), water (control) and GA_3 (GA) once (app 1) and twice (app 2). Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=72.

3.2 ASSESSING FREEZING TOLERANCE FOLLOWING COLD ACCLIMATION IN A CONTROLLED ENVIRONMENT

PHOTOSYNTHESIS

Actual photosynthesis was significantly affected by species, treatment and temperature (Table 5). There was an interaction for species by treatment and species by temperature (Table 5). The species by treatment interaction resulted from TE and control treatments showing significantly higher photosynthesis rates than the GA_3 treatment in AB, but not in CB, where there was no difference between any of the treatments (Figure 14). There were no significant differences for any of the CB treatments (Figure 14). For the species by temperature interaction, photosynthesis rates were higher in AB at 20°C and 10°C than CB but there was no difference between the two species at 2°C and -2°C (Figure 15).

CARBOHYDRATE STATUS

The main effect of species with all treatments and acclimation time points combined for HMW fructan concentration was significant (Table 6). Annual bluegrass had a significantly higher concentration of HMW fructan 2.8g/100g DW of plant material when compared to CB 2g/100g DW of plant material (Figure 16).

LT_{50} VALUES

For the determination of LT_{50} values, only time 1 of the experimental data was analyzed, as in time 2 all the turf died. There were no significant effects or interactions for the LT_{50} values (Table 7).

Table 5. Summary of analysis of variance (ANOVA) of actual photosynthesis rates at different acclimation temperatures (20°C, 10°C, 2°C and -2°C) (temperature) for a study assessing the effect of trinexapac-ethyl, water and GA_3 (treatment) on annual bluegrass and creeping bentgrass (species) during cold acclimation in a controlled environment.

Source	Actual Photosynthesis Pr>F
Species	<.0001
Treatment	0.0003
Temperature	<.0001
Species*Treatment	0.0312
Species*Temperature	0.0183
Species*Treatment*Temperature	0.8767
Treatment*Temperature	0.7086

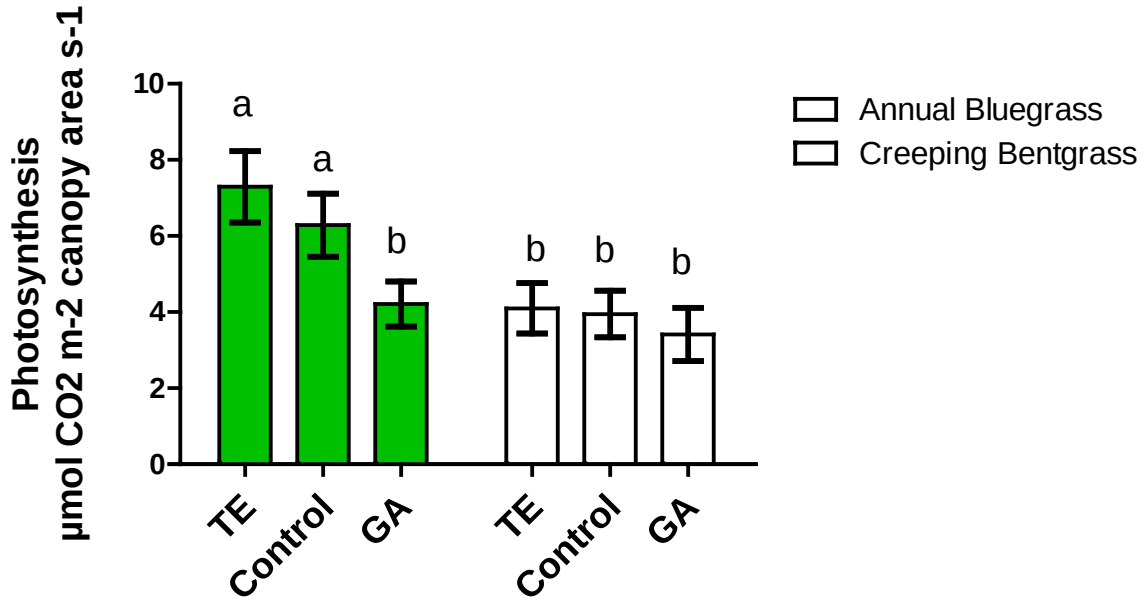

Figure 14. Actual photosynthesis rates of treatments Trinexapac-ethyl (TE), control (water) and GA_3 (GA) for the species by treatment interaction with all acclimation temperatures combined in the assessing freezing tolerance following cold acclimation in a controlled environment. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=16.

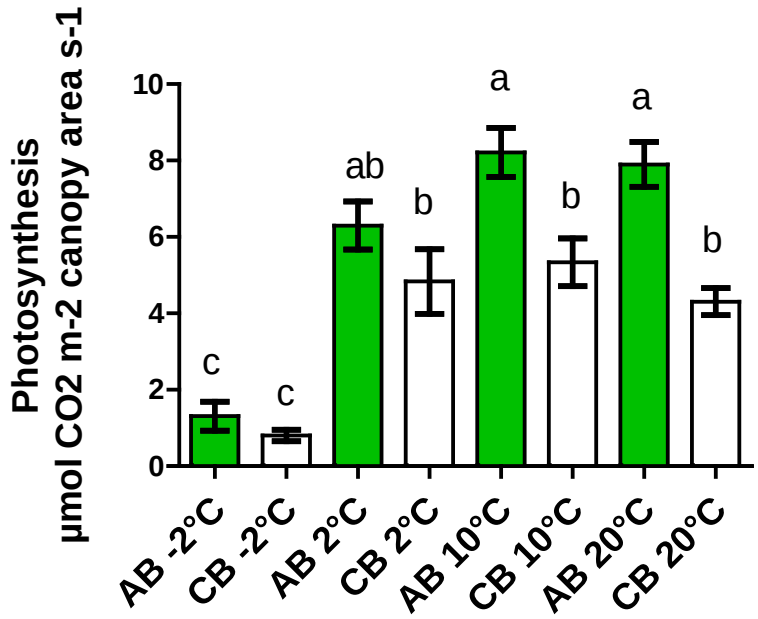

Figure 15. Actual photosynthesis rates of creeping bentgrass (CB) and annual bluegrass (AB) for the species by temperature interaction with all treatments combined in the assessing freezing tolerance following cold acclimation in a controlled environment. Bars not connected by same letter are significantly different, each data point represents the mean of the mean ± standard error, n=12.

