PHYSIOLOGICAL MECHANISMS AND MANAGEMENT STRATEGIES ASSOCIATED WITH ANNUAL BLUEGRASS RESISTANCE TO ICE ENCASEMENT STRESS

By

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ABSTRACT

Annual bluegrass (*Poa annua*) is a cool-season, managed turfgrass species that is susceptible to winter injury, particularly ice encasement. Development of winter preparatory practices in the fall is important for turfgrass managers to decrease the incidence and costs associated with ice encasement damage. In the first experiment, a two-year growth chamber study evaluated the effect of fall soil water content (SWC) at three different levels (8%, 12%, and 20%) on ice encasement survival. Plants were analyzed for relative water content, percent recovery, total nonstructural carbohydrates, and lipid peroxidation. Low SWC during cold acclimation improved recovery could be associated with higher root TNC content. In the second experiment, plant growth regulator (PGR) treatments in the fall season were used to evaluate their ice encasement recovery in controlled conditions and field spring recovery. Field plots were treated with mineral oil, aminoethoxyvinylglycine (AVG), ethephon [(2-chloroethyl)phosphonic acid)], and a control (water). Refraining from high levels of ethephon or reducing water inputs during the fall acclimation period may reduce annual bluegrass stand loss due to ice encasement. A third growth chamber experiment was conducted to observe the differences in gas accumulation between creeping bentgrass (*Agrosis stolonifera*; ice encasement tolerant) and annual bluegrass (ice encasement sensitive) during ice encasement and after melt. Annual bluegrass had reduced regrowth after ice encasement when compared to creeping bentgrass, which could be associated with higher amounts of ethylene and CO_2 in annual bluegrass plants and a decrease in glucose and fructose after 20 d of ice encasement. This research improves our understanding of the effects of ice encasement on turfgrass growth, winterkill preventative management practices, and mechanisms that may correlate to ice encasement resilience.

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LIST OF ABBREVIATIONS

ABG Annual bluegrass

CBG Creeping bentgrass

MDA Malondialdehyde

PGR Plant growth regulator

SC Storage carbohydrates

SWC Soil water content

TNC Total nonstructural carbohydrates

WSC Water soluble carbohydrates

NDVI Normalized difference vegetation index

LAI Leaf area index

CI Chlorophyll index

TQ Turf quality

CHAPTER 1: LITERATURE REVIEW

Annual bluegrass and Winterkill

Annual bluegrass (*Poa annua*) is a cool-season grass often found in pure or mixed grass stands in northern climates. In the turfgrass industry, it is often considered a weed when it invades surfaces consisting of other species, such as creeping bentgrass (*Agrostis stolonifera*) putting greens. Removal of annual bluegrass as a weed can be costly and ineffective resulting in management of annual bluegrass as the preferred perennial grass. Management of annual bluegrass requires knowledge of methods to suppress frequent flowering and promote survival during abiotic and biotic stress conditions (Goss 1964). With complex genetic and epigenetic traits, annual bluegrass can exist in perennial or annual forms and is prone to ecotype variation (LaMantia & Huff 2011; Benson et al. 2021). It can be sensitive to certain abiotic stresses such as winterkill associated stresses.

Winterkill stresses of annual bluegrass can include premature de-acclimation, freeze/thaw injury, crown hydration injury, winter desiccation, and ice encasement stress. Loss of grass cover on putting greens results in repair and restore costs associated with renovation, reseeding, and/or resodding. Costs are also incurred by golf facilities when winterkill of putting greens is severe, which would require temporary green construction, delayed opening of golf courses, and therefore reduced revenue from golf play (Frank 2014). To reduce costs associated with winterkill loss of annual bluegrass and to allow for winter preparatory management strategy recommendations to be created, more information is needed regarding physiological ramifications and methods to promote resilience of annual bluegrass over winter. Knowledge of cold acclimation traits and how they promote freezing tolerance are well known; however, how

plant external and internal factors during cold acclimation influence survival of specific winterkill stresses like ice encasement are poorly understood.

Plants Preparing for Winter via Cold Acclimation

Proper acclimation of important overwintering tissues, such as crown tissue in annual bluegrass, is necessary for plants to withstand winter stresses and avoid injury. During acclimation, metabolic and physiologic changes occur through multiple genetic processes (Li et al. 2018). Exposing plants to low temperatures will increase freezing tolerance while warmer temperatures will reduce freezing tolerance (Levitt 1980). During fall, a decrease in temperatures and shorter photoperiods begin to cause changes in the metabolic pathway of turfgrass. Photosynthesis and respiration slow down, and the plant accumulates metabolites in preparation for dormancy to protect against freezing temperatures. During the fall, grasses acclimate in preparation for cold stress, but it is not clear if acclimation associated changes may prepare plants for ice encasement stress.

Changes in Carbohydrates

Acclimation induces changes in carbohydrate metabolism and allocation within the plant to maintain cellular functions. Total nonstructural carbohydrates (TNC) increase in crown tissues of winter wheat (*Triticum aestivum*) during cold acclimation (Gao et al. 1983). Similar results were found in leaf tissues of over 100 cool- and over 50 warm-season Gramineae species where cooler temperatures generally increased the accumulation of TNC, fructan, sucrose, and starch (Chatterton et al. 1988). Creeping bentgrass and annual bluegrass both accumulate high molecular weight fructans during cold acclimation and levels are depleted during deacclimation, however, creeping bentgrass maintains higher levels of fructans (Hoffman et al. 2014). Fructans were found to be an important overwintering carbohydrate as they accumulate and are stored in

crown tissue, then can be transported between tissues in the form of sucrose (Tronsmo et al. 1993). Differences in how plants maintain both water soluble (WSC) and storage carbohydrates (SC) affect their ability to survive overwintering stresses. In spring and winter wheat, when exposed to low-temperature stress and cold acclimation, WSC such as sucrose, glucose, and fructose increased while SC such as starch decreased relative to control temperatures (Savitch et al. 1999). A greater increase in WSC, specifically sucrose, in perennial ryegrass (*Lolium perenne*) crown tissue during acclimation was found in a freezing-tolerant cultivar compared to a freezing-susceptible cultivar (Hoffman et al. 2010). Similar results were found in centipede grass (*Eremochloa ophiuroides*) where sucrose levels were higher in acclimated tissues when compared to non-acclimated or de-acclimated centipede grass (Fry et al. 1993).

Amino Acid Metabolism

Other metabolites accumulate in plant tissues during cold acclimation. These metabolites include amino acids and organic acids which can change in concentration throughout tissues depending on the rate of photosynthesis, energy metabolism, and antioxidant metabolism. Proline is an amino acid that can be utilized in a plant under stress through maintaining cellular homeostasis through osmolarity, preventing oxidative injury, and act as a signaling molecule in gene expression (Jiang et al. 2023). As an osmoprotectant, proline levels increased in rye (*Secale cereale L.*) after more than three weeks of cold acclimation (Koster and Lynch 1992). In response to exposure to 4 °C wheat seedlings accumulated glutamine, proline, alanine, aspartic acid, asparagine, glycine, valine, threonine, and isoleucine while decreasing in concentration of glutamic acid (Naidu et al. 1991). During cold acclimation of perennial ryegrass (*Lolium perenne*), glutamic acid, aspartic acid, and asparagine increased resulting in a significant association with the acclimation process (Bocian et al. 2015). After acclimation at subfreezing

temperatures, annual bluegrass ecotypes accumulated proline, glutamine, and glutamic acid (Dionne et al. 2001a).

Membrane fatty acids and other metabolites

During cold acclimation, fatty acid ratios shift from higher saturated fatty acids to unsaturated fatty acids. After a natural hardening period, a winter-hardy cultivar of white clover (*Trifolium repens*) had higher total amounts of unsaturated fatty acids when compared to a less winter-hardy cultivar with an emphasis on the 18:2 fatty acid (Dalmannsdottir et al. 2001). In early acclimation of winter rye, palmitic acid (16:0), linoleic acid (18:2), and linolenic acid (18:3) were the major phospholipid fatty acids in the plasma membrane (Uemura and Yoshida 1984). The phospholipid fatty acid ratio is important for survival throughout winter to maintain plant homeostasis. In perennial ryegrass, a freezing tolerant cultivar showed a higher ratio of membrane stabilizing lipids and unsaturated fatty acid content compared to freezing-susceptible cultivars after 21 d of cold acclimation (Hoffman et al. 2010).

Enzymes in the TCA cycle such as aconitase, pyruvate, and 2-oxoglutarate-dehydrogenase, are sensitive to oxidative stress causing changes in organic acid accumulation (Verniquet et al. 1991; Sweetlove et al. 2002). During acclimation, respiration rates increase in both light and dark, however increases more in the dark (Talts et al. 2003). Changes in respiration and photosynthetic rates alter the efficiency and rate of the TCA cycle, resulting in accumulation or utilization of various organic acids. These metabolites can function as many things including osmolytes to influence cellular water relations, compatible solutes to stabilize cellular components, optimizing membrane lipid composition to maintain proper membrane function, and as energy sources (Guy et al. 2007). After cold acclimation in two grape species (*Vitis amurensis* and *Vitis vinifera*), several organic acids increased in concentrations including

glycerate, ascorbate, citrate, succinate, malate, pyruvate, and fumarate (Chai et al. 2019). *Arabidopsis thaliana* accumulated organic acids such as ascorbic acid, citrate, and malic acid after 4 to 8 d of cold treatment at 4°C (Doerfler et al. 2013). The organic acid fumaric acid is essential for cold acclimation of metabolism in *Arabidopsis thaliana* (Dyson et al. 2016). Cold acclimated bermudagrass (*Cynodon dactylon*) had increased propanoic acid along with other sugars such as arabinose, mannose, glucopyranose, maltose, and turanose (Fan et al. 2015). Taken together, all changes associated with cold acclimation may lead to enhanced freeze tolerance but are not well correlated to resistance or sensitivity to ice encasement stress.

Ice encasement: A complex winterkill stress

Ice encasement, either a solid nonporous or a porous layer of ice covering the grass surface, can arise when snow melts and refreezes or during sleet and ice storms. Ice encasement is a complex stress that can cause necrosis of annual bluegrass if the ice encasement conditions persist for an extended duration. This stress is unique due to the environment that develops under prolonged ice cover. Plants under the ice may endure low temperature, anoxic or hypoxic conditions, and toxic gases and metabolites that may accumulate (Andrews 1996). On top of that, once ice melts and percolates through the soil, plants often experience a sudden burst of high concentration of oxygen and light that the plant would not be acclimated to and would need to adjust to (Crawford 2003).

Ice encasement survival of plants likely depends on various internal and external factors, but the mechanisms of survival are not fully understood. As the primary overwintering structure, survival of plant crown tissues is especially important over winter for perennial grasses that lack rhizomes or stolon structures. Leaf and root tissue often senesce or die back during cold acclimation and the winter dormancy period. While much is known regarding cold acclimation,

as discussed above, it is not clear whether cold acclimation associated changes offer protection during ice encasement stress for plant tissues and what mechanisms exist in plant organs to resist ice encasement induced damages.

Gas and metabolism changes associated with ice encasement

Under ice encasement, plants can experience hypoxic or even anoxic conditions where a lack of oxygen can cause oxygen deprivation injury (Schluter & Crawford 2003; Kalashnikov at al. 1994; Biemelt et al. 2000). There is often a switch to anaerobic respiration in plant tissues. Anaerobic respiration utilizes plant carbon stores but produces only a fraction of the ATP compared to aerobic respiration. Anaerobic respiration pathways produce end-products lactic acid, CO_2, and ethanol that are toxic to cells if they accumulate to a high enough level (Gudleifsson 1993). An increase in plant and microbial respiration results in increased CO_2 concentrations in soil environments during wintertime (Clein and Schimel 1995). In forage plants under experimental ice covers, CO_2 accumulated as much as 10%, from about .04% of atmospheric CO_2, while oxygen levels were reduced to as low as 4% of the atmosphere (Freyman 1967). CO_2 increased cell membrane permeability in cold-hardened winter wheat (*Triticum aestivum*) and, in combination with ethanol, reduced plant viability under ice encasement (Andrews and Pomeroy 1979). High levels of CO_2 can result in high levels of bicarbonate which inhibits membrane transport processes leading to an inability of plants to recover from ice encasement (McKersie and Leshem 1994). In wheat seedlings encased in ice, lactic acid did not accumulate, however, CO_2 accumulated at high levels suggesting that injury was likely due to CO_2 toxicity (Andrews and Pomeroy 1989). When CO_2 levels were increased to at least 20% under simulated ice encasement, the sub-arctic shrub species *Vaccinium vitis-*

idaea had 70% greater shoot mortality when compared to control conditions without increased CO_2 (Preece & Pheonix 2013).

As metabolites build-up under ice encasement, some are maintained throughout the ice encasement period while other metabolites are utilized. In timothy grass (*Phleum pratense L.*) plants encased in ice, metabolites such as CO_2, ethanol, malate, oxalate, citrate, fumarate, and pyruvate build up over time (Gudleifsson 1994). In a similar study, timothy grass plants, which are highly ice encasement tolerant, accumulated small amounts of citrate, fumarate, and shikimate and depleted levels of malonate (Gudleifsson 1997). After an extended period under ice encasement, lactate, butyrate, malate, formate, and tartarate build up. In crown tissue of winter cereal cultivars, malic acid did not accumulate after 7 d of ice encasement (McKersie et al. 1982). Amino acids are the building blocks of these metabolites. Anoxic conditions, maintained by utilizing a 1-L mason jar with ports to modify the atmosphere within, induce the accumulation of alanine and tyrosine in creeping bentgrass and annual bluegrass which could be attributed to an increase in the rate fermentation pathway as well as an acceleration of glycolysis or protein degradation (Castonguay et al. 2009). High alanine was associated with low damage levels in creeping bentgrass while tyrosine levels were higher in the susceptible annual bluegrass, indicating an association of these metabolites with tolerance to anoxic stress. For turfgrass species, little is known about the extent to which anaerobic metabolism impacts resilience to ice encasement conditions and is important to understand since anaerobic metabolism could impact carbohydrate usage and accumulation.

Fatty acid ratios may also be influenced by ice encasement conditions and can lead to damage to the plant membrane. Having more unsaturated fatty acids within a plant membrane may be useful during ice encasement conditions to maintain some membrane fluidity.

Unsaturated fatty acids and sucrose during ice encasement were found in plants that were more winter hardy (Dalmannsdottir 2001). Another study found a 2- to 4- fold increase in free fatty acid levels in the microsomal fraction in winter wheat crown tissue while also seeing only a minor change in fatty acid unsaturation during ice encasement (Hetherington et al. 1987). However, in annual bluegrass ice encasement did not change fatty acid composition of plant membranes when compared to low temperature treatments (Laskowski and Merewitz 2021). In annual bluegrass after chemical treatments, there was a shift in crown tissue fatty acids from saturated to unsaturated fatty acids that may play a role in survival of stands after simulated ice encasement (Laskowski et al. 2019). Since crown tissue is the important overwintering tissue, membrane fluidity and plant viability are important to maintain in this structure.

Re-aeration following ice encasement

In addition to metabolic damage and changes while ice encasement persists, an additional set of stresses can occur when ice melts. Soil may become saturated or waterlogged, toxic byproducts of anaerobic respiration may linger in the soil and plant system, and oxygen and light are suddenly at a much higher level of exposure to plant tissues. This sudden re-exposure to oxygen is known as post-anoxic reaeration and often causes reactive oxygen species or free radicals to be generated in tissues (Blokhina et al., 1999). Once plants are exposed to oxygen again, superoxide dismutase turns into hydrogen peroxide which causes injury to the plant due to membrane peroxidation (Santosa et al. 2007). Damage due to re-aeration may be worse for the plant than damage due to hypoxia or anoxia. For example, lipid peroxidation in the roots of wheat and rice seedlings developed during anoxia but intensified only during reaeration (Chirkova and Blokhina 1991). After a 6-hour aerobic post-thaw treatment of winter wheat crowns, leakage of electrolytes increased, and recovery of microsomal protein and phospholipid

decreased indicating continued degradation after being exposed to oxygen (Hetherington et al. 1987). This oxidative stress can affect the central metabolic pathways including the tricarboxylic acid cycle as well as causing a shift from anabolic to catabolic metabolism (Baxter et al. 2007).

Plant antioxidant systems may become activated to prevent excessive oxidative damage by reducing the amount of ROS in the plant (Blokhina et al. 2003). After ethylene-promotive treatments, a decrease in spring recovery after ice encasement was found to correlate with an increase in lipid peroxidation and a decrease in superoxide dismutase (SOD) and peroxidase (POD) antioxidant activity within annual bluegrass (Laskowski and Merewitz 2021). Lipid peroxidation occurs when ROS react and destroy lipids in the plant cells and can results in membrane rupture and cell death. Lipid peroxidation is often measured through the formation of malondialdehyde when unsaturated fatty acids react with thiobarbituric acid (Yagi 1998). During hypoxic or anoxic conditions, lipid peroxidation can represent overall membrane health of plant cells. Fatty acid ratios are ever-changing and can switch from unsaturated to saturated throughout temperature and other abiotic changes.

