PHYSIOLOGICAL AND METABOLIC FACTORS ASSOCIATED WITH COLD AND FREEZING TOLERANCE AND INDUCED RECOVERY IN COOL SEASON AND WARM SEASON GRASS SPECIES

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ABSTRACT OF THE THESIS

PHYSIOLOGICAL AND METABOLIC FACTORS ASSOCIATED WITH

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Low temperature stress is a major abiotic stress affecting many plants worldwide. Cool season and warm season grass species, that are used on high value turf areas, are susceptible to winter injury through chilling or freezing stress, or more commonly through the disruption of cold acclimation. Cold acclimation is a process in which plants are subjected to low temperatures before freezing temperatures to acquire low temperature tolerance. Fluctuating temperatures due to consistently warmer autumn and winter months, cause a disruption in cold acclimation or de-acclimation, resulting in a loss in cold tolerance. Little is known about the pathways of cold acclimation, but a better understanding of the physiological and biochemical changes during the acquisition of freezing tolerance will aid in the development of more tolerant grass species, or better management practices to induce cold tolerance. Research goals were to investigate the physiological and metabolic changes during cold stress that play roles in the acquisition of cold tolerance and induce recovery after freezing stress. This was accomplished in many studies that included a comparison in the physiological and metabolic responses of warm-season and cool-season grass species to cold stress, with foliar application of naturally occurring cold responsive compounds, calcium dichloride, glycine betaine, abscisic acid, sucrose and potassium phosphate, and their effects on plant productivity

and recovery from freezing stress. The collection of this information will help to expand our knowledge of cold acclimation and freezing tolerance in many grass species, and can be used for the development of more cold tolerance species or management practices to maintain plant health during low temperature or de-acclimation events.

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LITERATURE REVIEW

Introduction

Variation in environmental conditions, especially temperature, can greatly affect plant growth and development. During the past decade temperatures have continued to rise by approximately 0.6°C (Root et al., 2003). Climatic changes, especially in the fall and winter months, can adversely affect plant health and potentially led to plant death. Cold acclimation or the exposure to non-freezing temperatures prior to freezing stress, is a process that plants use to acquire freezing tolerance and mitigate cold injury (Janska et al., 2009). During this process plants redirect their metabolism to accumulate cryoprotectant compounds or stress responsive elements. Fluctuating or increased temperatures prior to freezing can disrupt cold acclimation processes and accelerate injury through cellular dehydration, reduced metabolic functions, or cellular membrane rupture. Damage sustained due to the disruption of acclimation, or de-acclimation, can then inhibit photosynthesis and other metabolic processes therefore limiting or preventing growth and development upon warmer temperatures (Park et al., 2004). De-acclimation and freezing injury has been observed in many different plant species, including turfgrasses (Webster and Ebdon, 2005; Espevig et al. 2014; Espevig et al. 2011). Winter injury can greatly diminish turfgrass performance for many species, and reestablishment of damaged turf can be costly (Chalmers and Schmidt, 1978; Webster and Ebdon, 2005). It is predicted that climate change will continue to occur at a dangerous rate and rapid de-acclimation can potentially lead to the extinction of plant species, which makes it necessary to better understand the cold acclimation process (Root et al., 2003). Increased knowledge on the

mechanisms in which C3 and C4 plants undergo cold acclimation can provide insight to aid in the development of more cold tolerant species, or provide improved management practices to reduce low temperature injury.

Turfgrasses

Turfgrasses are a part of the *Poaceae* family of plants, with approximately 600 genera and 7,500 species (Beard and Green, 1993). Turfgrass has been widely utilized recreationally, functionally, and aesthetically for centuries. They are commonly used in environmental enhancement for soil erosion control, flood control, soil or air improvement, temperature moderation and have many other beneficial contributions. Turfgrasses provide safe playing grounds for various recreational sports, and improve landscapes attractiveness for everyday life. The turfgrass species has made many modifications and evolutions over centuries, and the modern turfgrass industry contributes extensively to the economy resulting in a minimum of a 45-billion-dollar economic output annually as of 1993 (Beard and Green, 1993).