Table 6. Summary of analysis of variance (ANOVA) of high molecular weight (HMW) and low molecular weight (LMW) fructans concentration of annual bluegrass and creeping bentgrass (species) before and after acclimation (acclimation) following the application of the Trinexapac-ethyl, water and GA_3 (treatment) for the assessing freezing tolerance following cold acclimation in a controlled environment study.

Source	HMW Fructan Pr>F	LMW Fructan Pr>F
Species	0.0015	0.4616
Treatment	0.4864	0.9585
Species *Treatment	0.6508	0.9372
Acclimation	0.336	0.3035
Treatment *Acclimation	0.8629	0.8595
Acclimation *Species	0.3274	0.69
Acclimation *Species *Treatment	0.1071	0.9643

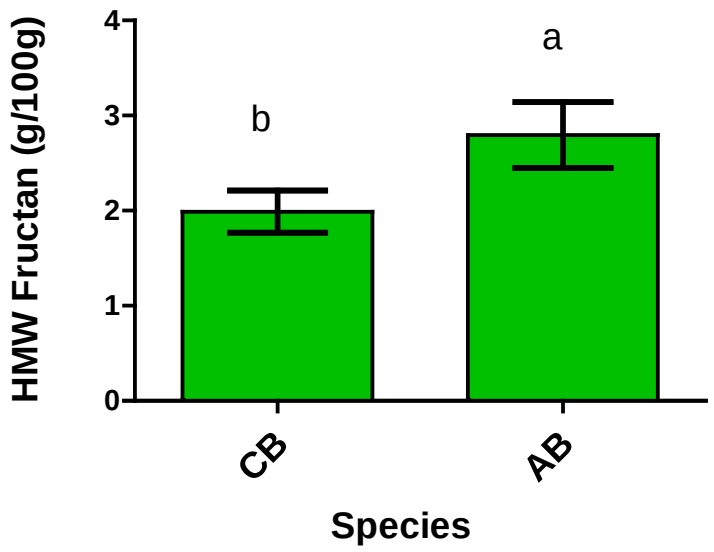

Figure 16. High molecular weight fructan (g/100g dried weight of plant material) (HMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and acclimation time points (pooled) for the species effect of annual bluegrass (AB) and creeping bentgrass (CB). For a controlled environment study conducted at the University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=48.

Table 7. Summary of analysis of variance (ANOVA) for LT_{50} (temperature that results in 50% loss of population) values from time 1 of annual bluegrass (AB), creeping bentgrass (CB) and treatments Trinexapac-ethyl, water and GA_3 (treatment) for the experiment assessing freezing tolerance following cold acclimation in a controlled environment.

Source	Pr>F
Species	0.3159
Treatment	0.9015
Species *Treatment	0.7543

Table 8. Summary of LT_{50} (temperature that results in 50% death of population) values for time 1 of annual bluegrass (AB) and creeping bentgrass (CB) for the experiment assessing freezing tolerance following cold acclimation in a controlled environment. The treatments were Trinexapac-ethyl (TE), GA_3 and control (water). Each data point represents the mean of the mean, n=8.

Treatment	Species	Mean LT_{50}
GA_3	AB	-13.39°C
TE	AB	-13.19°C
Control	AB	-13.08°C
GA_3	CB	-16.24°C
TE	CB	-16.32°C
Control	CB	-14.93°C

3.3 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS IN THE FIELD OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS

There was a species and date effect for HMW fructan, but no significant treatment effect or interactions (Table 9). With both dates and all treatments combined, CB had a HMW fructan concentration of 2g/100g of DW plant material while AB had a significantly higher concentration of 2.7g/100g DW plant material (Figure 17). There was a date effect for both HMW and LMW fructans (Table 9); on December 12th 2012, with all species and treatments combined, the concentration of HMW fructan was 7g/100g DW of plant material (Figure 18). On May 3rd 2013, the concentration of HMW fructan decreased significantly to 3g/100g DW of plant material (Figure 18). Low molecular weight fructan concentration on December 12th 2012 with all treatments and species combined was 2.5g/100g DW of plant material but the concentration significantly decreased to 1.2g/100g DW of plant material on May 3rd 2013 (Figure 19).

Table 9. Summary of analysis of variance (ANOVA) of high molecular weight (HMW) and low molecular weight (LMW) fructans concentration of annual bluegrass and creeping bentgrass (species) before and after acclimation (date) following the application of the Trinexapac-ethyl, water and GA_3 (treatment) in the Guelph Turfgrass Institute (GTI) field study.

Source	HMW Fructan Pr>F	LMW Fructan Pr>F
Species	0.0079	0.2608
Treatment	0.9926	0.9269
Date	<.0001	0.0003
Species *Treatment	0.1203	0.6953
Species *Treatment *Date	0.9842	0.619
Date *Species	0.0855	0.9838
Date *Treatment	0.3364	0.936

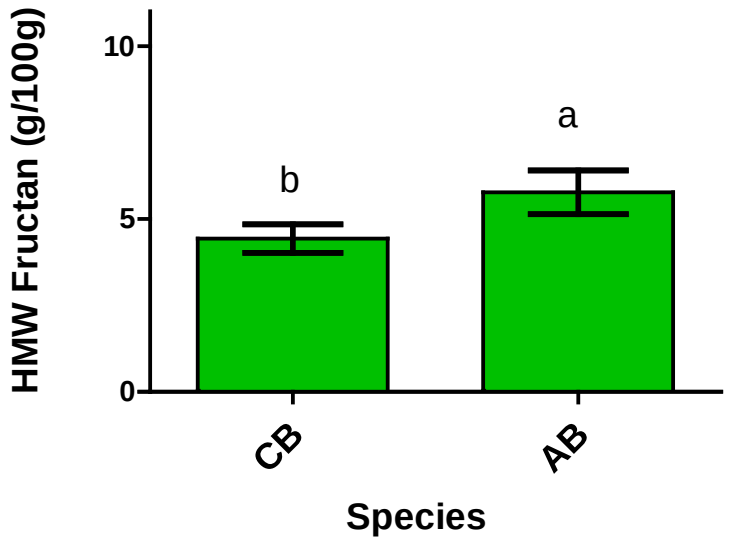

Figure 17. High molecular weight fructan (g/100g dried weight of plant material) (HMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and acclimation time points (pooled) for the species effect of annual bluegrass (AB) and creeping bentgrass (CB), for a field study conducted at the Guelph Turfgrass Institute (GTI), University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=48.