Current Management Strategies to Prepare Turfgrasses for Overwintering

Soil and plant water content influence the freezing tolerance of plants and the resulting spring recovery. Variability of soil water content (SWC) or overly dry or wet conditions during the fall may result in reduced plant survival of ice encasement stress during the winter months. Studies have shown that exposing a plant to an initial stress may result in higher tolerance to other stresses that use the same stress pathway, called stress preconditioning. (Hoffman et al. 2012; de Azevedo Neto et al. 2005; Jiang and Huang 2001). Reducing SWC before or during cold hardening may prepare plants to undergo ice encasement better than optimal and high SWC treatments. In infrequently irrigated creeping bentgrass, leaf tissue WSC, SC, and TNC levels

generally increased when compared to frequently irrigated creeping bentgrass which was believed to increase tolerance and improve recovery to drought stress (Fu and Dernoeden 2008). Whether stress preconditioning strategies are feasible or effective for promoting ice encasement resilience in turfgrasses is not known.

Putting greens are high input turfgrass surfaces that are highly managed with frequent irrigation, chemical treatments, and cultural practices. How these management strategies impact turfgrass acclimation to cold conditions and how these management strategies impact survival of winterkill stresses, such as ice encasement, are poorly understood and little information exists on these matters in peer reviewed literature. Few broadly applicable strategies exist for preventing winterkill and damage. The use of certain plant PGRs has been shown to increase unsaturated fatty acid ratios and increase recovery rates compared to untreated controls (Laskowski et al. 2019). These PGRs up- or down-regulate certain hormones within the plant potentially making them more winter hardy. The plant hormone ethylene plays a major role in plant cold acclimation; however, the literature is lacking information on how ethylene influences winter stresses such as ice encasement during dormancy (Szalai et al. 2000; Gaudet et al. 2011). Regulating ethylene, a gaseous plant hormone, may influence annual bluegrass winterkill survival. Previous studies have shown ethylene-promotive plant growth regulator (PGR) treatments decrease regrowth after an extended period under ice (Laskowski and Merewitz 2020). Similarly, ethylene-inhibitory treatments showed improved regrowth after a period of ice encasement. Ethylene promotive treatments decreased certain antioxidant activity resulting in poorer spring recovery (Laskowski and Merewitz 2021). Fall months are optimal for implementation of management strategies so PGR treatments may be feasible winter preparatory

treatments for larger turfgrass areas compared to current management strategies such as using protective covers.

Physical management strategies currently exist for superintendents such as digging water channels to move water away from putting greens during snowmelt in the wintertime (Quinn 1990). Physical removal of ice and snow from fields can be beneficial if ice accumulation is prolonged (Frank 2016). However, these physical management strategies can be laborious and potentially damaging to the putting greens if precautions are not taken. To further prevent ice accumulation, superintendents can topdress putting green surfaces with sand to reduce or melt current snow cover or use permeable or impermeable covers prior to snowfall (Frank 2016). Importantly, permeable covers allow for gas exchange while impermeable covers do not which would call for the need to vent the putting green periodically to prevent oxidative damage (Rochette et al. 2006). Permeable covers do not need to be vented; however, they are more susceptible to ice accumulation which reduces the effectiveness of the cover. Therefore, this research aims to provide superintendents with novel strategies to reduce the incidence of winterkill due to ice encasement.

Conclusion

Climate change may exacerbate winterkill stresses for perennial grasses and cause environmental fluctuations during fall cold acclimation periods and spring regrowth periods (Bélanger et al. 2002). There is currently high demand for management practices that could promote winter survival of cool season turfgrasses, but there is a lack of information that could lead to extension-based recommendations to turfgrass practitioners. There is a particular need for management strategies that can be used on a broad scale and knowledge of how environmental factors and species differences during cold acclimation impacts ice encasement survival and overwintering

survival, in general. Therefore, the goals of this thesis are to provide potential management strategies of annual bluegrass putting greens to prevent damage due to ice encasement as well as observe differences in metabolism following ice encasement between an ice encasement resilient and sensitive species.

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CHAPTER 2: LEAF, ROOT, AND CROWN TISSUE PHYSIOLOGY OF ANNUAL BLUEGRASS FOLLOWING COLD ACCLIMATION AT VARYING SOIL MOISTURE LEVELS AND ICE ENCASEMENT

Abstract

Annual bluegrass (*Poa annua*) is a turfgrass species prone to winterkill-induced damage such as from ice encasement stress. This research aimed to determine whether different levels of soil volumetric water content (SWC) influence cold acclimation and recovery from ice encasement. Annual bluegrass was exposed to 8%, 12%, and 20% SWC treatments during cold acclimation in growth chambers. After cold acclimation, plants were subjected to ice encasement by misting at –3 °C until a 2.5-cm ice layer was formed. On 0 (no ice encasement exposure), 40, and 80 days of treatment, plants were analyzed for recovery (percent green canopy cover over time), and leaf, crown, and root tissues were harvested for lipid peroxidation and total nonstructural carbohydrates (TNC) including storage carbohydrates and water-soluble carbohydrates (WSC). Low SWC during cold acclimation enhanced recovery from cold temperatures and ice encasement. Root carbohydrates were influenced by SWC regimes during cold acclimation since day 0 plant roots exposed to the 8% SWC treatment had 143.9% higher TNC and 137.6% higher WSC compared with day 0 plants exposed to 12% and 20% SWC. Root levels of carbohydrates and lipid peroxidation were least influenced by cold and ice encasement among the organs evaluated. Prolonged freezing conditions and ice encasement reduced leaf and crown tissue carbohydrates and increased lipid peroxidation compared with day 0 plants not exposed to freezing temperatures and ice encasement. After 40 days of ice encasement, plants exposed to the 8% SWC treatment recovered faster than plants that were cold acclimated at higher soil moisture levels. Average percent canopy cover after 36 days of recovery in the

greenhouse was 71.9% higher for 8% SWC treated plants than in 12% and 20% SWC treated plants. Turfgrass managers may benefit from annual bluegrass putting green management strategies to reduce fall soil moisture. Given that soil moisture did not significantly influence carbohydrate or lipid peroxidation results, except for in roots, additional research is needed to understand the mechanism associated with these findings.

Introduction

Cold temperature-induced changes associated with cold acclimation are critical for the winter survival of perennial grass species. Adequate cold acclimation or hardening requires major physiological changes, including changes in cellular water status, altered membrane fatty acid ratios, altered rates of carbohydrate metabolism, and carbohydrate allocation shifts (Thomashow 1999). Fall weather can be variable and does not always provide optimum conditions for cold acclimation processes, leaving plants susceptible to winter damage. Climate change may increase the incidence of variable conditions during cold acclimation calling for a need for an understanding of how critical environmental factors influence cold acclimation and winterkill stress survival. Low soil moisture in alfalfa (*Medicago media*) seedlings increased cold hardening and freezing survival (Paquin and Mehuys 1980). How soil moisture content influences cold acclimation and survival of severe winter stresses of perennial grass species has not been investigated.

For highly managed turfgrass areas, such as annual bluegrass putting greens, irrigation levels can be tailored to attempt to provide ideal soil moisture levels. Variable fall conditions and precipitation levels may cause soil moisture to be higher or lower than desired. An understanding of how soil water content influences winterkill-related stresses is needed to make recommendations to turfgrass managers related to fall irrigation practices or winter preparatory

strategies. Annual bluegrass is more susceptible to ice encasement damage compared to other cool-season putting green species such as creeping bentgrass [*Agrostis stolonifera* (Beard 1964)]. However, controlled research studies that have evaluated if irrigation or soil moisture content during the fall season influences cold acclimation and overwintering of turfgrasses are lacking. Additionally, knowledge of the effects of soil moisture content on physiological changes in turfgrass species during acclimation could reveal novel strategies to promote acclimation or prime plants for enhanced acclimation and winter tolerance, such as through stress pre-conditioning.

Cold acclimation-induced changes in freezing tolerance of perennial grasses have been associated with the level of total non-structural carbohydrates (TNC) and specific carbohydrates in various species (Fry et al. 1993; Hoffman et al. 2010; Zhang et al. 2011). In annual bluegrass, fructans were found at high levels following non-freezing cold acclimation conditions and sucrose content in crown tissues was associated with freezing tolerance (Dionne et al. 2001). Perennial ryegrass (*Lolium perenne*) crown tissue water-soluble carbohydrates (WSC) increased during cold acclimation to a greater extent in a freezing-tolerant cultivar compared to a susceptible one (Hoffman et al. 2010). Determining whether soil moisture conditions factor into differential impacts on carbohydrate production and reserves during cold acclimation is needed.

The carbohydrate content of plant tissues may also influence recovery from ice encasement stress and ice encasement may influence available carbohydrates during stress recovery. McKersie et al. (1981) found that crown tissue of winter cereals such as winter wheat (*Triticum aestivum)* with higher tolerance to ice encasement had greater levels of total available carbohydrate and reducing sugars compared to cultivars less tolerant to ice encasement. The sensitive cultivar used in the study did not lose or utilize carbohydrates; thus, the loss of viability

was not associated with the depletion of carbohydrate reserves. In a different study, cold acclimation increased TNC levels in crown tissues of winter wheat, which were then decreased following 7 d of ice encasement (Gao et al. 1983). Evaluating the impact that ice encasement has on carbon availability and whether carbohydrate content of plant tissues may be associated with enhanced recovery is important to understand the physiology associated with annual bluegrass susceptibility to ice encasement stress.

Lipid health and degree of saturation in plant membranes play major roles in plant overwintering. Fatty acid ratios shift to higher levels of unsaturated compared to saturated fatty acids during cold acclimation of plant tissues (Baird et al. 1998; Hoffman et al. 2010; Shang et al. 2006). Whether cold acclimation and ice encasement influence lipid health associated with lipid peroxidation has not been thoroughly evaluated for turfgrass species. Cold acclimation elicits an increase in lipid peroxidation, particularly in photosynthetic structures (Wise and Naylor 1987). Levels of hydrogen peroxide have been found to increase during cold acclimation, which can lead to oxidative stress (Prasad et al. 1994). Anoxic conditions, often found during prolonged ice encasement, can cause lipid peroxidation, particularly following plant re-aeration, and less lipid peroxidation is associated with greater tolerance of anoxic conditions (Blokhina et al. 1999, 2001). In annual bluegrass, Laskowski et al. (2021) found that leaf and crown tissues had similar levels of lipid peroxidation following cold acclimation and overwintering stresses. Lipid peroxidation levels increased in both tissues following 80 d of cold temperatures or ice encasement treatment. Annual bluegrass plants showing low levels of recovery had high levels of lipid peroxidation in crown and leaf tissues, indicating that lipid peroxidation damage of crown tissue may be a good indicator for overwintering recovery of annual bluegrass.

Therefore, the objective of the study was to expose annual bluegrass plants to low, optimum, and high soil moisture levels during cold acclimation to compare plant carbohydrate content and carbon allocation of plant tissues, lipid peroxidation levels, and recovery percentages following cold acclimation and following cold and ice encasement stress. Based on limited background literature, we hypothesized that drier soil moisture conditions may increase plant recovery and could have lower associated levels of lipid peroxidation and higher levels of carbohydrates compared to plants exposed to optimum or higher levels of soil moisture.

Materials And Methods

Plant material and growth conditions

Annual bluegrass sod plugs were collected from the Hancock Turfgrass Research Center, East Lansing, MI, USA from a mature, perennial-type research putting green field mown at 0.3 cm. The plugs (10.2 cm diameter) were cut free of roots (1.3 cm deep) for optimal root regeneration during potting and were brought to a greenhouse on 6 Aug 2021 and 3 May 2022. Greenhouse conditions were maintained at an average day/night 23/16 ºC, 14 h photoperiod, average 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) supplemented with high pressure sodium lamps. Each sod piece was cut into four pieces and placed in a sandy loam soil (65.9% sand, 14.9% silt, 19.2% clay; Typic Hapludult) in a deep pot (6.0 cm diameter by 35.0 cm deep) to allow for adequate root growth. The plants were allowed to become established and spread to the entire diameter of the pot in the greenhouse for approximately 60 d. There was a total of 144 pots for sufficient plants to test three soil moisture regimes with eight replications for three sampling dates and two experiments (year 1 and 2). The plants were fertilized weekly with half-strength Hoagland's solution (Hoagland and Arnon 1950) during greenhouse establishment. Plants were trimmed to maintain a putting green height of 1.3 cm (to allow for sufficient leaf tissue for

analysis) and watered as needed. Following pot establishment, all pots were moved to a growth chamber for cold acclimation treatment.

Experimental treatments during acclimation

For cold acclimation, plants were transferred from the greenhouse to a low-temperature growth chamber (LTCB-19; Biochambers, Winnipeg, Manitoba, Canada). The cold acclimation periods lasted for approximately 40 d for year 1 (3 Sep to 15 Oct 2021) and 33 d for Year 2 (16 Jul to 17 Aug 2022). Plants were fertilized once halfway through the 10 ºC cold acclimation period with half-strength Hoagland's solution. For both experiments, growth chamber (LTCB-19) conditions were maintained at an average day/night 18/16 ºC, 12 h photoperiod, average 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR initially and the chamber settings were changed to 4 ºC with a 10 h photoperiod and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR for the last 2 weeks of cold acclimation.

Water content treatments

Soil volumetric water content (SWC) was recorded and regulated for each pot to maintain the surface soil moisture at approximately 8%, 12%, and 20% during cold acclimation, measured daily using time domain reflectometry (TDR) using a handheld soil moisture meter (TDR 150; Spectrum Technologies Inc., Aurora, IL, USA) with rods (3.8 cm) completely inserted into the soil. Daily SWC was used to determine the amount of water added to plants to maintain similar treatment levels, with the upper 3.8 cm of soil measured. Plants were watered with deionized water as follows: 8% SWC watered every other day with 10 mL, 12% SWC watered daily with 10 mL, and 20% SWC watered daily with 20 mL to maintain proposed treatment SWC levels. Since root length was determined during sampling to be approximately 30.0 cm long for day 0 plants and a 3.8 cm SWC probe was used, our treatments may be interpreted as a soil surface

drying treatment for the low moisture condition. Leaf relative water content (RWC) was only determined in 2022 by standard methods from Barrs and Weatherley (1962).

Ice encasement treatments.

Following the cold acclimation and water treatment periods, plants were exposed to –3 ºC temperatures, 10 h photoperiod, with a 200s µmol·m^{-2}·s^{-1} light level and were then separated into ice and no ice (0 d) treatments within the growth chamber (LTCB-19). Ice treatment began on 3 Dec 2021 for year 1 and 18 Aug 2022 for year 2. Day 0 plants were not subjected to freezing temperatures since they were cold hardened to 4 ºC. The ice encasement-treated plants received mist with deionized water every 30 min until a 2.5 cm ice layer was formed. The ice layer was maintained for the duration of the study by misting plants as needed. After 0, 40, or 80 d of treatment, pots were cut in half. Half of the plant was destructively sampled to separate leaf, crown, and root tissues and were immediately frozen in liquid nitrogen and stored at –80 ºC until further analysis. The other half was put at 4 ºC for 2 d and then placed in the greenhouse (24 ºC) for recovery analysis. Greenhouse conditions were the same as those described above.

Figure 2.1 Annual bluegrass plants in a -3 °C growth chamber with an approximate 1.27 cm ice layer. Plants were potted and clear acetate sheets were wrapped around the top to allow an ice layer to form above the leaf tissues.

Recovery analysis

Digital images were taken daily during the recovery period to determine percent regrowth. The percent regrowth was estimated by the percentage of canopy green cover using digital analysis software [Canopeo (Patrignani and Ochsner 2015)]. For plants sampled on 40 d of ice encasement for year 1, images were taken from 4 Jan to 3 Mar 2022, for a total of 43 d of recovery. For plants exposed to 80 d of ice encasement in year 1, readings were taken from 22 Feb to 4 Apr 2022, for a total of 41 d of recovery. For year 2, images of day 0 plants, not exposed to ice encasement, were taken from 17 Aug to 25 Aug 2022, for a total of 9 d of recovery. For plants exposed to 40 d of ice encasement, images were taken from 27 Sept to 1 Nov 2022, for a total of 36 d of recovery. For plants exposed to 80 d of ice encasement, images were taken from 5 Nov to 15 Dec 2022, for a total of 40 d of recovery.

Total nonstructural carbohydrate analysis

Previously frozen crown, leaf, and root tissues were analyzed for total nonstructural carbohydrates (Chatterton et al. 1989, Westhafer et al. 1982). Tissues were dried initially at 100 °C for at least 1 h to prevent metabolism in plant material and then maintained in the oven at 70 °C for 72 h. About 50 mg of tissue was placed into 2 mL tubes and was ground using a tissue homogenizer (1600 MiniG; SPEX SamplePrep LLC, Metuchen, NJ, USA). For extraction, 1 mL of 92% ethanol was added to each tube and centrifuged (5430R; Eppendorf North America, Enfield, CT, USA) for 10 min at 14,000 gn at room temperature. The supernatant was transferred to a clean 15 mL tube and the pellet was resuspended two more times in 1 mL of 92% ethanol resulting in a total of 3 mL extraction solution, which was diluted to 10 mL with deionized water. The remaining pellet of plant tissue was placed in the oven at 70 °C to dry overnight for storage carbohydrate analysis.

Reducing sugar content was determined as in Ting (1956) and Smith (1969). A 0.2 mL aliquot of ethanol extraction solution was added to 1.25 mL of alkaline ferricyanide solution and 0.8 mL of deionized water in a large test tube. This mixture was heated to 100 °C for 10 min and then immediately cooled in an ice bath. Then, 2.5 mL of 2N sulfuric acid was added to the cooled solution and the test tube was shaken vigorously. Arsenomolybdate solution (1 mL) was added to this solution and then it was diluted to 25 mL with deionized water.

For sucrose hydrolysis, 2 mL of the extraction solution was added to 2 mL of 4% sulfuric acid (w/v). This solution was mixed and boiled at 100 °C for 15 min. Solutions were then allowed to cool at room temperature and 1 mL of 1N NaOH was added to neutralize the solution. The preparation for the quantification of sucrose hydrolysis was performed as described above for reducing sugars.

To determine the total storage carbohydrates, the dried tissues from the ethanol extraction were resuspended in 0.5 mL deionized water and heated at 100 ºC for 10 min then allowed to cool to room temperature. Acetate buffer (0.4 mL, 200 mM, pH 5.1) and 0.1 mL enzyme solution including amyloglucosidase and alpha-amylase (Fu and Dernoeden 2008) was added to the tube. Tubes were vortexed and incubated at 55 ºC for 16 h (Thermomixer; Eppendorf North America, Inc.). The next day, samples were centrifuged at 14,000 gn for 20 min until a solid pellet was formed. The supernatant was poured into a 15 mL centrifuge tube and diluted to 10 mL with deionized water.

To determine starch content, 0.9 mL of the water extraction solution and 0.1 mL 1N sulfuric acid were added to a test tube. This tube was then mixed and boiled at 100 °C for 15 min and then was left to cool to room temperature. Sodium hydroxide (0.1 mL 1N NaOH) was added to neutralize the solution. For quantifying all sugar extractions, absorbances were measured at

515 nm using a spectrophotometer (Genesys 10S UV-VIS; Thermo Fisher Scientific Inc., Waltham, MA, USA). Sugar extractions were quantified based on a glucose standard curve from methods of Ting (1956).

Lipid peroxidation

Lipid peroxidation was determined based on malondialdehyde (MDA) content using modified methods from Dhindsa et al. (1981) and Zhang and Kirkham (1994). Previously separated and frozen crown, leaf, and root turfgrass samples were weighed to about 200 mg and ground using an automated tissue homogenizer (1600 MiniG). Samples remained frozen using liquid nitrogen throughout the entire process and MDA was extracted using a 50 mM phosphate buffer with a pH of 7.0 along with 1% polyvinylpolypyrrolidone; 0.8 mL of this solution was used with 0.4 mL 20% trichloroacetic acid (w/v) and 0.5% thiobarbituric acid (w/v), then heated at 95 °C for 30 min. The absorbance at 532 and 600 nm was measured, and MDA content was calculated.

Experimental design and statistical methods

The experimental design was a split-plot design within one growth chamber with soil moisture treatment as the whole plot and ice encasement or no ice encasement (0 d) split within each whole plot with 8 replications per treatment. The sampling time was completely randomized within the whole plot. The experiment was repeated using the same growth chamber. A similar experimental design was used previously (Laskowski et al. 2018). Normality was assessed using visual analysis of residual plots and Levene's test for homogeneity of error. The MDA and TNC results did not conform to the assumption of normality and were transformed using the square root of x; data presented are the untransformed means whereas P values are from the transformed analysis. Data resulting from all measured parameters were subjected to analysis of variance (ANOVA) using RStudio software (version 4.1.0, Boston, MA, USA) using a linear mixed

model to determine the main and interacting effects of the experimental factors. Mean separations were performed using Fisher's protected least significant difference test at the $p \leq 0.05$ level. The duration of ice encasement and SWC treatment were held as fixed effects. Interactions associated with years were not significant; therefore, data were pooled together across years for all measured parameters.

Results

Soil water content and leaf relative water content

Soil water content was achieved to the approximate desired experimental levels of 8%, 12%, and 20% since the average soil moisture level for low, medium, and high levels were 8.27%, 12.37%, and 24.75%, respectively (Fig. 2.1). The RWC of leaf tissues taken from plants exposed to 12% and 20% SWC treatment remained at an average of 79.7% and 81.2%, respectively, throughout the duration of the study (Fig. 2.2). The initial RWC of leaf tissues from the plants exposed to the 8% SWC treatment averaged 81.1% for the first 2 weeks and then decreased to an average of 72.4% for the following 3 weeks. The RWC for the 8% SWC treated plants was significantly lower than those for the 12% and 20% SWC treatments on 14, 21, and 28 d of acclimation (Figure 2.2; Table 1).

Figure 2.1 Soil water content percent (SWC) of annual bluegrass plants watered to achieve a treatment level of 8%, 12%, or 20% SWC during growth chamber simulated cold acclimation. Least significant difference (LSD) values are indicated on dates when significance was detected, and LSD values are represented by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment. Means from 2021 and 2022 are pooled together.

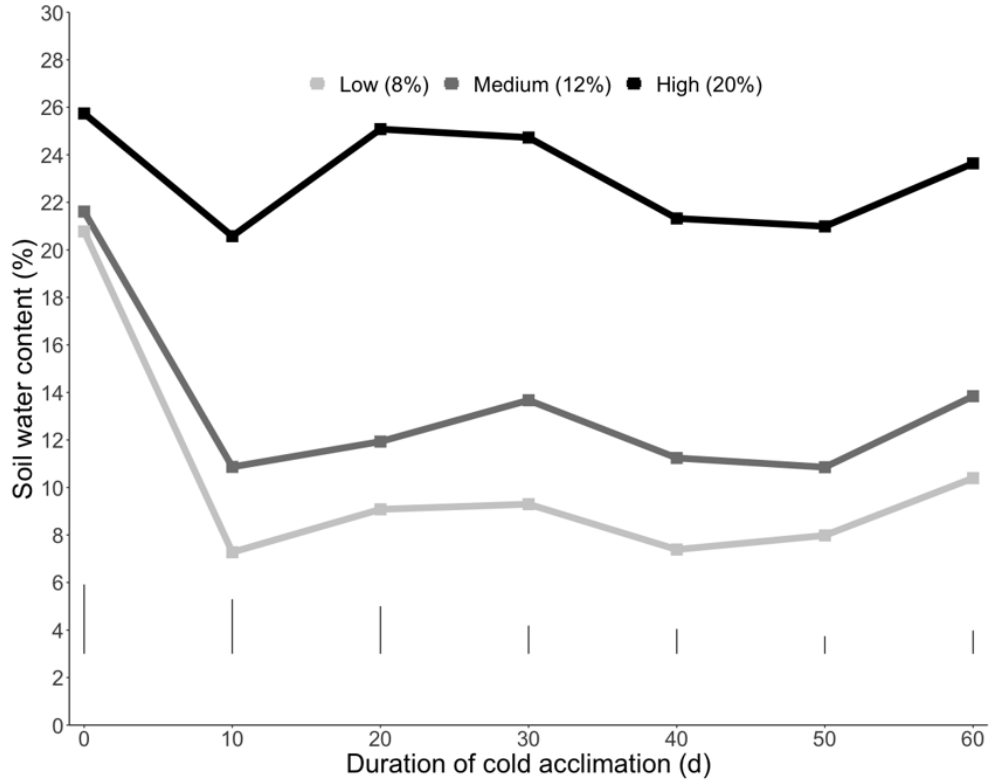


Fig. 2.2. Percent relative water content from 2022 of leaf tissues during cold acclimation for annual bluegrass plants exposed to 8%, 12%, or 20% soil water content treatments. Least significant difference (LSD) values are indicated on dates when significance was detected and LSD values are represented by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment.

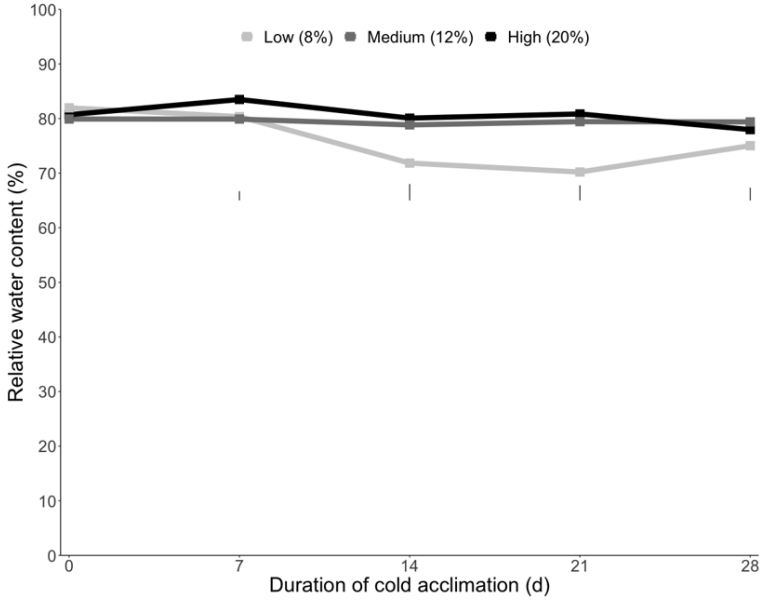


Table 2.1 – Analysis of variance (ANOVA) for the main treatment factors of water treatment (WT), time (T), and their interactions for relative water content (RWC), soil volumetric water content (SWC), and recovery of annual bluegrass following different SWC treatments during cold acclimation and following ice encasement treatments in growth chambers and recovery in a greenhouse. Results from the years 2021 and 2022 were pooled together. The factor T represents duration of cold acclimation for RWC and SWC values, whereas T for recovery represents the duration of the recovery period. Recovery periods were not the same for 0, 40, and 80 d.

Effect[i]			Recovery		
			Time under ice (d)		
	RWC	SWC	0	40	80
WT	***	***	***	**	ns[ii]
T	***	***	***	***	***
WT × T	***	***	*	*	ns

[i] All data were subjected to ANOVA using a mixed model procedure.
[ii] ns = not significant ($P > 0.05$); * = significant ($P \leq 0.05$); ** significant ($P \leq 0.01$); *** significant ($P \leq 0.001$)

Lipid peroxidation

Soil water content treatment level did not influence MDA content, but the duration of cold and ice encasement did (Table 2). In crown tissues, MDA content increased from levels observed for day 0 plants after 80 d of cold and ice encasement by 32.1%. Leaf tissue MDA content increased in response to cold and ice encasement exposure of 40 or 80 d compared to day 0 plants (Figure 2.3). However, prolonged ice encasement duration did not result in any differences in leaf MDA

content (40 d not different than 80 d). Root tissues exhibited fewer changes associated with MDA content due to cold and ice encasement treatment and had less MDA content than crown and leaf tissue on all dates measured (Table 3). For instance, day 0 plant roots had 14.01 nmol·g^{-1} DW compared to 38.44 and 46.63 nmol·g^{-1} DW of MDA for crown and leaf day 0 plants, respectively.

Figure 2.3 Lipid peroxidation as expressed by malondialdehyde (MDA) content of annual bluegrass leaf, crown, and root tissues after 0, 40, or 80 d of ice encasement at (-3 °C) in growth chamber conditions following soil moisture treatments during cold acclimation. Means from 2021 and 2022 are pooled together. Capital letters indicate statistical differences within a given plant organ (leaf, crown, or root). All statistical letters are derived from LSD tests ($P \leq 0.05$). The same letters indicate values that are not significantly different ($P \leq 0.05$).

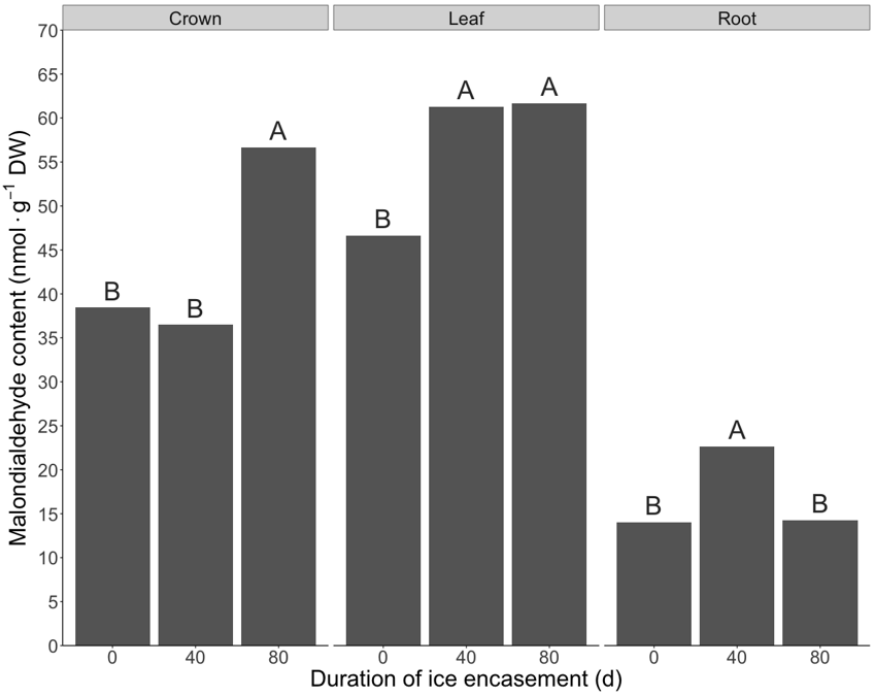


Table 2.2 - Analysis of variance (ANOVA) for main treatment factors, water treatment (WT) and duration of cold temperature and ice encasement (ID), and their interactions for parameters measured including crown (Cr), leaf (L), and root (R) malondialdehyde (MDA) content, water soluble carbohydrates (WSC), storage carbohydrates (SC), and total nonstructural carbohydrates (TNC) of annual bluegrass following different SWC treatments during cold acclimation and following cold and ice encasement treatments in growth chambers in 2021 and 2022.

Effect[i]	MDA			WSC			SC			TNC		
	Plant organ			Plant organ			Plant organ			Plant organ		
	Cr	L	R	Cr	L	R	Cr	L	R	Cr	L	R
WT	ns[ii]	ns	ns	ns	ns	***	ns	*	ns	ns	ns	**
ID	**	**	***	***	***	***	***	***	ns	***	***	***
WT × ID	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns

[i] All data were subjected to ANOVA using a mixed model procedure.
[ii] ns = not significant ($P > 0.05$); * = significant ($P \leq 0.05$); ** significant ($P \leq 0.01$); *** significant ($P \leq 0.001$)

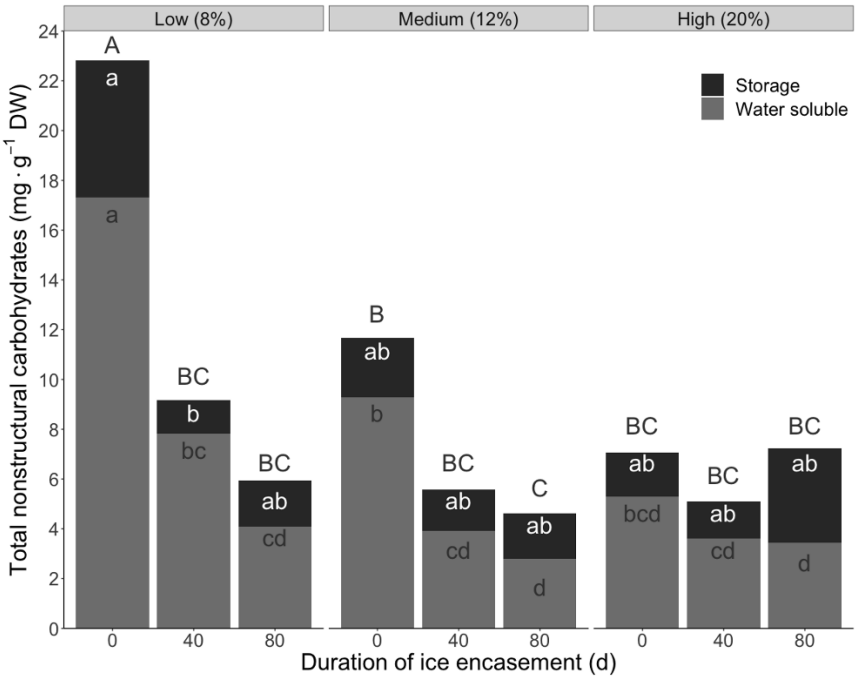
Total nonstructural carbohydrates

The TNC contents (sum of SC and WSC) of the crown or leaf tissue were not influenced by SWC treatments (Table 2), but a significant interaction of SWC treatment and duration of ice encasement was found for the TNC of root tissues (Figure 2.4). The day 0 plant roots exposed to 8% SWC had higher levels of TNC and WSC compared to the day 0 plants exposed to 12% and 20% SWC. For day 0 plants, SC was not significantly different among plants treated with different levels of SWC. On other sampling days, fewer differences associated with SWC were detected for TNC, SC, and WSC for 40 or 80 d plants.