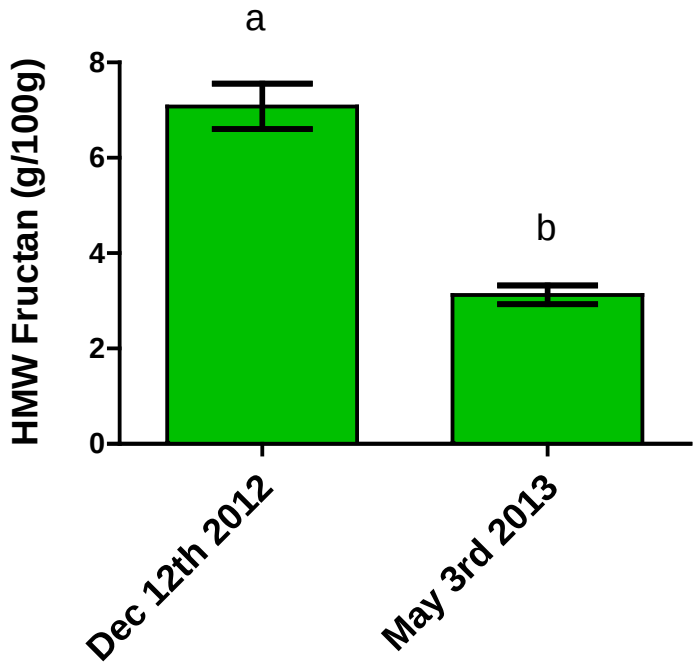

Figure 18. High molecular weight fructan (g/100g dried weight of plant material) (HMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and species annual bluegrass (AB) and creeping bentgrass (CB) (pooled) for the date effect, for a field study conducted at the Guelph Turfgrass Institute (GTI), University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=48.

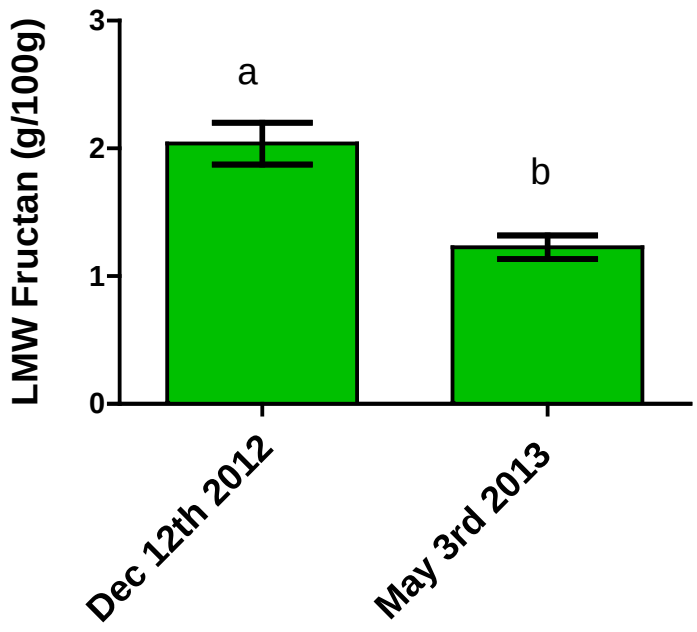

Figure 19. Low molecular weight fructan (g/100g dried weight of plant material) (LMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and species annual bluegrass (AB) and creeping bentgrass (CB) (pooled) for the date effect, for a field study conducted at the Guelph Turfgrass Institute (GTI), University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean ± standard error, n=48.

3.4 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS IN THE FIELD OF SEVEN CEREALS WITH VARYING WINTER HARDINESS

Due to the number of samples and time constraints, concentrations for both HMW and LMW fructans were determined for the first sampling date December 17th 2012 and the last sampling date April 12th 2013. Date was the only significant factor for both HMW and LMW fructans (Table 10). Fructan concentration was significantly higher on December 17th 2012 when compared to April 12th 2013. High molecular weight fructan concentration was 0.9g/100g DW of plant material on December 17th 2012, but by April 12th 2013 the concentration had decreased significantly to 0.25g/100g DW of plant material (Figure 20). Low molecular weight fructan concentration was 0.42g/100g DW of plant material on December 17th 2012, and by April 12th 2013 the concentration had decreased to 0.19g/100g DW of plant material (Figure 21).

Table 10. Summary of analysis of variance (ANOVA) of high molecular weight (HMW) and low molecular weight (LMW) fructans concentration of seven different cereal crops (species) before and after acclimation (date) following the application of the Trinexapac-ethyl, water and GA_3 (treatment) in the Arkell research station, University of Guelph field study.

Source	HMW Fructan Pr>F	LMW Fructan Pr>F
Species	0.6616	0.6559
Date	0.0065	0.0007
Treatment	0.784	0.9353
Date *Species	0.6048	0.6059
Date *Treatment	0.9887	0.2545
Date *Treatment *Species	0.7566	0.7737
Treatment *Species	0.8337	0.5272

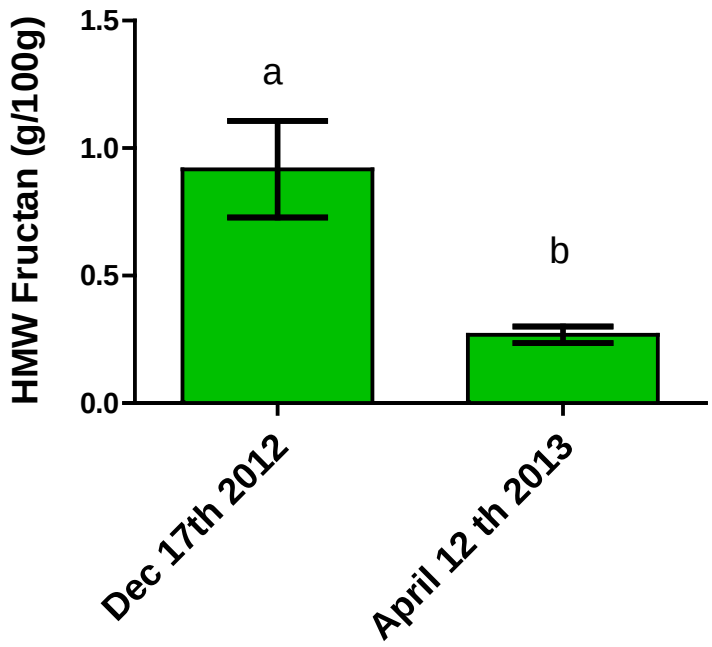

Figure 20. High molecular weight fructan (g/100g dried weight of plant material) (HMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and species of cereal crop (pooled) for the date effect. The study was conducted at the Arkell Research Station, University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean of fructan concentration, for the December 17th 2012 date n=21, for the April 12th 2013 date there was 1 missing value, so n=20.

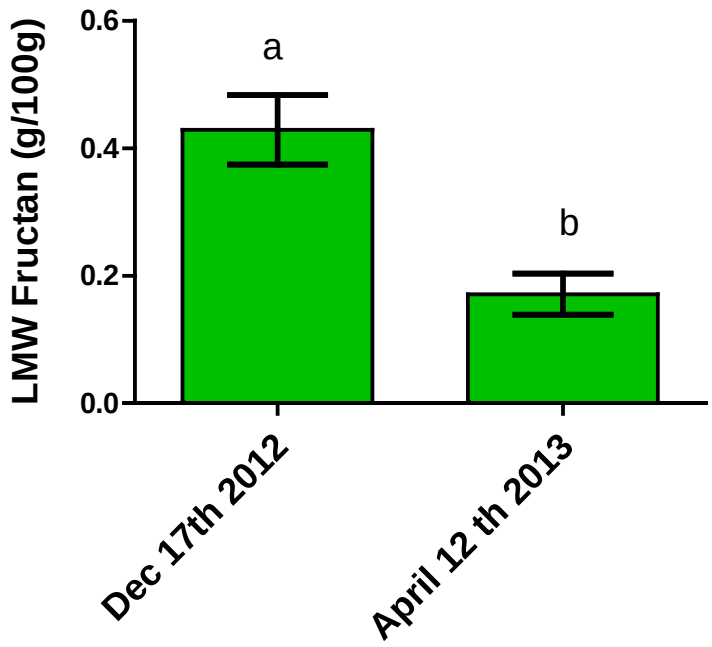

Figure 21. Low molecular weight fructan (g/100g dried weight of plant material) (LMW) concentrations with all treatment, Trinexapac-ethyl (TE), control (water) and GA (GA_3) and species of cereal crop (pooled) for the date effect. The study was conducted at the Arkell Research Station, University of Guelph. Bars not connected by same letter are significantly different, each data point represents the mean of fructan concentration, for the December 17th 2012 date n=21, for the April 12th 2013 date there was 1 missing value, so n=20.

4.0 DISCUSSION

4.1 ASSESSING THE EFFECT OF TRINEXAPAC-ETHYL AND GA_3 ON ANNUAL BLUEGRASS AND CREEPING BENTGRASS UNDER GREENHOUSE CONDITIONS

The objective of the study was to determine if there are different physiological responses (specifically photosynthesis and chlorophyll content) in an unstressed environment between AB and CB. It was predicted that TE, a GA inhibitor, would increase chlorophyll content based on previous research (Ervin and Koski 2001; Arghavani et al. 2012). Our study was in agreement with the previous findings; two applications of TE at 3.8ml/100m^2 increased chlorophyll content (Figures 3 and 5).

There have been a number of studies that have shown, or suggested, that TE can improve or increase photosynthesis of turfgrass species (Ervin and Koski 2001; Ervin and Zhang 2007; McCann and Huang 2008). Our study is the first to our knowledge to have used GA_3 as a treatment to give a gibberellic acid treatment or range effect. Although there was no significant effect on photosynthesis when comparing the TE treatment to the control, there was significance when comparing the TE to the GA_3 and when comparing the control to the GA_3 application. Trinexapac-ethyl significantly increased photosynthesis when compared to GA_3 following one application and two applications, and when data from the first and second applications were combined (Figures 8, 9 and 10).

This study highlighted some of the physiological differences between the two turfgrass species AB and CB. After the second application of all treatments, AB had a significantly higher

rate of photosynthesis than CB (Figure 11). This effect on photosynthesis rates highlights the complexity of the interspecies competition between the two species.

4.2 ASSESSING FREEZING TOLERANCE FOLLOWING COLD ACCLIMATION IN A CONTROLLED ENVIRONMENT

The results in the stressed environment were different from the unstressed; application of TE yielded higher photosynthesis rates for AB as compared to the GA_3 treatment and compared with all of the CB treatments, and the GA_3 treatment on AB, with all the acclimation temperatures combined (Figure 14).

Photosynthesis rates were significantly higher for AB than CB at 20°C and 10°C during the cold acclimation procedure and this is the first time, to our knowledge, that AB has shown increased photosynthesis rates at low temperatures when compared to CB (Figure 15). The results for the carbohydrate status tie together with the photosynthesis results. The only effect for carbohydrate status was species; AB had a greater concentration (by 0.8g/100g DW plant material) of HMW fructan than CB (Figure 16). Because photosynthesis rates were higher for AB throughout the experiment, AB had a higher concentration of HMW fructan. Hoffman et al. (2014) found CB had a greater concentration of HMW fructan compared to both ecotypes of AB; however, this study showed AB to have a greater concentration of HMW fructan than CB. It is possible that AB grows better in the growth chamber conditions than CB.

For the determination of LT_{50} values, all of the plant material in time 2 of the experiment died following the freezer rotations; therefore, there was not enough replication for statistical analysis of the whole experiment. One of the time points of the experiment takes

8 months to complete from establishment of the plant material to determining LT_{50} values and for this reason, another time point was not completed. The LT_{50} values generated from two replications of the experiment for CB treatments were -16.24°C for GA_3, -14.93°C for the control and -16.32°C for TE. For AB, the LT_{50} values were -13.39°C for GA_3 treatment, -13.08°C for the control and -13.19°C for TE. Although there was no significance based on the two replications of the study, another two replications of the study could give a greater statistical power to the experiment and LT_{50} values. It is not clear why all the plant material died in the second replication in time. The growth chamber conditions were consistent at time one and two, and both species died, so it impacted both species similarly. The acclimation protocol was based on a number of studies and personal communication with Dr. Michelle DaCosta who is an Associate Professor at the University of Massachusetts Amherst (Gay and Eagles 1991; Hoffman et al. 2010; Espevig et al. 2011).