Table 2.3 – Main effects for tissue type for malondialdehyde content (MDA) in nmol • g^{-1}, total nonstructural carbohydrates (TNC) in mg • g^{-1}, water-soluble carbohydrates (WSC) in mg • g^{-1}, and storage carbohydrates (SC) in mg • g^{-1} of annual bluegrass for leaves, crowns, and roots. Analysis of variance (ANOVA) and Fisher's protected least significant difference test at a 0.05 P level were used to detect differences between treatment means. Means from 2021 and 2022 are pooled together. Within each measured parameter for each tissue type, means followed by the different letters are statistically different ($P \leq 0.05$).

	MDA	WSC	SC	TNC
Leaf	56.6 a	30.2 a	27.0 a	57.1 a
Crown	43.9 b	17.1 b	12.1 b	29.2 b
Root	17.0 c	6.4 c	2.4 c	8.8 c

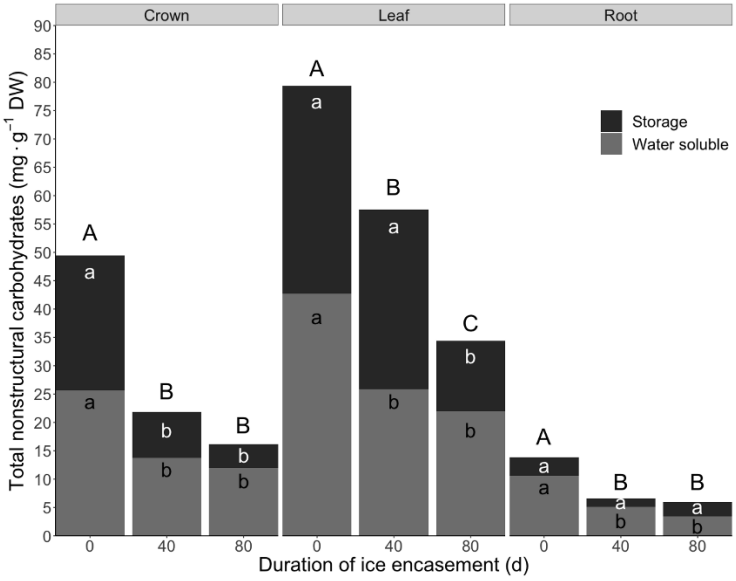
Figure 2.4 Total nonstructural carbohydrates (TNC) as a sum of water-soluble carbohydrate (WSC) and storage carbohydrate (SC) fractions in root tissues of annual bluegrass exposed to soil water content levels of 8%, 12%, or 20% during cold acclimation. Means from 2021 and 2022 are pooled together. Different capital black letters indicate statistically different means based on least significant difference values for TNC across sampling days ($P \leq 0.05$). Different white lowercase letters indicate statistical differences in SC and different gray lowercase letters indicate statistical differences for WSC.



Cold and ice encasement for 40 and 80 d at -3 °C significantly decreased the amount of TNC and the respective fractions of SC and WSC in crown and leaf tissues compared to day 0 plants (Figure 2.5). Plants exposed to 80 d of cold and ice encasement had similar levels of crown and root TNC, WSC, and SC compared to plants exposed to 40 d of ice encasement. Leaf tissue TNC and SC were lower for day 80 plants compared to day 40 plants. Root tissue TNC was less influenced by cold and ice encasement duration since root TNC and SC did not significantly

change over sampling days, but WSC was lower after 40 or 80 d of ice encasement compared to day 0 plant roots.

Figure 2.5 Total nonstructural carbohydrates (TNC) as a sum of water-soluble carbohydrate (WSC) and storage carbohydrate (SC) fractions in leaf, crown, and root tissues of annual bluegrass as it relates to 0, 40, or 80 d of ice encasement. Means from 2021 and 2022 are pooled together. Different capital black letters indicate statistically different TNC means, white letters are for SC, and gray letters are for WSC ($P \leq 0.05$) within a plant organ and statistical letters are derived from least significant difference tests ($P \leq 0.05$).



Percent recovery

The recovery of plants was estimated by measuring the percentage of green cover over time. Recovery was significantly influenced by cold and ice encasement duration (Figure 2.6; Table 1) since 40 and 80 d of ice encasement reduced the rate of recovery and maximum percent cover achieved compared to day 0 plants. For instance, after 40 d of ice encasement and a 36 d recovery period plants had an average of 47.35% green cover, whereas after 80 d of ice encasement and a 40 d recovery period plants had an average of approximately 31.15% green

cover. All day 0 annual bluegrass plants fully recovered to an average of 93.82% green cover after a recovery period of 9 d in the greenhouse. Of the 0 d plants, those that were maintained at 8% SWC started at a lower percentage of green canopy cover and had significantly less green cover than the 12% and 20% SWC treated plants for 6 d out of the 9-d recovery period. Plants treated with 8% SWC had a faster rate of recovery after 40 d under ice encasement. After a 36-d greenhouse recovery period, 8% SWC-treated plants had an average green cover of 65.67% while 12% and 20% soil moisture-treated plants averaged 40.56% and 35.81% green cover, respectively. No differences in recovery rate were detected among plants differing in SWC treatments following 80 d of ice encasement.

Figure 2.6 Recovery analysis as measured by green canopy cover (%) of annual bluegrass plants exposed to 8%, 12%, and 20% soil water content treatments after (A) 0 d, (B) 40 d, and (C) 80 d of ice encasement in low temperature (-3 °C) growth chamber conditions following a regrowth period in a greenhouse. Least significant difference (LSD) values are indicated on dates when significance was detected and LSD values are represented by vertical bars ($P \leq 0.05$) for treatment comparisons on a given day of treatment. Means from 2021 and 2022 are pooled together. Note that scales are different on each x-axis.

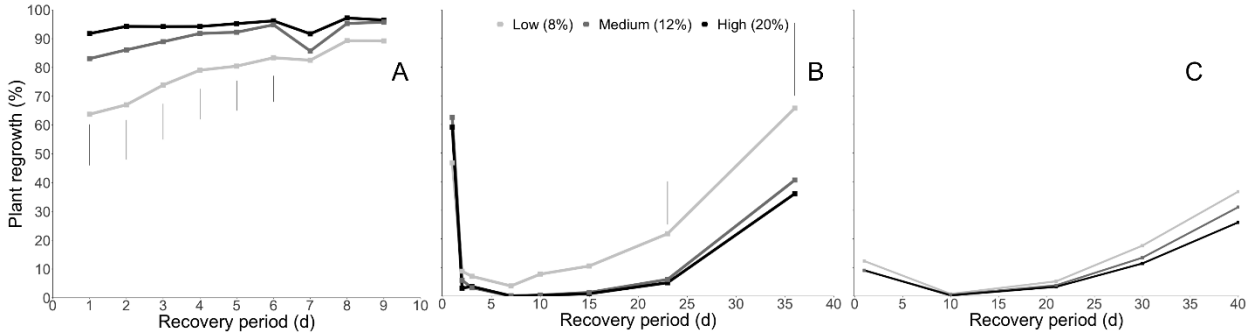
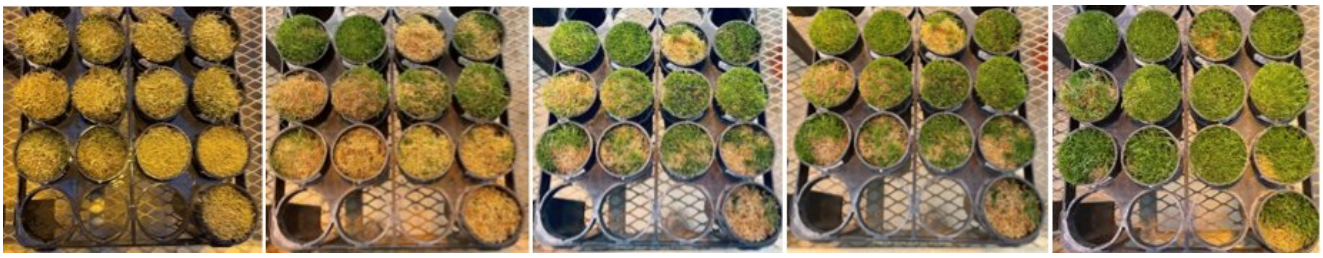


Figure 2.7 ABG plants throughout the recovery period after 40 d of ice encasement. Regrowth was assessed during a recovery period in a greenhouse. Plants were trimmed regularly and watered daily.



Discussion

Annual bluegrass plants sampled at 0 d experienced only cold acclimation conditions (4 °C) followed by de-acclimation and recovery in the greenhouse. During the cold acclimation or hardening process, high molecular weight sugars such as starches are typically hydrolyzed into monosaccharides to promote freezing tolerance (Tronsmo et al. 1993). Water soluble carbohydrates such as sucrose and nonstructural storage carbohydrates such as fructans have been found to accumulate following cold acclimation, but neither correlated to the freezing tolerance of annual bluegrass ecotypes (Dionne et al. 2001). The latter contrasts with findings in various other grass species (Livingston 1996; Suzuki and Nass 1988). Hoffman et al. (2014) report that higher levels of high molecular weight fructans may contribute to enhanced freezing tolerance of some annual bluegrass ecotypes for crown tissues. Our day 0 plant results expand the understanding of carbohydrate allocation in annual bluegrass following cold acclimation by including a comparison of leaf, root, and crown tissues. Storage carbohydrate quantities determined in our study can be ranked as leaf > crown > root for day 0 plants (Table 2). Low levels of photosynthesis at or near-freezing conditions could still be occurring in leaf tissues (Levitt 1980), causing leaf carbohydrates to be higher than in crowns or roots. Additional cold

acclimation regimes, pre-acclimation measurements, and cold acclimation durations would be needed to explain and further elucidate organ-level carbohydrate partitioning in annual bluegrass.

Ice encasement and cold duration reduced crown TNC and WSC dramatically compared to day 0 plants. The experimental design does not allow for explicit linkage of carbohydrate loss specifically to ice encasement stress, but rather the combination of the duration of low temperature and ice encasement as a single treatment. Ice encasement stress has been associated with a reduction in carbohydrates (Gao et al. 1983). McKersie et al. (1981) found that in crown tissue of winter wheat cultivars with higher tolerance to ice encasement had greater levels of total available carbohydrates and reducing sugars compared to cultivars less tolerant to ice encasement. The sensitive cultivar used in that study did not lose or utilize carbohydrates and thus the loss of viability was not associated with the depletion of carbohydrate reserves. It is not clear from our study if the loss of TNC over time was associated with stress or maintenance respiration during cold dormancy. Future research to identify individual sugars associated with organ-level changes in annual bluegrass along with tissue viability indicators may be warranted.

As meristematic regions essential to spring regeneration, crown and root tissue locations at or below the soil level facilitate the protection and insulation of crowns by foliage, thatch, and soil particles. While obvious, these are particularly important factors of grass plant survival of winterkill stresses. The lower levels of lipid peroxidation (oxidative stress), which indicates greater membrane health, in root tissues compared to leaf and crown tissues of cold-acclimated only (day 0) plants were likely associated with the protected location of the roots. The lack of damage to root tissue membranes observed here and the enhanced levels of carbohydrates observed during dry soil conditions compared to greater levels of soil moisture may warrant a need for more investigations of root acclimation, survival, and overwintering in annual

bluegrass, particularly in field conditions. In leaf tissue of plants exposed to 40 and 80 d of ice encasement at -3 °C, MDA content was higher compared to in 0 d plants not exposed to ice encasement and prolonged time at -3 °C. Our results are consistent with the increase in MDA content found in crown and leaf tissues of annual bluegrass in response to low-temperature duration with no ice encasement and in ice-encased plants (Laskowski and Merewitz 2021). Cold acclimation and extended durations of plant tissues in cold temperatures can cause reactive oxygen species generation and lipid peroxidation, particularly since cold temperatures influence membrane fluidity, stability, and dynamic regulation of fatty acid metabolism (Gill and Tuteja 2010). Crown tissue maintained low levels of MDA until 80 d in ice encasement and root tissues maintained low levels throughout the study. Leaf tissue had significantly higher MDA than day 0 plants at 40 d of cold and ice encasement. Thus, crown and root tissues appear to be more resilient to lipid peroxidation than leaf tissues; therefore, they may be protected by the soil when compared to leaf tissue.

During greenhouse recovery, cold-acclimated only plants (day 0) had a rapid resumption of leaf growth and canopy coverage, as expected. The 8%, 12%, and 20% SWC treatment levels during cold acclimation influenced annual bluegrass recovery following 40 d of cold temperature (-3 °C) and ice encasement. After 40 d under ice, the initial percent green canopy was high and then decreased to almost 0% after 2 d in the greenhouse. This is commonly seen in our ice encasement experiments and is likely due to the cold temperature preservation of chlorophyll and other leaf structures followed by post-hypoxic reaeration and light exposure conditions causing tissue browning. Plants that cold-acclimated in the 8% SWC treatment recovered quicker and to a greater level of green coverage than the plants cold acclimated at higher soil moisture contents. This supports the hypothesis that maintaining low soil moisture before cold temperatures or ice

encasement may result in quicker recovery after cold temperatures or ice encasement. Our analysis of TNC and MDA content in different organs shed some light on why this hypothesis was supported. Lipid peroxidation levels were not significantly influenced by soil moisture levels in any of the plant organs. Levels of TNC were not influenced by soil moisture during cold acclimation for crown or leaf tissues but were for root tissue. Root tissue levels of TNC, SC, and WSC were higher for day 0 plants in low soil moisture conditions compared to TNC levels of medium and high soil moisture-treated plants. Root carbohydrate content before overwintering may play an important role in annual bluegrass winter stress resilience.

Exposing a plant to a mild stress before a more severe or prolonged stress can precondition the plant and enhance survival. Drought preconditioning led to enhanced freezing tolerance in perennial ryegrass (Hoffman et al. 2012). In this study, leaf RWC was indicative of a mild drought stress imposition on the 8% SWC-treated annual bluegrass plants before ice encasement and low-temperature exposure. As stress response mechanisms often overlap for various abiotic stresses, the pathways or mechanisms activated by mild drought stress could have increased overwintering potential. It is also possible that plants with drier soil had more available oxygen in open soil pore spaces. Oxygen limitation plays a major role in ice encasement damage. Further research into soil moisture conditions in field conditions and drought preconditioning mechanisms that may influence cold acclimation or winterkill survival of annual bluegrass is needed.

Regarding applied turfgrass management strategies, our findings suggest that preventative measures such as reducing water inputs during the fall cold acclimation period or improving drainage prior to fall acclimation period may reduce annual bluegrass stand loss due to ice encasement. Other management strategies such as cultivation techniques such as core or solid

tine aerification or removal of snow from putting greens during the fall may assist with promoting drier soil conditions. These preventative measures should be used in conjunction with measuring and recording soil moisture levels, which could assist with forecasting potential damage associated with a given winter.

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CHAPTER 3: INFLUENCE OF CONTROLLED CONDITION ICE ENCASEMENT OF CREEPING BENTGRASS AND ANNUAL BLUEGRASS ON PLANT RECOVERY, GAS EVOLUTION, AND METABOLITES

Abstract

Two cool-season putting green turfgrass species, annual bluegrass and creeping bentgrass, are differential in ice encasement tolerance. Physiological mechanisms associated with creeping bentgrass ice encasement tolerance and annual bluegrass susceptibility are not understood. The objectives were to evaluate oxygen, ethylene, and CO_2 content within the upper soil space of the plants while frozen and immediately after ice melt after 0, 5, 10, 20, and 28 days of ice encasement (2.54 cm of ice) in growth chamber conditions. Following ice melt, plant samples were separated into leaf, crown, and root tissues and used to evaluate carbohydrate and amino acid content. Annual bluegrass exhibited higher damage (slower recovery rates) on most sampling days compared to creeping bentgrass. The organs that were most damaged, and exhibited a differential principal component analysis snapshot, were the leaf and crown tissues. Creeping bentgrass may preserve leaf and crown tissues for post-winter recovery whereas significant metabolic changes occur in annual bluegrass leaves and crowns. Creeping bentgrass retained total amino acids in leaves following ice encasement whereas total leaf amino acid levels declined in annual bluegrass. Specific carbohydrates and amino acids such as the ability to maintain high levels of fructose, asparagine, and proline may be important indicators of the tolerance to ice encasement stress. Based on more prominent carbohydrate and amino acid loss in leaves and crowns and higher levels of CO_2 evolution, annual bluegrass may exhibit a higher metabolism and/or tissue damage during ice encasement compared to creeping bentgrass, which could reduce spring recuperative potential.