Hoffman et al. (2013) published a study assessing freezing tolerance following a period of cold deacclimation of CB and AB. The study concluded CB had a significantly lower LT_{50} value of -21.2°C when compared to the AB LT_{50} value of -17.7°C. Hoffman et al. (2014) also published another study assessing freezing tolerance and carbohydrate status of CB and two ecotypes of AB - AB-T, which is freeze tolerant and AB-S, which is sensitive to freezing during deacclimation. The study showed greater freezing tolerance of CB over both AB ecotypes during the deacclimation period and the order of freezing tolerance, as represented by LT_{50} values, was AB-S<AB-T<CB at each stage of deacclimation. The study also found HMW fructan depleted during the deacclimation period and it was noted that as the HMW fructan was depleting the LT_{50} values were decreasing (Hoffman et al 2014). My studies are congruent with Hoffman et al

results of CB having greater freezing tolerance than AB, although results of this study were not significant likely due to statistical power being lost because of the dead plant material.

The objective of this study was to determine if GA inhibitors and/or exogenous GA relate with carbohydrate status and/or LT_{50}, and to determine if there are different physiological responses at different acclimation temperatures between AB and CB. The controlled environment study could not completely answer the questions we had; however, physiological differences between the two species during acclimation and carbohydrate status gives us an insight into what is occurring at low temperatures and during stressful periods.

4.3 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS IN THE FIELD OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS

The species effect was similar to the results in the controlled environment; AB had a 0.7g/100g DW of plant material (Figure 17) greater concentration of HMW fructan than CB. In the controlled environment the difference between AB and CB was 0.8g/100g DW of plant material. The results of the controlled experiment and field experiment were consistent; however, there is a different species effect from the Hoffman et al. (2014) study. Both HMW and LMW fructan decreased over the winter; HMW fructan decreased by 4g/100g DW of plant material (Figure 18) and LMW fructan decreased by 1.3g/100g DW of plant material (Figure 19) during the winter months. Dionne et al. (2001) also found HMW fructan to be the dominant carbohydrate and in the highest concentrations in acclimated AB.

The objective of the study was to assess the physiological effects of TE and GA_3 on CB and AB during cold acclimation in the field, and to determine if hormone manipulation relates

to changes in carbohydrate status in the spring. Carbohydrate status in the spring was not affected by hormone manipulation in the fall. As mentioned above, the study did identify physiological differences between the species.

4.4 ASSESSING THE EFFECTS OF HORMONE MANIPULATION ON CARBOHYDRATE STATUS IN THE FIELD OF SEVEN CEREALS WITH VARYING WINTER HARDINESS

The objective of the study was to assess the physiological effects of TE and GA_3 on cereal crops in the field over the winter months and to determine if hormone manipulation in the fall would relate to changes in carbohydrate status in the fall following treatments and in the spring.

The main effect in the cereal crop field experiment was date for both HMW and LMW fructans. High molecular weight fructan decreased during the winter months by 0.65g/100g DW of plant material (Figure 20). Low molecular weight fructan decreased during the winter months by 0.23g/100g DW of plant material (Figure 21). Olien and Clark (1993) published a study looking at the carbohydrate depletion during winter months of field-grown and growth chamber grown-cereal crops. In general, the results were similar to this study in that the concentration of fructan decreased in concentration from December to March/April (Olien and Clark 1993).

5.0 SUMMARY

Trinexapac-ethyl, is a GA inhibitor regularly used in the turfgrass industry to reduce mowing and improve playing quality of golf course greens. Cold acclimation in the fall and cold deacclimation in the spring are stressful periods for plant growth and development. Gibberellic acid has been shown to play a critical role in stress tolerance of plants (Vettakkorumakankav et al. 1999), and GA inhibitors have shown potential to improve stress tolerance (Kraus et al. 1995; Sarkar et al. 2004).

This research was set up to address the following objectives; to determine if there are different physiological responses in an unstressed environment between AB and CB, to determine if GA inhibitors and/or exogenous GA relate to carbohydrate status and LT_{50}, to determine if there are different physiological responses at different acclimation temperatures between AB and CB, to determine if turfgrass species can be used as a model system for similar physiological responses in cereal crops, and to determine if GA inhibitors and/or exogenous GA relate to carbohydrate status in different cereal species with different genetic tolerance to cold tolerance.

Four experiments were set up to test these objectives: The first experiment was conducted in the research greenhouse at the Bovey Building, Department of Plant Agriculture, University of Guelph to simulate a unstressed environment. The second experiment was conducted in the controlled environment facilities in the Bovey building, Department of Plant Agriculture, University of Guelph. This experiment was conducted in a controlled environment to simulate cold acclimation. The third experiment was a field experiment conducted at the

Guelph Turfgrass Institute (GTI), University of Guelph, from September 2012 - May 2013. Each experiment compared AB and CB, two species commonly found on golf greens, for physiological responses in relation to improving stress tolerance. The fourth experiment was a field experiment conducted at the Arkell Research Station, University of Guelph, from September 2012 - May 2013. Seven cereal crops known to have different genetic tolerance levels for cold tolerance were compared for physiological changes in relation to stress tolerance. In each of the experiments treatments were made to manipulate hormone levels, the treatments were GA_3, control (water) and TE. These treatments were similar to the turfgrass experiments.

In general, the hormone manipulation did not affect the carbohydrate status of the turfgrass species or the cereal species, in either the fall or the spring. There was a treatment effect for the turfgrass species in both the unstressed and stressed environment; TE increased the photosynthesis rates when compared with the GA_3 treatment. In general, AB had a greater concentration of HMW and LMW fructan in the controlled experiment, and a higher concentration of HMW fructan in the field experiment. There was a date effect for the controlled experiment and both of the field experiments with the highest concentration of carbohydrates at the end of acclimation or start of winter, and the lowest concentration at the end of deacclimation or the end of winter.

The experiments set up to address the objectives of this thesis were inconclusive in terms of finding a relationship with freezing tolerance and hormone manipulation in the fall. One of the most interesting findings of the thesis is how manipulating GA can impact the relationship between the two turfgrass species AB and CB, by improving stress tolerance of AB.

These findings could potentially change the way turfgrass managers manage a mixed sward putting green containing both AB and CB.

Understanding the effects of using GA inhibitors in the fall and how this impacts stress tolerance and winter survival is important for turfgrass growers here in Ontario and other areas of the world that experience sub-freezing temperatures during winter. Once the causation of the physiological changes that occur when GA inhibitors are applied to turfgrass are fully understood, there is the potential to more easily manipulate and improved winter acclimation and survival. Carbohydrate concentration in the fall, and in the spring, is critical for winter survival and specifically in the grasses, the most important carbohydrate is fructan (D. P. Livingston, Hincha and Heyer 2009) and it is present in two forms: high and low molecular weight. For the experiment that involved an acclimation period (section 3.2), HMW fructan concentrations were higher after the acclimation period and for the field experiments (sections 3.3 and 3.4); the concentration of fructan significantly decreased by the spring. Photosynthesis rates in the greenhouse increased with the application of TE; it is known that an increase in photosynthesis and reduction in growth results in increased concentration of carbohydrates (Qian and Engelke 1999; Steinke and Stier 2004; Arghavani et al. 2012). Although my studies did not result in a treatment effect for carbohydrate status, this area needs further work to identify the correct number of applications, TE application rates and time period required for a treatment effect during the cold acclimation of cool season turfgrass species.

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APPENDIX

DETERMINATION OF HIGH AND LOW MOLECULAR WEIGHT FRUCTAN

To determine the concentration of high and low molecular weight fructan before acclimation 7 conetainers were harvested from each experimental unit and 20 conetainers were harvested after acclimation.

1. Take 100mg of freeze dried sample, extract twice with 90% ETOH (5mL) at 70°C for 20 min then centrifuge at 4000 rpm for 5 minutes, decant and collect the supernatant (low molecular weight fraction).

2. Using the same pellet, extract twice with deionised water (dH_2O) (5mL) at 80°C for 20 min then centrifuge at 4000 rpm for 5 minutes, decant and collect the supernatant (high molecular weight fraction).

3. High molecular fraction or dH_2O fraction adjust to 50mL with dH_2O.

4. Low molecular fraction or ETOH fraction is evaporated to dryness, then solubilised with 50mL of dH_2O.

5. In a glass test tube (16 x 100 mm) add 200µL of the extract and 200µL of enzyme A and incubate at 40°C (water bath) for 30min.

6. Following incubation add 200µL of reagent 3 (alkaline borohydride solution) vortex, then incubate at 40°C (water bath) for 30min. This step is to reduce all the reducing sugars to sugar alcohols.

7. Add 500µL of reagent 4 (200 mM acetic acid), vortex. A vigorous effervescence (bubbling) should be observed (this step removes excess borohydride and adjusts the pH to approx 4.5). The resulting solution is known as solution S.

8. Solution S - Remove a 200µL aliquot (3 times) and place into the bottom of 3 glass test tubes (16 x 100mm).

9. Add 100µL of enzyme B (fructose solution) to two of the tubes (smaples). Add 100µL of 0.1M sodium acetate to the third (sample blank).

10. Incubate the tubes at 40°C for 60min to effect complete hydrolysis of fructan to D-fructose and D-glucose. Seal the tubes with Parafilm during incubation.

11. Add 5mL of PAHBAH working reagent to all samples and controls (see controls and precautions 2a, 2b and 3), then incubate in 100°C water bath for 6min.

12. Remove from water bath and immediately place in a ice/cold water bath (18-20°C).

13. Measure the absorbance of all solutions at 410nm against the reagent blank. Measure the absorbance values as soon as possible after cooling the tubes. The PAHBAH color complex will fade with time.

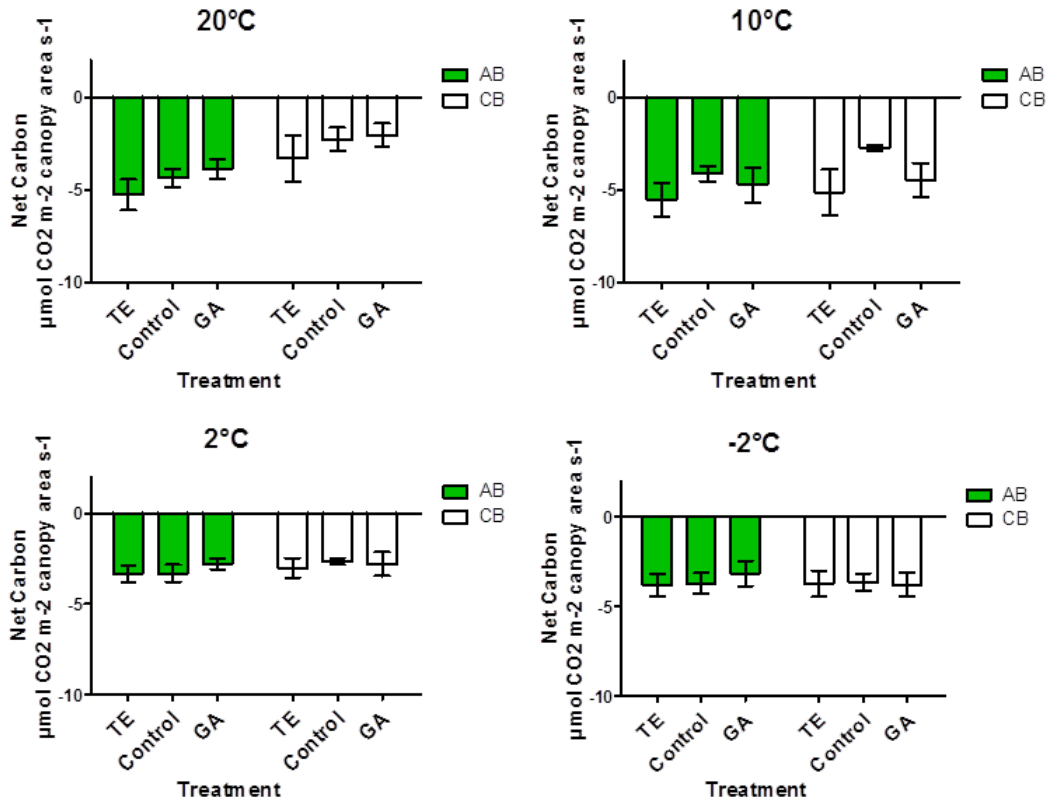

Figure 1. Net carbon assimilation in the dark of Annual bluegrass (AB) and Creeping bentgrass (CB) (Species) as affected by Trinexapac-ethyl (TE), control (water) and GA_3 (GA) (Treatment) during cold acclimation temperatures (20°C, 10°C, 2°C and -2°C). Each data point represents the mean of the mean ± standard error, n=4.