Introduction

Ice encasement on golf course putting greens is problematic in northern areas and could occur more frequently due to thawing and refreezing events that could lead to ice encasement in the future (Cohen et al, 2018). Ice encasement is when there is a thick layer of ice extending from the soil through the plant canopy, with leaves embedded in the ice. If the ice is nonpermeable, it can block gas exchange from the soil/plant to the atmosphere and is more stressful than snow cover, which still allows for gas exchange (Andrews, 1996). Creeping bentgrass (*Agrostis stolonifera*) and annual bluegrass (*Poa annua*) are two putting green species that differ significantly in their resistance to ice encasement, with creeping bentgrass being more resistant, recorded to withstand 120 days under ice, and annual bluegrass surviving approximately half of that duration (Beard, 1964; Tompkins et al., 2004; Vargas and Turgeon, 2004). Creeping bentgrass is a stoloniferous species while annual bluegrass relies primarily on tillering for lateral growth (Beard, 1978). The two plant species also have different competitive strategies since annual bluegrass spreads by tillering, competes by seed production, and new plant establishment, while creeping bentgrass relies more on vegetative survival and lateral vegetative growth via stolons (Lush, 1989; Jonsdottir, 1991). Other than differing morphology that could potentially lead to differences in stress resistance, it is not clear how creeping bentgrass is more winter resilient than annual bluegrass. A better understanding of the turfgrass physiology and viability during winter stresses, such as ice encasement, will assist with turfgrass breeding and management strategies that promote winter survival.

The mechanisms associated with the disparity in ice encasement resistance between these two turfgrass species, even when grown in a mixed stand under the same conditions, are not known. During winter dormancy, plants require carbohydrates to produce metabolic energy to

maintain tissue viability via respiratory pathways. Ice encasement can cause hypoxia or anoxia leading to the buildup of toxic anaerobic metabolic gases in the canopy and soil such as CO_2, butyrates, ethanol, and others, all while the plants may also be enduring low-temperature stress and be experiencing carbohydrate depletion (Aamlid et al. 2007; Clein and Schimel 1995). In grass species following ice encasement, anaerobic byproducts were higher in wheat (*Triticum aestivum*) compared to timothy grass (*Phleum pratense*), which is a more ice encasement resistant species, and timothy grass accumulated various organic acids that were not increased in wheat (Andrews 1997). Maintenance of carbohydrates by low levels of respiration or prevention of electrolyte leakage may be major determinants of ice encasement resistance and recuperative ability. Ice encasement was shown to reduce total non-structural carbohydrate levels in crown tissues of winter wheat due to 7 d of ice encasement (Gao et al. 1983). Glycolytic rates and activities of respiratory enzymes such as pyruvate decarboxylase are lower in ice-encasement-resistant grasses compared to those more susceptible (Andrews 1996), which could lead to major differences in total carbohydrates and other metabolites. Carbohydrate content of plant crown tissues was found to influence recovery from ice encasement stress of wheat plants contrasting in ice encasement resilience (McKersie et al. 1981). Evaluating the impact that ice encasement has on carbon stores and whether carbohydrate content is differentially altered due to ice encasement stress between creeping bentgrass and annual bluegrass during ice encasement will help elucidate survival mechanisms. It is unknown whether carbohydrate contents or metabolic rates of annual bluegrass and creeping bentgrass differ during winter dormancy or ice encasement for various plant organs.

Similarly, little is known about how other important metabolites like amino acids may play a role in ice encasement stress. Ice encasement is known to cause amino acid leakage due to

membrane damage (Pomeroy et al. 1983). The accumulation of some amino acids during stress can reduce the incidence of membrane damage, promote antioxidant responses, and regulate water potential of cells (Hayat et al. 2012; Hildebrandt et al. 2015). Following ice encasement, the sudden re-exposure to oxygen can cause major tissue damages and oxidative stress (Blokhina et al., 2002). Thus, characterizing total and individual amino acids between creeping bentgrass and annual bluegrass before and after ice encasement in leaves, crowns, and roots will assist with our understanding of ice encasement resistance mechanisms. It is possible that amino acids will be alternately metabolized or differentially lost through membrane leakage in the two species.

In addition to carbohydrates and other metabolites, hormone regulation plays a major role in influencing stress survival; however, major knowledge gaps exist for hormonal responses during ice encasement. Ethylene plays a major role in regulating plant cold acclimation (Szalai et al. 2000; Gaudet et al. 2011), is a major factor in plant responses to low oxygen conditions such as waterlogging (Hartman et al. 2019), and regulates respiration rates in grass species (Laskowski and Merewitz 2020). Whether ethylene affects stress incidence during plant dormancy and release from dormancy during low oxygen conditions at low temperatures is not known. Ethylene-promotive treatments resulted in decreased regrowth of annual bluegrass after extended time exposed to ice encasement while ethylene-inhibitory treatments resulted in improved regrowth (Laskowski and Merewitz 2020). Hormone changes during acclimation and cold tolerance of wheat varieties differing in frost tolerance have been reported (Kosova et al. 2012), but few conclusions could be made regarding ethylene. Therefore, the objectives of the study were to expose creeping bentgrass and annual bluegrass, a resistant and sensitive species, to ice encasement conditions in growth chambers to analyze differences in plant recovery duration and rate, gas evolution, and the content of sugars and amino acids in crown, leaf, and

root tissues. We hypothesized that annual bluegrass ice encasement damage could be associated with greater loss or utilization of metabolites and greater gas evolution compared to creeping bentgrass.

Materials and Methods

Plant material, Growth conditions, and Ice Encasement Treatments

Two experiments were performed to evaluate sod plugs (10.16 cm) of creeping bentgrass 'Penn-A4' and annual bluegrass (*Poa annua* var. reptans 'Two-Putt'). These were collected from fields at the Hancock Turfgrass Research Center, East Lansing MI. Plugs were collected on 6 Dec 2021 for experiment 1 and 22 Nov 2022 for experiment 2 to ensure natural acclimation and planted into plastic pots (15.24 cm diameter) and placed in a low temperature growth chamber (LTC-37; BioChambers Inc., Winnipeg, Manitoba, CA) at 4 ºC with fluorescent lighting with a 10 h photoperiod and 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR). The creeping bentgrass field was seeded in 2017 and the annual bluegrass field was seeded in 2021. All fertilizer, cultural practices, and soil type were equivalent for the fields. Plants were held at low temperature dormancy for longer for experiment 1 compared to experiment 2 due to societal issues in 2021 but the health of the plants did not seem to be effected. Ice encasement treatment was imposed on four replicate plants for different durations with destructive sampling. Plants on day 0 (17 Mar 2022 in experiment 1 and 7 Dec 2022 in experiment 2), day 5 (22 Mar 2022 in experiment 1 and 12 Dec 2022 in experiment 2), day 10 (27 Mar 2022 in experiment 1 and 17 Dec 2022 in experiment 2), day 20 (6 Apr 2022 in experiment 1 and 27 Dec 2022 in experiment 2), and day 28 (14 Apr 2022 in experiment 1 and 4 Jan 2023 in experiment 2). Clear acetate sheeting was wrapped around the pots to enable visualization of ice and to create a uniform ice layer above the plant surface. Ice encasement was imparted by misting deionized water over the

pots every 30 minutes in a –3 ºC chamber until an ice layer about 2.54 cm thick was formed and extended from soil to above the plant canopy, with leaves imbedded in the ice. The ice layer was maintained by daily misting, as needed, throughout the study.

Carbon dioxide, oxygen, and ethylene analysis

Just after planting in pots, each pot was modified by drilling a small hole and inserting a rubber stopper with a septum (8-9 mm). The stopper filled the hole and was located just at the canopy/soil interface and would be just below where the ice layer would lie. Just prior to ice encasement treatment imposition, all drainage holes on the bottom of the pot were covered with polypropylene tape. Gas measurements were made on days 0, 5, 10, 20 and 28 while ice was still present by taking gas samples from pots while in the low temperature growth chamber (-3 ºC). Plants on a respective sampling day, as described above, were taken out of the low temperature chamber and moved to a growth chamber (ENR Growth Room; BioChambers Inc.) set to 10 °C overnight. To gather gas samples while the pots and ice were still frozen, the rubber stopper/septum port was pierced with a syringe. To gather gas samples following melt, aluminum foil was tightly wrapped around each pot while melt was occurring overnight at 10 °C. In the morning, a syringe was used to pierce through the aluminum foil to obtain headspace gas samples.

Carbon dioxide and oxygen were measured using a combination of an infrared gas analyzer (model 225-MK3; Analytical Development Co., Hoddesdon, UK) and a paramagnetic oxygen analyzer (Series 1100; Servomex Co., Crowborough, UK) linked in series and operated in a flow-through mode with N_2 as the carrier gas (150 mL • min^{-1}) as described in Beaudry et al. (1992). A standard gas sample was used to calibrate the instruments and calculate the percentage of carbon dioxide and oxygen in the gas samples. Gas concentrations were calculated using a

certified gas standard (Matheson Gas Products, Montgomeryville, PA) containing 0.979 $\mu L \cdot L^{-1}$ ethylene, 4.85% CO_2, and 1.95% O_2, balanced with N_2. Since differences were likely between species in the amount of live tissue capable of respiration, which would influence respiratory CO_2 levels, data was normalized or divided by percent green tissue at the time of sampling for each species.

Ethylene gas was measured from gas samples using a gas chromatograph (GC; Carle Series 400 AGC; Hach Co., Loveland, CO). The GC was fitted with a 6-m long, 2-mm I.d. stainless steel column packed with activation alumina F-1 (80/100 mesh) and was equipped with flame ionization detector. An ethylene standard of 1 ppm was used to calibrate the instrument. A 1-mL gas sample was taken from the plant pots and then injected into the GC. The concentration of ethylene was calculated based on the standard. The detection limit was 0.005 $\mu L \cdot L^{-1}$.

Tissue sampling and recovery analysis

After ice encasement plants were moved to a 10 ºC growth chamber overnight for the ice layer to thaw and melt. Then destructive sampling was performed by cutting the turfgrass plugs in half and separating one half into crown, leaf, and root tissues, which were then immediately frozen in liquid nitrogen and stored in a freezer at –80 °C until they were analyzed for metabolites. The remaining half was repotted, de-acclimated at 10 ºC and 4℃ each for 1 week and moved to a greenhouse at an average day/night 23/16 ºC, 14 h photoperiod, average 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR supplemented with high pressure sodium lamps for recovery analysis. Digital images were taken throughout the recovery period and analyzed with digital analysis software [Canopeo (Patrignani and Ochsner 2015)] to determine the total percent green cover. Images were taken until full recovery (>= 80-90%) or until 30 d of recovery in the greenhouse. The recovery rate was calculated as the increase in percent green cover over the amount of time to reach 80% green

cover for either species. This 80% value was determined to be the average value where all recovery rates began to plateau over time.

Metabolite analysis

Stored and frozen leaf, crown, and root tissue were ground in liquid nitrogen using a tissue homogenizer (1600 MiniG; SPEX SamplePrep LLC, Metuchen, NJ, USA) and extracted by methods in Roessner et al. (2000) and Rizhsky et al. (2004). Metabolites were extracted by transferring 50 mg of frozen, ground tissue into 2-mL tubes. Internal standard cocktails consisting of succinate-d_4 (2,2,3,3), pyruvate-$^{13}C_3$, ribitol (10 µl, 5 mg • ml^{-1}), and labeled amino acids were added to each sample. Then 600 µL of methanol/chloroform (1:2 v/v) + 1% formic acid and 0.01% BHT was added to each tube and were homogenized in a lab shaker (Thermomixer; Eppendorf North America, Inc.) for 3 minutes. Next, 500 µL of Milli-Q water was added and tubes were vortexed for 15 seconds. Then tubes were incubated on ice for 1 h. Tubes were then centrifuged (5430R; Eppendorf North America, Enfield, CT, USA) at 13500 x g for 15 minutes at room temperature. The upper aqueous layer was transferred to a new 2-mL tube and set to the side. To the original tube, another 500 µL of Milli-Q water was added and tubes were vortexed again for 15 seconds and then centrifuged at 13500 x g for 15 minutes at room temperature. The upper aqueous layer was again transferred to the same microcentrifuge tube as before. In a new glass vial, 500 µL of the upper aqueous solution was transferred in preparation to be dried. The upper aqueous layer was evaporated to dryness in a SpeedVac (Savant SC110; Thermo Fisher Scientific, Waltham, MA, USA). Once the solvents fully evaporated, the glass vials went on to the derivatization process.

Derivatization of the amino acids was done through methoximation and trimethylsilylation to prepare for GC-MS analysis. To each vial for metabolite analysis, 50 µL of

methoxyamine-HCl/pyridine solution was added. The glass vials were heated at 60 °C for 24 hours and then allowed to cool to room temperature. Then 50 µL of N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) containing 1% trimethylsilyl chloride (TMSCl) was added to each glass vial. These glass vials were heated again at 60 °C for 24 hours and then allowed to cool to room temperature. Finally, 50 µL of the reaction mixture was transferred to an autosampler vial equipped with a low volume insert.

Gas chromatography-mass spectrometry

The reaction mixture of derivatized extracts were analyzed with a gas chromatograph coupled with a mass spectrometer (Agilent 5975 Series MSD; Agilent Technologies, Santa Clara, CA, USA). For amino acid analysis, a 1 µL aliquot of the extracts were injected into a 30m VF5 column (Agilent J&W GC Columns; Agilent Technologies, Santa Clara, CA, USA). The inlet temperature was set at 230 °C. After a 5.5 min solvent delay, the initial GC oven temperature was set at 40 °C; 1 min after the injection, the GC oven temperature was raised to 80 °C for 1 min then to 240 °C for 16 min and finally to 320 °C for 9 min. Helium was used as a carrier gas with a constant flow rate set a 1 mL min^{-1}. For sugar analysis, a 1 µL aliquot of the extracts were injected into a 30m VF5 column. Injected aliquot was divided into a 40 split ratio. The inlet temperature was set at 230 °C. After a 4 min solvent delay, the initial GC oven temperature was set at 80 °C; 0 min after the injection, the GC oven temperature was raised to 190 °C for 2.75 min then to 320 °C for 7.25 min. Helium was used as a carrier gas with a constant flow rate set a 1 mL min^{-1}. Compounds were identified based on retention time and comparison with reference spectra in mass spectral libraries. Relative content is utilized and acknowledged throughout the manuscript.

Experimental design and statistical analysis

The experimental design was a split-plot design within one growth chamber with ice encasement duration as the whole plot and species (annual bluegrass or creeping bentgrass) split within each whole plot with 4 replications per treatment. The experiment was repeated using the same growth chamber. Normality was assessed using visual analysis of residual plots and Levene's test for homogeneity of error. If measurements did not conform to the assumptions of normality, the data were transformed. The data presented are the back-transformed means whereas P values are from the transformed analysis. Data resulting from all measured parameters were subjected to analysis of variance (ANOVA) using RStudio software (version 4.1.0, Boston, MA, USA) using a linear mixed model to determine the main and interacting effects of the experimental factors. Mean separations were performed using Fisher's protected least significant difference test at the $p \leq 0.05$ level. Due to lack of significant effect, years are pooled together for all parameters. Analysis of CO_2 and ethylene were normalized using the recovery analysis data by determining the amount of gas per amount of percent green cover for each species. Lactose data were square root-transformed to meet the assumptions for ANOVA and later back-transformed to the original scale for interpretation.

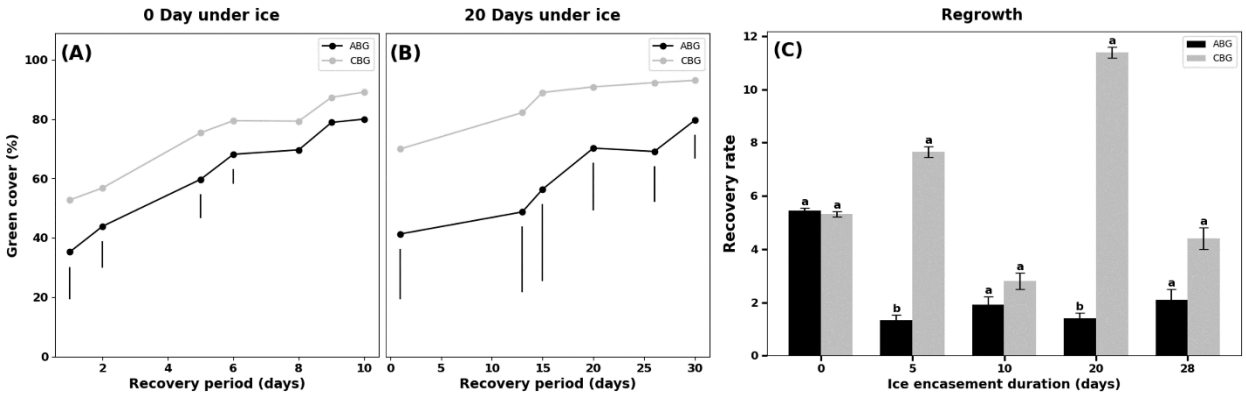
Results

Recovery analysis

Turfgrass plants that were sampled on day 0, which were cold acclimated but not treated with an ice encasement layer, recovered quickly for both species. The day 0 plants took approximately 8 days to reach 70 to 80% canopy coverage (Figure 4.1A). All durations of ice encasement extended the recovery period compared to day 0 plants, but more so for annual bluegrass compared to creeping bentgrass. For example, an ice encasement treatment period of 20 d

required 13 d of recovery for creeping bentgrass to reach 80% recovery but took 30 d of recovery for annual bluegrass (Figure 4.1B). Since the species differ in initial percent green cover just after ice encasement, the recovery rates were calculated and compared between the grass species of plant sets for each treatment duration (Figure 4.1C). Plants that had ice encasement for 0 days had similar rates of recovery, whereas 5 through 28 days of ice encasement caused a numerically lower rate of recovery for annual bluegrass compared to creeping bentgrass; however, only 5 d and 20 d were statistically higher rates for creeping bentgrass compared to annual bluegrass (Figure 4.1C).