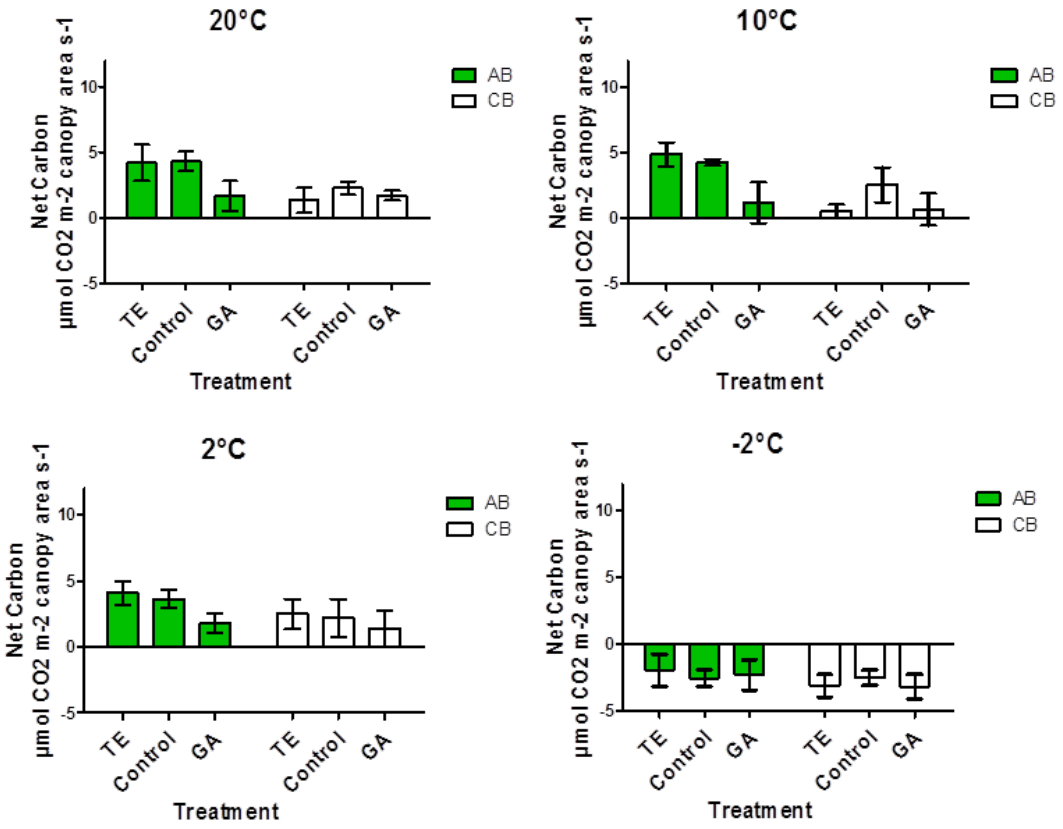

Figure 2. Net carbon assimilation in the light of Annual bluegrass (AB) and Creeping bentgrass (CB) (Species) as affected by Trinexapac-ethyl (TE), control (water) and GA_3 (GA) (Treatment) during cold acclimation temperatures (20°C, 10°C, 2°C and -2°C). Each data point represents the mean of the mean ± standard error, n=4.

Table 1. Analysis of variance (ANOVA) for photosynthesis rates following the first application of treatments; for the study assessing the effect of trinexapac-ethyl and GA_3 (Treatment) on annual bluegrass and creeping bentgrass (Species) under greenhouse conditions.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
species	1	0.34	0.09	0.7603
treatment	2	40.77	5.63	0.0055*
species*treatment	2	1.64	0.23	0.7979
Error	66	238.78		
Total	**71**	**281.11**		

Table 2. Analysis of variance (ANOVA) for photosynthesis rates following the second application, of the treatments (Application); for the study assessing the effect of trinexapac-ethyl and GA_3 (treatment) on annual bluegrass and creeping bentgrass (species) under greenhouse conditions.

Source	DF	Sum of Squares	F Ratio	Prob > F
species	1	31.13	5.36	0.0238*
treatment	2	179.79	15.47	<.0001*
species*treatment	2	10.50	0.90	0.4101
Error	65	377.64		
Total	**70**	**596.34**		

Table 3. Analysis of variance (ANOVA) for photosynthesis rates following the first and second applications (pooled) of the treatments (Application); for the study assessing the effect of trinexapac-ethyl and GA_3 (Treatment) on annual bluegrass and creeping bentgrass (Species) under greenhouse conditions.

Source	DF	Sum of Squares	F Ratio	Prob > F
species	1	19.09	4.06	0.0461*
treatment	2	195.84	20.81	<.0001*
Application	1	50.78	10.79	0.0013*
species*treatment	2	10.12	1.08	0.3441
species*treatment*Application	2	2.11	0.22	0.7991
Application*treatment	2	24.97	2.65	0.0742
species*Application	1	12.58	2.67	0.1044
Error	131	616.41		
Total	**142**	**930.26**		

Table 4. Analysis of variance (ANOVA) for photosynthesis rates before the first application and following the first application and the second application of the treatments (Application); for the study assessing the effect of trinexapac-ethyl and GA_3 (Treatment) on annual bluegrass and creeping bentgrass (Species) under greenhouse conditions.

Source	DF	Sum of Squares	F Ratio	Prob > F
species	1	9.84	2.23	0.137
treatment	2	146.54	16.60	<.0001*
Application	2	70.97	8.04	0.0004*
species*treatment	2	2.68	0.30	0.7382
species*treatment*Application	4	12.39	0.70	0.5914
Application*treatment	4	77.37	4.38	0.002*
species*Application	2	22.40	2.5386	0.0816
Error	197	869.28		
Total	**214**	**1210.74**		

Table 5. Analysis of variance (ANOVA) for total chlorophyll content of a study assessing the effect of trinexapac-ethyl and GA_3 (Treatment) on annual bluegrass and creeping bentgrass (Species) under greenhouse conditions with 2 application dates (Date).

Source	DF	Sum of Squares	F Ratio	Prob>F
Species	1	0.00	15.22	0.0003*
Treatment	2	0.00	21.82	<.0001*
Date	2	0.00	7.84	0.001*
Species*Treatment	2	0.00	0.27	0.7682
Species*Treatment *Date	4	0.00	0.97	0.4337
Date *Treatment	4	0.00	4.60	0.0028*
Error	55	0.00		
Total	**70**	**0.00**		

Table 6. Analysis of variance (ANOVA) for chlorophyll A content of a study assessing the effect of trinexapac-ethyl and GA_3 (Treatment) on annual bluegrass and creeping bentgrass (Species) under greenhouse conditions with 2 application dates (Date).

Source	DF	Sum of Squares	F Ratio	Prob>F
Species	1	0.00	21.13	<.0001*
Treatment	2	0.00	22.57	<.0001*
Date	2	0.00	11.58	<.0001*
Species*Treatment	2	0.00	0.30	0.7419
Species*Treatment *Date	4	0.00	1.13	0.3532
Date *Treatment	4	0.00	4.94	0.0018*
Error	55	0.00		
Total	**70**	**0.00**		

Table 7. Analysis of variance (ANOVA) for chlorophyll B content of a study assessing the effect of trinexapac-ethyl and GA_3 (treatment) on annual bluegrass and creeping bentgrass (species) under greenhouse conditions with 2 application dates (Date).

Source	DF	Sum of Squares	F Ratio	Prob>F
Species	1	0.00	0.38	0.5386
Treatment	2	0.00	6.60	0.0027*
Date	2	0.00	1.22	0.3019
Species*Treatment	2	0.00	0.13	0.8819
Species*Treatment *Date	4	0.00	0.20	0.9348
Date *Treatment	4	0.00	1.35	0.2616
Error	55	0.00		
Total	**70**	**0.00**		

Table 8. Analysis of variance (ANOVA) of actual photosynthesis rates at different acclimation temperatures (20°C, 10°C, 2°C and -2°C) (temperature) for a study assessing the effect of trinexapac-ethyl and GA_3 (treatment) on Annual bluegrass and Creeping bentgrass (species) during cold acclimation in a controlled environment.

Source	DF	Sum of Squares	F Ratio	Prob > F
Species	1	106.76	32.89	<.0001*
Treatment	2	59.63	9.19	0.0003*
Temperature	3	483.92	49.70	<.0001*
Species*Treatment	2	23.62	3.64	0.0312*
Species*Temperature	3	34.69	3.56	0.0183*
Species*Treatment*Temperature	6	7.79	0.40	0.8767
Treatment*Temperature	6	12.20	0.63	0.7086
Error	48	57.40		
Total	**95**	**962.29**		

Table 9. Analysis of variance (ANOVA) of high molecular weight fructan concentration of annual bluegrass and creeping bentgrass (Species) before and after acclimation (Acclimation) following the application of the Trinexapac-ethyl and GA_3 (Treatment) for the assessing freezing tolerance following cold acclimation in a controlled environment study.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Species	1	38.23	11.77	0.0015*
Treatment	2	4.78	0.74	0.4864
Species *Treatment	2	2.83	0.43	0.6508
Acclimation	1	3.09	0.95	0.336
Treatment *Acclimation	2	0.96	0.15	0.8629
Acclimation *Species	1	3.20	0.99	0.3274
Acclimation *Species *Treatment	2	15.46	2.38	0.1071
Error	36	117.00		
Total	**47**	**185.55**		

Table 10. Analysis of variance (ANOVA) of low molecular weight fructan concentration of annual bluegrass and creeping bentgrass (Species) before and after acclimation (Acclimation) following the application of the Trinexapac-ethyl and GA_3 (Treatment) for the assessing freezing tolerance following cold acclimation in a controlled environment study.

Source	DF	Sum of Squares	F Ratio	Prob > F
Species	1	0.37	0.55	0.4616
Treatment	2	0.06	0.04	0.9585
Species *Treatment	2	0.09	0.07	0.9372
Acclimation	1	0.73	1.09	0.3035
Treatment *Acclimation	2	0.20	0.15	0.8595
Acclimation *Species	1	0.11	0.16	0.69
Acclimation *Species *Treatment	2	0.05	0.04	0.9643
Error	36	24.12		
Total	**47**	**25.73**		

Table 11. Analysis of variance (ANOVA) of high molecular weight fructan concentration of annual bluegrass and creeping bentgrass (Species) before and after acclimation (Date) following the application of the Trinexapac-ethyl, water and GA_3 (Treatment) in the Guelph Turfgrass Institute (GTI) field study.

Source	DF	Sum of Squares	F Ratio	Prob > F
Species	1	21.52	7.90	0.0079*
Treatment	2	0.04	0.01	0.9926
Date	1	187.63	68.89	<.0001*
Species *Treatment	2	12.24	2.25	0.1203
Species *Treatment *Date	2	0.09	0.02	0.9842
Date *Species	1	8.52	3.13	0.0855
Date *Treatment	2	6.12	1.12	0.3364
Error	36	98.05		
Total	**47**	**334.20**		

Table 12. Analysis of variance (ANOVA) of low molecular weight fructan concentration of annual bluegrass and creeping bentgrass (Species) before and after acclimation (Date) following the application of the Trinexapac-ethyl and GA_3 (Treatment) in the Guelph Turfgrass Institute (GTI) field study.

Source	DF	Sum of Squares	F Ratio	Prob > F
Species	1	0.65	1.31	0.2608
Treatment	2	0.08	0.08	0.9269
Date	1	7.89	15.88	0.0003*
Species *Treatment	2	0.36	0.37	0.6953
Species *Treatment *Date	2	0.48	0.49	0.619
Date *Species	1	0.00	0.00	0.9838
Date *Treatment	2	0.07	0.07	0.936
Error	36	17.89		
Total	**47**	**27.42**		

Table 13. Analysis of variance (ANOVA) of high molecular weight fructan concentration of seven different cereal crops (Variety) before and after acclimation (Date) following the application of the Trinexapac-ethyl and GA_3 (Treatment) in the Arkell research station, University of Guelph field study.

Source	DF	Sum of Squares	F Ratio	Prob > F
Variety	1	0.10	0.20	0.6616
Date	1	4.28	8.58	0.0065*
Treatment	2	0.24	0.25	0.784
Date *Variety	1	0.14	0.27	0.6048
Date *Treatment	2	0.01	0.01	0.9887
Date *Treatment *Variety	2	0.28	0.28	0.7566
Treatment *Variety	2	0.18	0.18	0.8337
Error	29	14.47		
Total	**40**	**19.77**		

Table 14. Analysis of variance (ANOVA) of low molecular weight fructan concentration of seven different cereal crops (Variety) before and after acclimation (Date) following the application of the Trinexapac-ethyl and GA_3 (Treatment) in the Arkell research station, University of Guelph field study.

Source	DF	Sum of Squares	F Ratio	Prob > F
Variety	1	0.01	0.20	0.6559
Date	1	0.69	14.45	0.0007*
Treatment	2	0.01	0.07	0.9353
Date *Variety	1	0.01	0.27	0.6059
Date *Treatment	2	0.14	1.44	0.2545
Date *Treatment *Variety	2	0.02	0.26	0.7737
Treatment *Variety	2	0.06	0.65	0.5272
Error	29	1.38		
Total	**40**	**2.32**		