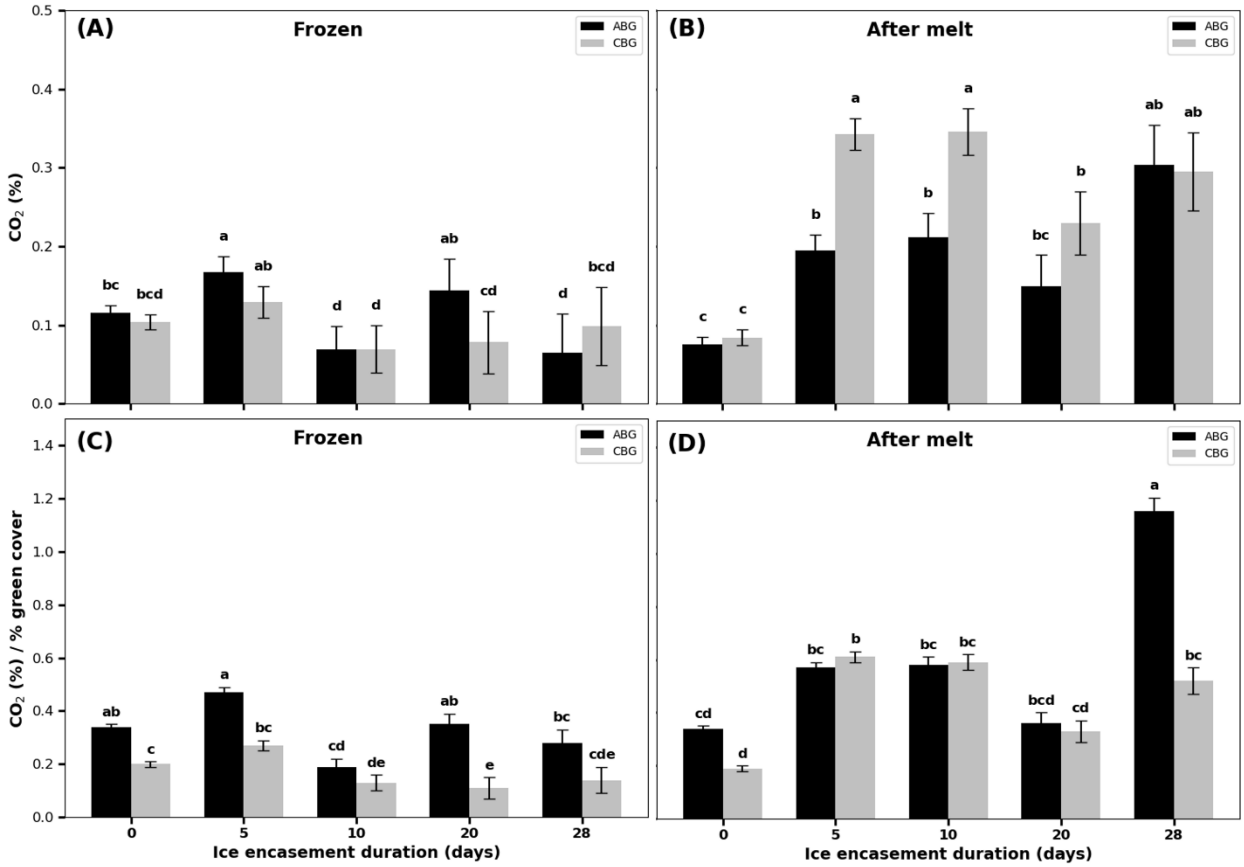
Figure 4.1 Recovery over time for plants subjected to A) 0 d of ice encasement (cold acclimation only) and B) 20 d of ice encasement. C) The rate of recovery calculated as the change in percent green cover over time of annual bluegrass (ABG) and creeping bentgrass (CBG) plants after 0, 5, 10, 20, and 28 d of ice encasement. Different lowercase letters indicate statistical differences in rate of recovery within each duration of ice encasement and comparing species. Letters of significance are based on Fisher's protected least significant difference values ($P \leq 0.05$).



Carbon dioxide and oxygen content

Oxygen content was not significantly different among treatments for any main or interacting effects before or after ice layers melted (data not shown). CO_2 concentration was similar between species and across durations, while the plants were frozen, except for day 20 samples, the CO_2 levels were higher in annual bluegrass compared to creeping bentgrass (Figure 4.2A). When gas samples were taken following melt, the CO_2 levels were higher with increasing durations of ice encasement (Figure 4.2B). On 5 and 10 d samples, creeping bentgrass had higher CO_2 levels than in annual bluegrass. Since the differences in the amount of live tissue capable of respiration would influence the gases resulting from plant metabolism such as CO_2, data was normalized or divided by the amount of percent green tissue at the time of sampling for each species (Figure 4.2C, D). Following this normalization, annual bluegrass had higher CO_2 concentrations compared to creeping bentgrass on most dates while frozen and after 28 d of ice encasement when samples were taken while melted (Figure 4.2C).

Figure 4.2 Carbon dioxide (CO_2) levels sampled from potted creeping bentgrass (CBG) and annual bluegrass (ABG) plants with different durations of ice encasement treatments. (A) The frozen samples were taken from below the ice layer. (B) After the ice was melted, air samples were taken from the headspace of the pot. (C and D) are normalized values based on the percent green cover of each species and at each time point. Different lowercase letters indicate statistical differences in CO_2 levels across duration of ice encasement and species. Letters of significance are based on Fisher's protected least significant difference values ($P \leq 0.05$).

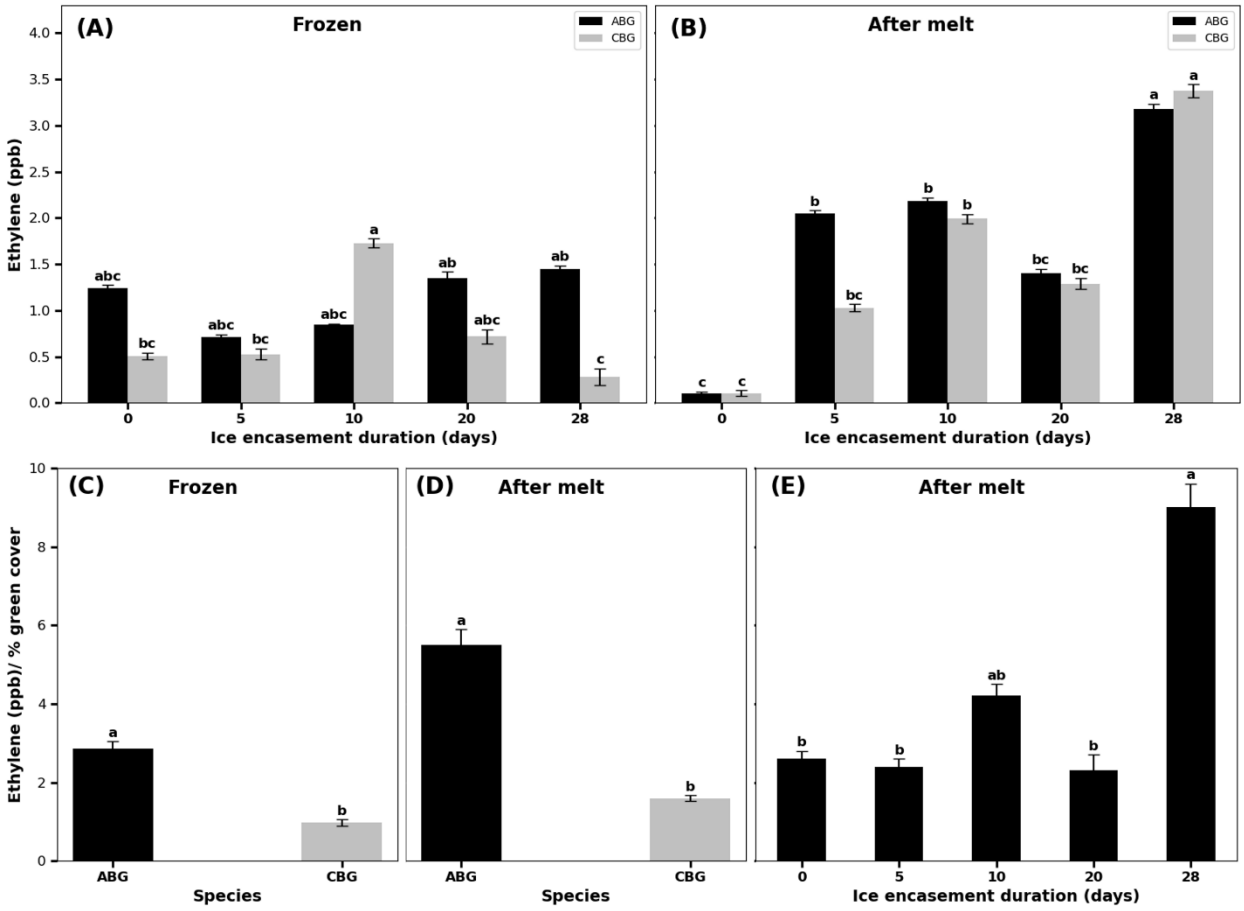


Ethylene content

Ethylene content was relatively unchanged by ice encasement duration and was consistent between species when assayed while samples were still frozen from the soil and plant interface under the ice layer (Figure 4.3A). After samples melted, ethylene concentration in the headspace

was higher in plants that experienced longer durations of ice encasement treatment. For instance, day 0 plants had approximately 0.1 ppb ethylene whereas plants exposed to 28 d of ice encasement had approximately 3.0 to 3.5 ppb of ethylene (Figure 4.3B). To account for differences in live tissue, the ethylene data was normalized by percent green cover at the time of sampling (Figure 4.3C, D, E). With normalization, ethylene main effects of species were significant and indicated that annual bluegrass had higher ethylene per percent green cover compared to creeping bentgrass when samples were taken while frozen and after melting. The main effect of ice encasement duration showed that ethylene content was 71.5% greater for both species at 28 d of ice encasement compared to day 0 levels. The interaction of species and duration was not significant for this parameter ($p > 0.05$).

Figure 4.3 Ethylene content of a potted creeping bentgrass or annual bluegrass plant with different durations of ice encasement treatments. Pots were sealed with foil and allowed to equilibrate for one hour prior to gas sampling from the headspace. Different lowercase letters indicate statistical differences in ethylene levels across duration of ice encasement and species (A and B) or across species or durations (C, D, E). Only main effects are shown for values normalized by % green cover (C, D, E) since the interactions of species x duration was not significant in the ANOVA. Letters of significance are based on Fisher's protected least significant difference values ($P \leq 0.05$).



Carbohydrates

Relative contents of glucose, fructose, sucrose, and lactose were analyzed since these were the primary and most abundant sugars found during GC/MS chromatogram analysis. In creeping bentgrass, leaf and crown tissues generally had higher levels of glucose and fructose compared to root tissue (Table 4.1). The total sugar content across organs declined in annual bluegrass due to 20 d of ice encasement but was not significantly reduced in creeping bentgrass (Table 4.2).

Table 4.1 Main effects for tissue type for relative content of glucose, fructose, and sucrose, of annual bluegrass and creeping bentgrass for crowns, leaves, and roots. Analysis of variance (ANOVA) and Fisher's protected least significant difference test at a 0.05 P level were used to detect differences between treatment means. Within each measured parameter for each species, means followed by different letters are statistically different (P ≤ 0.05). ABG, annual bluegrass (*Poa annua*). CBG, creeping bentgrass (*Agrostis stolonifera*).

	ABG			CBG		
	Crown	Leaf	Root	Crown	Leaf	Root
Glucose	34.0 b	95.2 a	38.7 b	122.2 a	101.8 a	40.1 b
Fructose	176.1 ns	158.5 ns	54.8 ns	244.5 a	282.8 a	64.1 b
Sucrose	16.94 ns	16.63 ns	5.85 ns	23.83 a	14.77 ab	9.10 b

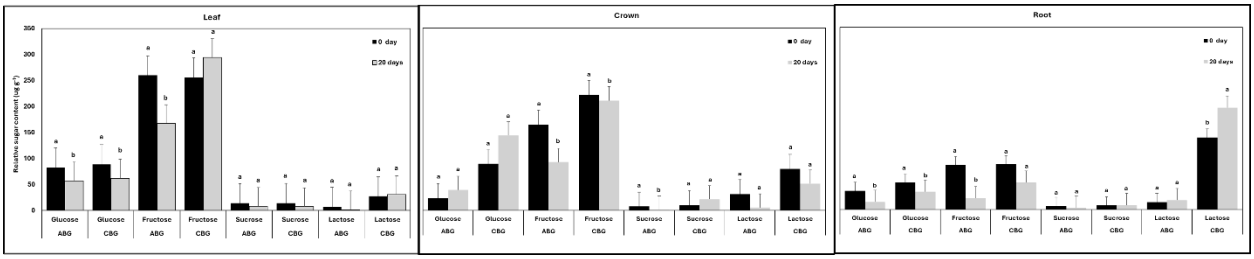
Table 4.2: Variation in total sugar content (µg g^{-1}) and total amino acid content (µg g^{-1}) in the leaves, crowns, and roots of two different cool-season turfgrass species (Annual bluegrass - ABG and Creeping Bentgrass - CBG) for two different durations of ice encasement (0 day and 20 days) during the controlled environmental study conducted in East Lansing, Michigan in 2021 and 2022. Means sharing a common letter within each metabolite were not significantly different from each other (P ≤ 0.05).

Ice encasement duration (days)	Metabolite			
	Relative total sugar content (ug g^{-1})		Relative total amino acid content (ug g^{-1})	
	ABG	CBG	ABG	CBG
0	2858.3 a	3070.5 a	15404.7 a	10151.4 b
20	1445.8 b	2820.3 ab	7382.1 b	7093.6 b

In annual bluegrass, glucose was higher in leaf tissue compared to crowns and roots. Comparing species, leaf tissue sugars exhibited similar changes due to 20 do of ice encasement in both species, except that fructose content declined in annual bluegrass due to ice encasement whereas this did not occur in creeping bentgrass (Figure 4.4a). Ice encasement reduced relative glucose and fructose content in annual bluegrass in leaf tissues since the concentration decreased from day 0 to day 20 plants by 70.0% and 78.4% in leaf tissue, respectively (Figure 4.4a). Annual bluegrass crowns had 84.7% lower levels of glucose than in creeping bentgrass crowns after 20 d of ice encasement (Figure 4.4b). For annual bluegrass crowns there was a loss of 91.2% fructose (Figure 4.4b) and a loss of 68.5% fructose in root tissues (Figure 4.4c). Annual bluegrass leaf tissue was 81.5% lower and crown tissue was 84.7% lower in fructose in annual bluegrass compared to creeping bentgrass plants within 20 d of ice encasement samples. Amongst organs, lactose content was higher in roots compared to crowns and leaves within a species (Figure 4.4

a,b,c). In all tissue types, annual bluegrass had lower lactose content compared to creeping bentgrass. For example, the lactose content in annual bluegrass was 31.7%, 77.0%, and 78.7% lower than in creeping bentgrass leaves, crowns, and roots, respectively.

Figure 4.4 Relative abundance of carbohydrates detected in annual bluegrass (ABG) and creeping bentgrass (CBG) (A) leaves, (B) crowns, or (C) root tissues exposed to 0 d of ice encasement (cold acclimation only) and 20 d of ice encasement during the 2021 and 2022 controlled environmental study in East Lansing, Michigan. Relative abundance is based on comparison with ribitol (10 µl, 5 mg • ml^{-1}) as an internal standard and total area of peaks. Different lowercase letters indicate statistical differences within each sugar within each species and indicate the difference due to duration of ice encasement within a species. Letters of significance are based on Fisher's protected least significant difference values ($P \leq 0.05$). An asterisk indicates a statistical difference between species at a given duration of ice encasement. NS indicates not significant within a given species for a given carbohydrate.



Amino Acids

Eighteen amino acids were detectable in creeping bentgrass and annual bluegrass leaf, crown, and root tissues at 0 and 20 d of ice encasement and exhibited significant variation due to treatments (Table S2). Root tissues generally had a lower abundance of amino acids compared to leaf and crown tissues (Table 4.1). The total relative amino acid content across all organs was

higher in 0 d annual bluegrass compared to 20 d annual bluegrass organs and creeping bentgrass organs at 0 and 20 d of ice encasement (Table 4.2).

For leaves, average total amino acid content was unchanged in creeping bentgrass leaves but was reduced in annual bluegrass leaves due to 20 d of ice encasement (Figure 4.5). There was a significant interaction between duration and amino acids in leaves (Table S1, Figure 4.6); however, no three-way interaction was observed among amino acids, duration, and species for leaves. In annual bluegrass, the abundance of different amino acids in leaves ranged from 22.10 ug g^{-1} (methionine) to 2538.19 ug g^{-1} (glutamine). The abundance of glutamine was significantly higher than all other amino acids (Table S1). In creeping bentgrass, amino acid abundance ranged from 25.08 ug g^{-1} (histidine) to 1232.18 ug g^{-1} (glutamine). The most abundant amino acid was glutamine, followed by proline (435.77 ug g^{-1}). The abundance of alanine, arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine were not different from each other. Some leaf amino acid abundances were higher in annual bluegrass compared to creeping bentgrass such as histidine, isoleucine, methionine, tryptophan, and tyrosine.

In crown tissues, amino acid content was significantly affected by both ice encasement duration and species. A three-way interaction was observed among duration, species, and amino acids (Table S1, Figure 4.7). Following cold acclimation and for plants with no ice encasement (0 d plants), annual bluegrass had higher levels of several amino acids compared to creeping bentgrass, including alanine, asparagine, phenylalanine, threonine, tyrosine and valine. Conversely, comparing 0 d plants, creeping bentgrass had higher levels of arginine than annual bluegrass. Since these differences in amino acids were found between species in 0 d samples for several amino acids, the results will primarily focus on the change in each amino acid in

response to ice encasement within a given species. In annual bluegrass crowns, ice encasement caused a significant reduction in 15 amino acids (alanine, asparagine, aspartic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine, and valine) whereas in creeping bentgrass ice encasement caused a reduction in 11 amino acids (asparagine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalaine, threonine, tyrosine, valine). The greatest loss of amino acids in annual bluegrass crowns occurred for asparagine, glutamine, and proline.

Roots of both species were generally less influenced by ice encasement for changes in amino acid contents (Figure 4.8). Ice encasement duration caused a loss of only root aspartic acid and glutamic acid when averaged across both species (Figure 4.8A). Creeping bentgrass roots generally had higher levels of arginine and glutamic acid compared to annual bluegrass whereas annual bluegrass had higher levels of asparagine and glutamine compared to creeping bentgrass roots. There was no major influence of duration or species on the other root amino acids observed.

Figure 4.5. Relative abundance of total amino acids detected in annual bluegrass (ABG) and creeping bentgrass (CBG) for (A) leaves (B) crowns and (C) roots. Relative abundance is based on comparison with ribitol (10 µl, 5 mg • ml^{-1}) as an internal standard and total area of peaks. Bars represent means ± standard error and bars not sharing a common letter within a given species are considered significantly different (P ≤ 0.05).

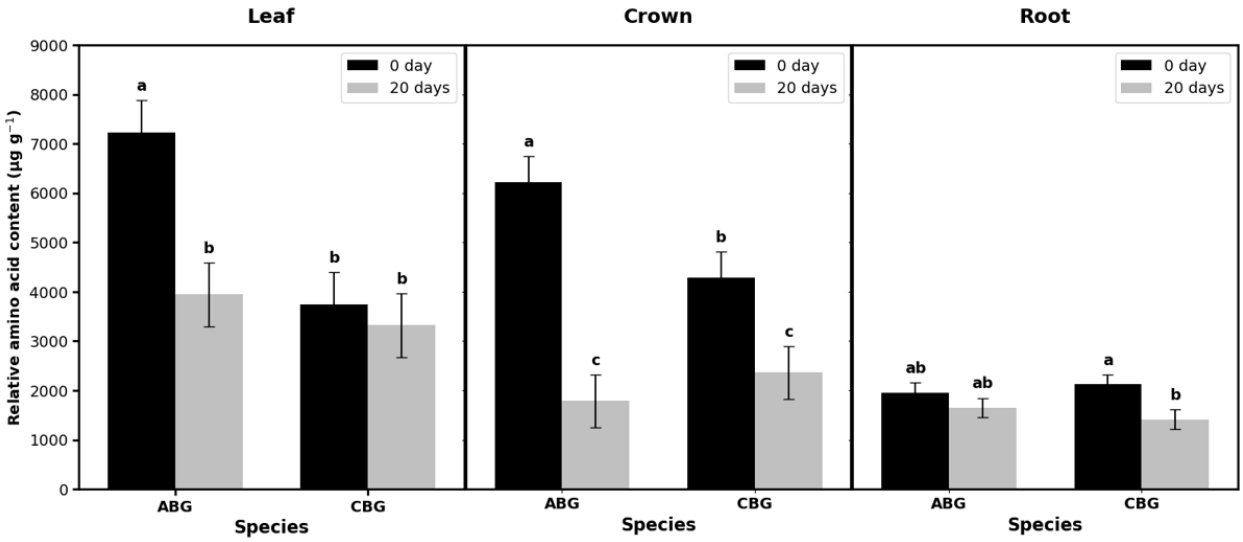


Figure 4.6. Main effect of species on relative abundance of various amino acids (µg g^{-1}) in leaf tissue of two different cool-season turfgrass species [annual bluegrass (ABG) and creeping bentgrass (CBG)] during a 2021 and 2022 controlled environmental study in East Lansing, Michigan. Relative abundance is based on comparison with ribitol (10 µl, 5 mg • ml^{-1}) as an internal standard and total area of peaks. Years are pooled together. Bars represent means ± standard error and bars not sharing a common letter within a given species are considered significantly different (P ≤ 0.05).

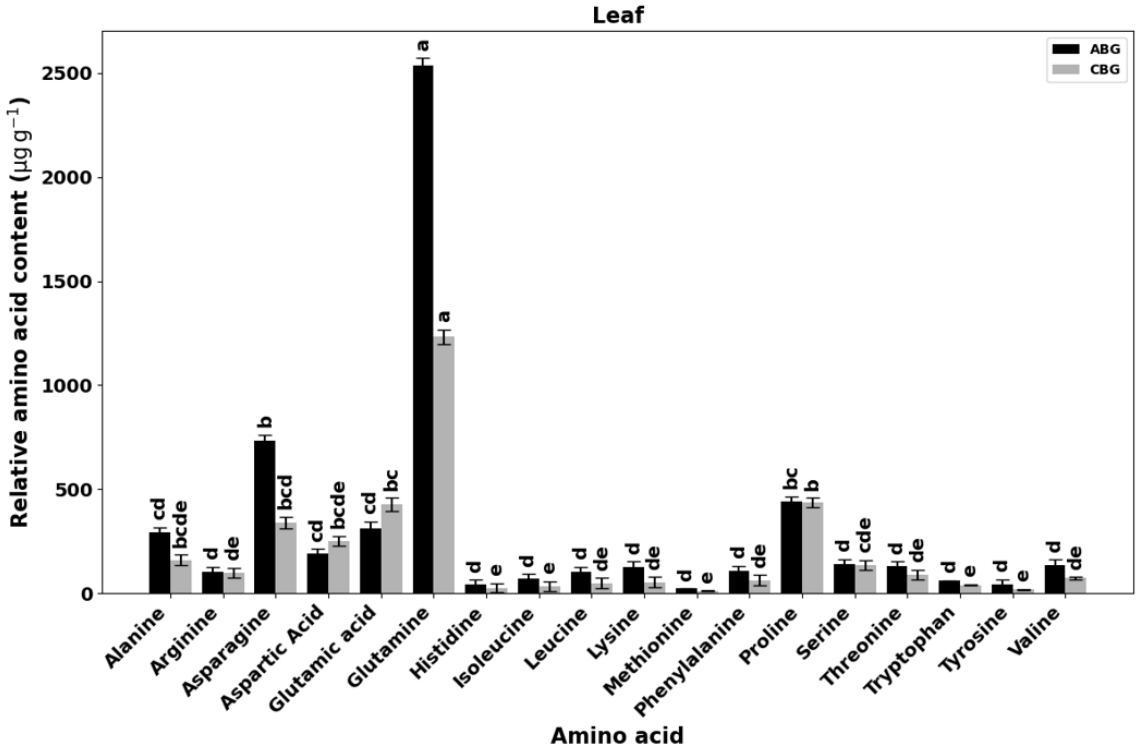


Figure 4.7. Relative abundance of various amino acids (µg g^{-1}) in crown tissue of two different cool-season turfgrass species [annual bluegrass (ABG) and creeping bentgrass (CBG)] in response to species and duration of ice encasement at 0 or 20 d during a 2021 and 2022 controlled environmental study in East Lansing, Michigan. Relative abundance is based on comparison with ribitol (10 µl, 5 mg • ml^{-1}) as an internal standard and total area of peaks. Years are pooled together. Bars represent means ± standard error and bars not sharing a common letter within a given species are considered significantly different (P ≤ 0.05).

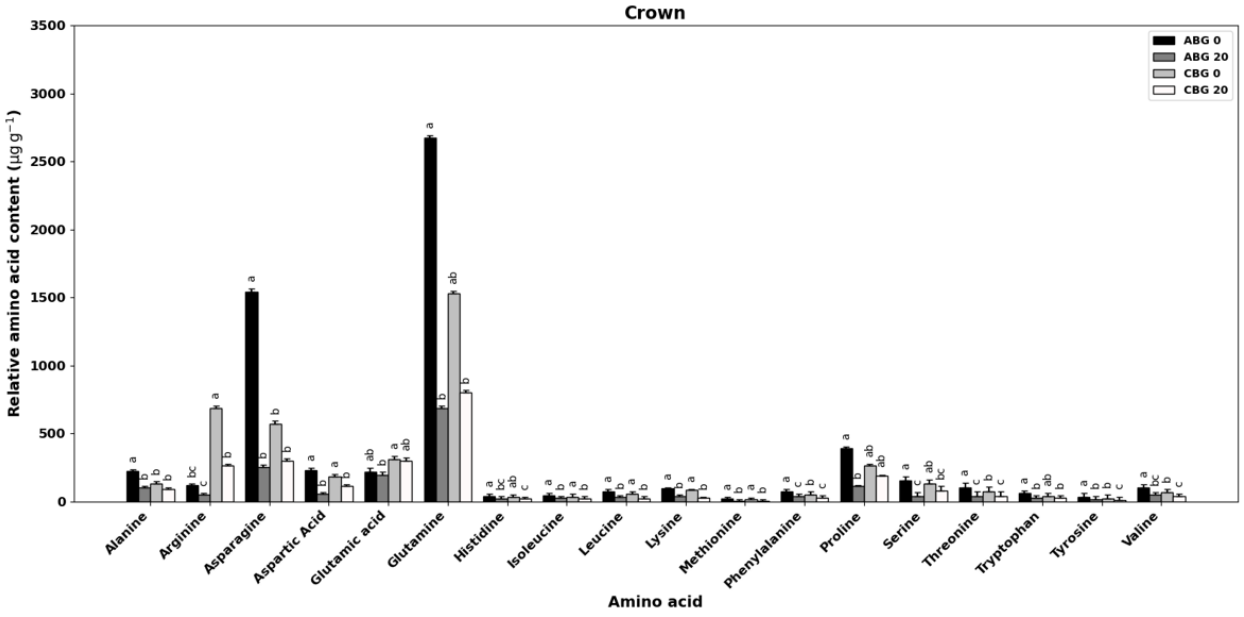
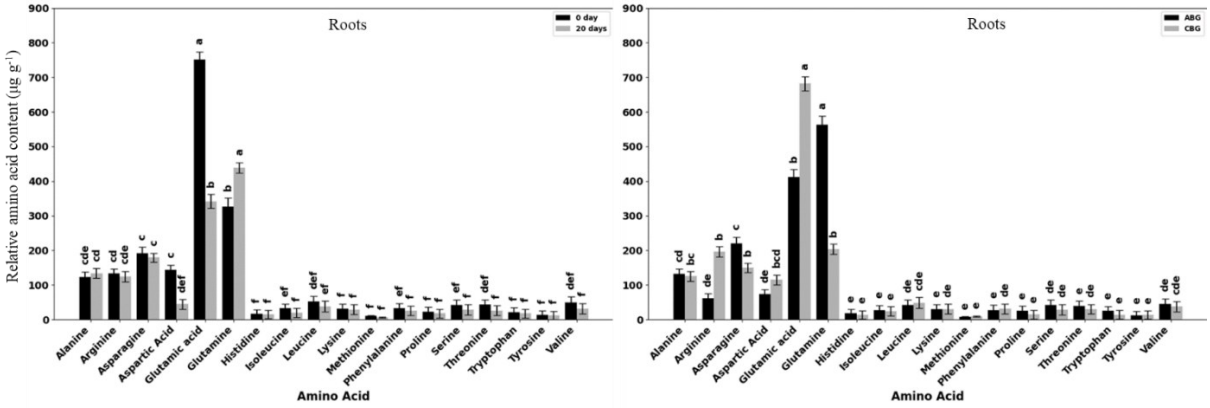


Figure 4.8. Relative abundance of various amino acids (µg g-1) in root tissue of two different cool-season turfgrass species [annual bluegrass (ABG) and creeping bentgrass (CBG)] in response to (A) duration of ice encasement at 0 or 20 d or (B) species during a 2021 and 2022 controlled environmental study in East Lansing, Michigan. Relative abundance is based on comparison with ribitol (10 µl, 5 mg • ml-1) as an internal standard and total area of peaks. Years are pooled together. Bars represent means ± standard error and bars not sharing a common letter within a given species are considered significantly different ($P \leq 0.05$).

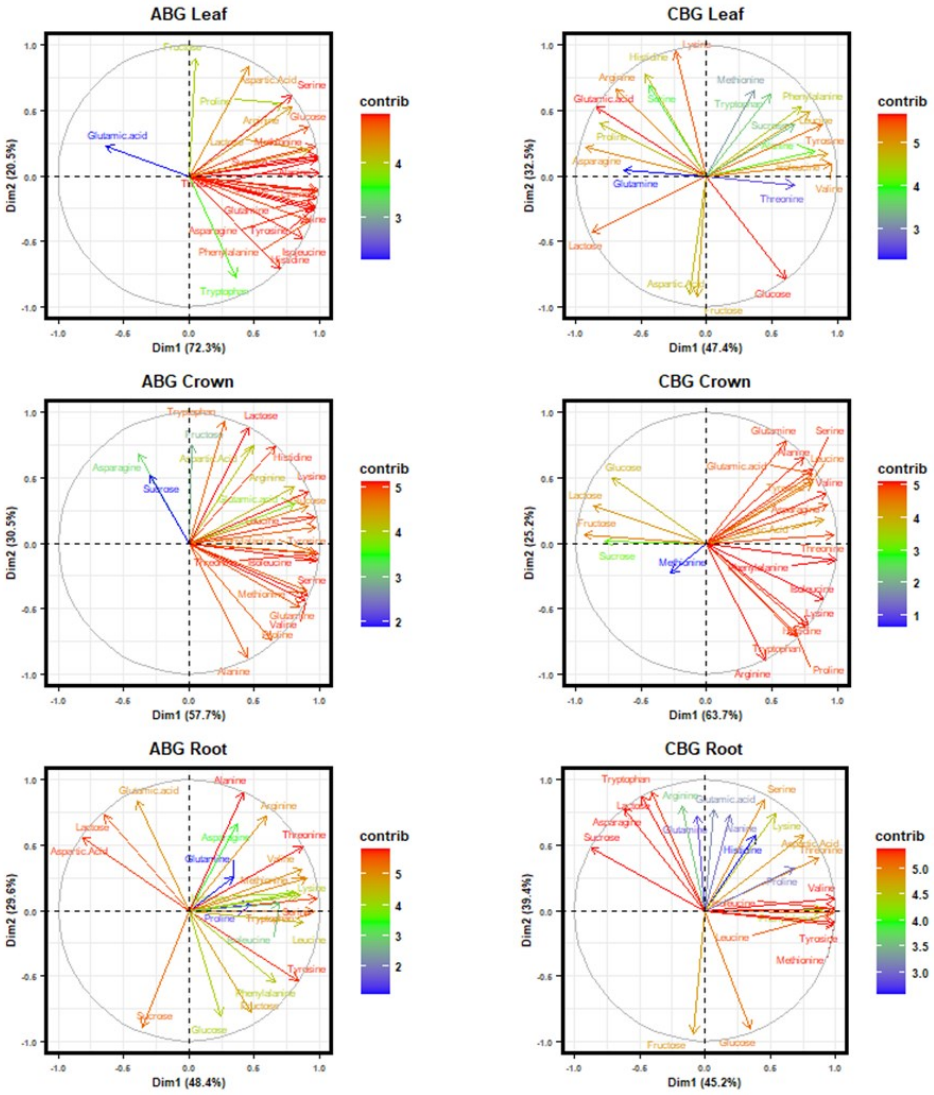


Principal Component and Correlation Analyses

Principal component analysis indicates that amino acids such as serine, methionine, threonine, alanine, lysine, leucine, glutamine, tyrosine, valine, asparagine, phenylalanine, isoleucine, and histidine, along with sugars glucose and sucrose, contributed most to the variation in annual bluegrass leaves, whereas glutamic acid contributed least to the variation (Figure 4.9). In contrast, in creeping bentgrass leaves, glutamic acid and glucose contributed most to the variation, while glutamine and threonine contributed least. Most amino acids and sugars were positively correlated in annual bluegrass leaves, whereas amino acids and sugars did not show positive correlation in creeping bentgrass leaves. Lactose and sucrose were negatively correlated

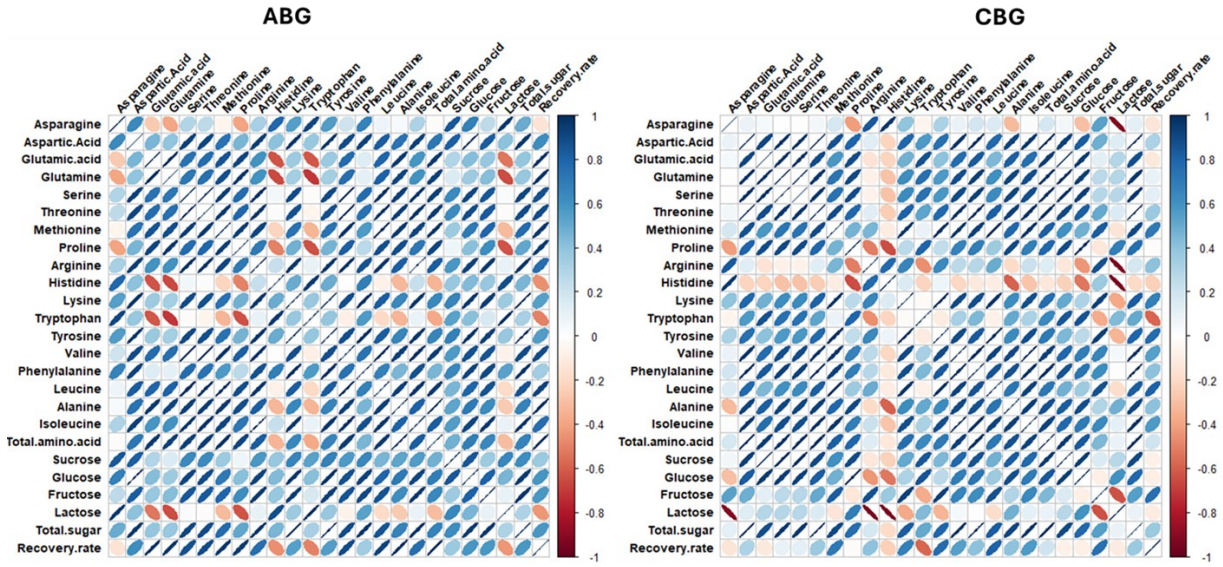
in creeping bentgrass leaves. In crown tissues, histidine, lysine, leucine, threonine, isoleucine, serine, and valine, along with sugars lactose, contributed most to the variation in annual bluegrass crowns, whereas sucrose contributed least to the variation. In contrast, in creeping bentgrass crowns, amino acids glutamine, alanine, serine, glutamic acid, valine, asparagine, threonine, phenylalanine, isoleucine, lysine, histidine, tryptophan, proline, and arginine contributed most to the variation, whereas methionine contributed least. All the sugars were positively correlated with each other and all the amino acids except methionine were positively correlated with each other in creeping bentgrass crowns. However, sugars and amino acids in general were negatively correlated with each other. In root tissues, alanine, threonine, serine, tyrosine, and aspartic acid contributed most to the variation in annual bluegrass roots, whereas glutamine and proline contributed least to the variation. Conversely, in creeping bentgrass roots, sucrose, asparagine, lactose, tryptophan, valine, isoleucine, leucine, tyrosine, and methionine contributed most to the variation. In annual bluegrass roots, sucrose and glucose were positively correlated, whereas lactose and fructose were negatively correlated with each other. However, in creeping bentgrass roots, lactose and sucrose, as well as fructose and glucose, were positively related to each other, but lactose and glucose were negatively correlated (Figure 4.9).

Figure 4.9 Principal component analysis of the leaves, crowns, and roots of annual bluegrass (ABG) and creeping bentgrass (CBG) under two different durations of ice encasement on the relationships of 18 different amino acids (alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and 4 different sugars (glucose, fructose, sucrose, and lactose) in 2021 and 2022. Traits with high contributions to the principal components are shown in red color.



In annual bluegrass, a strong positive correlation (r = 0.99) was found between total amino acids and the recovery rate, whereas in creeping bentgrass, a weaker positive correlation (r =0.20) was observed (Figure 4.10). In annual bluegrass, glutamic acid, glutamine, serine, threonine, methionine, proline, valine, leucine, alanine, and total amino acid showed a strong positive correlation ($0.8 \leq r \leq 1$) with the recovery rate, while aspartic acid, arginine, lysine, tyrosine, isoleucine, glucose, fructose, and fructose showed a moderate positive correlation ($0.5 \leq r < 0.8$) with the recovery rate. In creeping bentgrass, leucine showed a strong positive correlation with the recovery rate, whereas methionine, lysine, tyrosine, valine, isoleucine, and fructose showed a moderate positive relationship with the recovery rate. Tryptophan showed moderate negative correlation with the recovery rate in both species.

Figure 4.10 Correlation analysis of the abundances of 18 different amino acids (alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and 4 different sugars (glucose, fructose, sucrose, and lactose) in the leaves, roots, and crowns of annual bluegrass and creeping bentgrass under two different durations of ice encasement (0 day under ice and 20 days under ice) during the 2021 and 2022 controlled environmental study in East Lansing, Michigan, with the recovery rate following ice encasement stress.



Discussion

Annual bluegrass is more susceptible to ice encasement than creeping bentgrass since it has been found to survive after persisting under ice encasement for 80 d more than annual bluegrass (Beard 1964). Our recovery rate results are consistent with previous reports regarding higher resistance to ice encasement in creeping bentgrass compared to annual bluegrass. The short duration of the experiment for O_2 or insufficient sampling methods likely did not allow for hypoxic or anoxic conditions to be measured, since oxygen content was around atmospheric

levels at approximately 400 ppm. Studies using real time sensors to track oxygen levels may be important in future controlled condition research on ice encasement. Elevated levels of CO_2 were found due to ice encasement treatment, particularly by 28 d. Non-normalized CO_2 levels were generally higher in annual bluegrass when pots were frozen and on 28 d of ice encasement when normalized by percent green cover. CO_2 levels were higher in creeping bentgrass plants on a few dates following melt, when not adjusted for percent canopy cover. A study analyzing annual bluegrass compared to creeping bentgrass exposure to simulated anoxia using contained methods to expose turfgrass plants to specific gas concentrations compared at low temperatures found that annual bluegrass was more susceptible to higher CO_2 levels (Castonguay et al. 2009). While highly informative, the presence of actual ice has a major influence on cell physiology, such as causing membrane whorls, which could not be replicated with gas treatments alone (Pomeroy and Andrews, 1979). While frozen and under ice, minimal photosynthesis may occur, and elevated CO_2 could be the result of higher levels of respiration in annual bluegrass tissues. In the samples following melt and at above freezing temperatures and while exposed to light in the growth chambers, photosynthesis could have been taking in CO_2 at the same time as respiration using CO_2 thus, normalized values for percent green cover may be the most correct. Regardless, more assessments would be needed to compare these two species directly for CO_2 evolution.

While ice was still frozen and in non-normalized ethylene values, there was not much difference in ethylene evolution, which is likely related to the low level of metabolism at temperatures below freezing. Ethylene values following melt and when normalized reflect metabolism being reactivated and ethylene being released. This occurred earlier for annual bluegrass and at an overall higher level for annual bluegrass compared to creeping bentgrass. Ethylene levels typically increase during abiotic and biotic stresses and can be induced by

damage and senescence processes (Hall et al. 1977). Annual bluegrass accumulated more ethylene which could indicate an earlier release from dormancy, increased stress response, or active senescence occurring in this species compared to creeping bentgrass. Annual bluegrass requires a lower threshold temperature to induce de-acclimation and exhibits greater losses of freezing tolerance when compared to creeping bentgrass (Hoffman and DaCosta 2014). Additional time course evaluations of ethylene in this type of controlled ice encasement system may be of value since ethylene can be a transient response and was influenced by temperature in this study.

Carbon dioxide fixation and utilization directly ties into carbohydrate metabolism, which is important during overwintering, ice encasement, and the ability to regrow during spring, particularly for crown tissue. There were positive relationships between sugar contents and the rate of recovery following ice encasement stress in both species. Crown tissue levels of glucose and sucrose were relatively unchanged for both species, but fructose was lost in annual bluegrass crowns but not in creeping bentgrass crowns. Similar results were found for leaf and root tissues. These results are consistent with a previous study on annual bluegrass that showed a decline in water soluble sugars and storage carbohydrates in annual bluegrass organs during ice encasement (Gendjar and Merewitz 2023). For loss of measured free sugars, four possibilities exist, which are not mutually exclusive, 1) sugars are utilized by respiratory pathways for energy creation 2) sugars are lost due to electrolyte leakage and membrane damage, 3) there is a change in the amount of fructan hydrolysis or 4) there is an alternate source of these sugars (photosynthesis in either species or stolons in creeping bentgrass). In the current study, fructose was the sugar among the highest content in all plant organs analyzed. Fructose may be lost in leaf, crown, and root tissues more readily in annual bluegrass than creeping bentgrass. Fructans, or fructose

polymers, are known to be the major storage carbohydrate for grass species and are known to act as energy reserve and a source of fructose during overwintering in grasses (Yoshida et al., 1998). Cold stress can induce enzymes to break down fructans into fructose monomers, likely to prevent freezing stress and prevent membrane damage (Valluru and Van den Ende, 2008) and fructan hydrolysis is associated with consumption for energy production and survival during the winter (Yoshida 2021). During cold acclimation, sugars such as fructose, glucose, and sucrose can also accumulate as protective osmolytes (Savitch et al. 1999). Plants of both species at 0 d of sampling likely had high levels of fructose due to the cold hardening process and by 20 d of dormancy and ice encasement, metabolism in annual bluegrass started to utilize fructose. For crown tissues, since only fructose and sucrose were lost but glucose was unchanged, it is more likely that annual bluegrass crowns may have an active respiratory metabolism or change in fructan hydrolysis during dormancy rather than the explanation that only some sugars were lost due to electrolyte leakage. It is possible that morphology could play a role in these sugar metabolic differences found since annual bluegrass lacks stolons, whereas creeping bentgrass has them, which could be a source of sugars during dormancy or stress. Previous literature for crown tissue such as Hoffman and DaCosta (2014) supports the concept of active metabolism in annual bluegrass, as discussed above. In leaves, there were reductions in glucose, fructose, and the sucrose levels, although sucrose was not statistically significant. This could indicate less photosynthetic production and/or leaf damage could have caused electrolyte leakage and loss of sugars. The reduction of sugars in root tissue were most likely due to reduced source production due to lack of photosynthetic processes and utilization of sugars via respiration during dormancy and ice encasement. However, additional research is needed to confirm these mechanisms.

Lactose was higher in creeping bentgrass crown and root tissue compared to annual bluegrass. As one of the more enigmatic sugars in plants, lactose is not well understood (Toba et al., 1991). Lactose promoted growth of creeping bentgrass in tissue culture (Asano et al., 1994), was found in a *Hypoxis* species corms during dormancy (Mofokeng et al., 2022), and increased due to heat stress conditions in creeping bentgrass (Jespersen et al., 2015). Lactose was also found to be positively associated with salt tolerance in rice (*Oryza sativa*) seedlings (Zhao et al., 2014). However, relative to other sugars, there is relatively little to no literature associated with lactose and stress resistance or overwintering. Therefore, additional investigations into the metabolic importance of lactose in grass species are warranted.

Leaf, crown, and root tissue lost amino acids in response to the dormancy and ice encasement treatments for both species. Roots were the least prone to loss of amino acids compared to the other organs, likely due to the protective properties of the soil and lack of detected soil hypoxic or anoxic conditions in the experimental conditions. It is possible that root leakage of sugars and amino acids may have occurred, or root metabolism utilized these metabolites. Since, other than lactose, there were not major differences between species in what metabolites were lost from the roots, root viability or maintenance of free sugars or amino acids may not be an important factor dictating the major differences in recuperative potential following ice encasement in these two species in this experiment.

There were large differences between species in the amino acids that were lost from crown and leaves due to dormancy and ice encasement conditions. Creeping bentgrass leaves and crowns were able to maintain amino acids to a greater extent than annual bluegrass leaves and crowns. This could be an indicator of enhanced viability or less damage in these organs in creeping bentgrass, a reduced catabolism of these amino acids in these organs, or potentially, in

creeping bentgrass, stolons could serve as an additional source of these amino acids. Annual bluegrass also generally had a higher level of several amino acids following cold acclimation, which could indicate differences in cold acclimation mechanisms. Differences in amino acid metabolism or maintenance could play a major role in the difference in resistance against ice encasement between these two species; however, additional research specifically measuring amino acid leakage and viability would be needed to clarify these specific mechanisms.

Changes in specific amino acids and their functions may also help elucidate overwintering and winter stress differences between creeping bentgrass and annual bluegrass. Glutamine was a major amino acid in these grasses since it was found to be in the highest amount compared to the other amino acids in leaves and crowns. Annual bluegrass clearly produces a lot of glutamine following cold acclimation and loses much of this amino acid during overwintering in both leaves and crowns. This occurred also in creeping bentgrass but to a lesser extent. Similar results were discussed in Sagisaka (1993) for other herbaceous plants and perennial grasses during winter, such as timothy grass (*Phleum pratense*) and redtop bentgrass (*Agrostis gigantea*). Their study revealed that some overwintering plants accumulated arginine, arginine and proline, or glutamate and glutamine just prior to winter during cold acclimation and then exhibit a loss of these amino acids by spring. Proline and arginine were also at high levels prior to winter and were reduced following dormancy and ice encasement in both creeping bentgrass and annual bluegrass crowns, but were maintained at a higher level in creeping bentgrass. Proline was not lost to a significant extent in creeping bentgrass crowns. Proline is a well-studied amino acid for its role in abiotic stress tolerance and signaling (Kaur and Asthir 2015). The survival of stolons of centipedegrass (*Eremochloa ophiuroides*) during freezing stress was correlated with carbohydrate content and the content of proline (Cai et al. 2004). Asparagine

was high in abundance in annual bluegrass crowns following cold acclimation and then was reduced by a large extent in annual bluegrass crowns. This loss did not occur in creeping bentgrass crowns. Asparagine is a proteogenic amino acid that also plays a role in nitrogen transport and tillering in grasses (Luo et al., 2018). Further research on specific amino acid changes and overall amino acid abundance during overwintering and winterkill stresses like ice encasement are needed to elucidate critical winter resistance mechanisms and pathways in turfgrass species.

Principal component and correlation analysis indicate that amino acid responses were more apparent in annual bluegrass compared to creeping bentgrass. The organs that were most damaged and exhibited a differential principal component analysis "fingerprint" were the leaf and crown tissues. Creeping bentgrass may better preserve leaf and crown tissues for post-winter recovery whereas significant metabolic changes, likely due to damage, occur in annual bluegrass leaves and crowns. Accumulation of free amino acids in response to higher levels of abiotic stress have been observed in various studies across different turfgrass species (Bian et al., 2009; Jiang et al., 2023; Merewitz et al., 2012). In both species, leucine, lysine, tyrosine, isoleucine, and fructose were found to have positive correlation to the recovery rate, whereas tryptophan and lactose showed a negative correlation with the rate of recovery. A strong positive correlation between glutamic acid, glutamine, serine, proline, threonine, methionine, valine, leucine, alanine, and total amino acids in annual bluegrass with the rate of recovery following stress, whereas only leucine content was strongly correlated with the recovery rate in creeping bentgrass. Further study is needed to characterize the changes in amino acids in response to ice encasement stress using longer durations of ice encasement and under field conditions.

In conclusion, annual bluegrass had reduced regrowth after ice encasement when compared to creeping bentgrass, which could be associated with greater and earlier ethylene and CO_2 release, which indicates a more readily activated metabolism compared to creeping bentgrass. It is possible that plant and/or soil respiration and metabolic rates may differ in annual bluegrass compared to creeping bentgrass within and following winter dormancy and following ice encasement stress. This study has pinpointed specific metabolites that may play a role in annual bluegrass susceptibility and creeping bentgrass tolerance of ice encasement. Fructose was lost in annual bluegrass crowns but not in creeping bentgrass crown and in general, amino acid levels were better maintained in creeping bentgrass leaves and crowns compared to annual bluegrass. Additional research is needed to determine what plant, soil, or microbial factors directly contribute to these findings in each plant species growth system. Potential mechanisms identified here, such as key carbohydrates or amino acids, should be evaluated in future detailed winterkill studies of grass species.

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