Turfgrass species include C3, cool season species, and C4 or warm season species, differing in carbon dioxide assimilating mechanisms (Monson et al., 1984). *Agrostis* is a genus of cool season grasses, which includes a commonly used species creeping bentgrass, *Agrostis stolonifera*. Creeping bentgrass has a fine texture and is well adapted to low mowing heights which makes it a popular species used on high quality golf course greens and fairways (Casler and Duncan, 2003). *Cynodon dactylon*, or Bermudagrass, a warm season turfgrass that spreads through rhizomes and stolons, is also commonly used in high value turf areas due to its high density, wear tolerance and rapid recovery (Trenholm et al., 2000). Although creeping bentrgrass and bermudagrass participate in different carbon

metabolism systems, they are both adapted to economically valuable turf areas, especially golf courses and recreational landscapes. These C3 and C4 grasses also differ in their ability to withstand abiotic stresses, but are both commonly susceptible to cold injury and de-acclimation. Understanding the physiology and molecular mechanisms responsible for cold tolerance and recovery after freezing stress will allow for the development of improved cultivars with enhanced abiotic stress tolerance.

Physiological and Metabolic Responses to Low Temperature

Many different metabolic and physiological changes occur in a response to low temperature for cool season and warm season grass species. These changes include the alteration in membrane stability, osmotic adjustment, accumulation of protective compounds like amino acids, sugars, carbohydrates or antioxidant enzymes (Hoffman et al., 2010; 2014; Shi et al. 2014). Low temperature impacts plant growth and development by limiting the rate of photosynthesis and contributing to the degradation of cell components like chlorophyll. Also, typical symptoms of freezing stress are intracellular or extracellular ice formation, which can lead to membrane damage or cellular dehydration (Shi et al. 2014). Low temperature can cause damage or denaturing to other important cell components like proteins and enzymes, and can lead to the production of reactive oxygen species (McKersie and Bowley, 1997). Phenotypically, decreasing temperatures result in reduced leaf expansion, wilting, chlorosis, necrosis and damage to tissues like reproductive organs (Mahajan and Tuteja, 2005). These symptoms of cold or freezing stress dramatically affect turf quality, overall plant performance and can potentially lead to plant death. With a better understanding of the physiological and metabolic impacts of cold stress, we can

try to develop more tolerant plants or maintenance practices to help alleviate the effects of fluctuating temperatures and the disruption of cold acclimation on plant productivity.

Cold Acclimation

Cold acclimation is a process that plants use to acquire low temperature tolerance through the subjection to low non-freezing temperatures before periods of freezing stress. For example, non-acclimated rye is killed by temperatures of -5°C, but after exposure to low non-freezing temperatures it can survive to temperatures of -30°C (Thomashow, 1999). Plants also have been shown to benefit and gain tolerance from exposure to sub-zero temperatures before periods of freezing stress. Some plants need not only low temperatures but short photoperiods during acclimation to obtain cold tolerance (Janska et al., 2010). Low temperature tolerance gained from cold acclimation can be lost due to temperature or photoperiod increases, causing deacclimation and cold injury (Juntilla et al., 1999). During cold acclimation plants redirect their metabolism toward the synthesis of cryoprotectant molecules like soluble sugars, sugar alcohols and low molecular weight nitrogen compounds like proline or glycine betaine (Janska et al., 2010). The increase in freezing tolerance during cold acclimation can be due to changes in lipid composition for more unsaturated fatty acids, as well as the accumulation of sucrose or other sugars which may also stabilize membranes (Anchordoguy et al., 1987). During cold acclimation there is also an increase in cold regulated proteins and other stress induced proteins like dehydrins that are involved in stabilizing cell constituents. Also during this process antioxidant enzyme are induced that are involved in the scavenging of reactive oxygen species (Janska et al., 2010).

Cold acclimation results in many different metabolic changes and it has been reported that increases in calcium, glycine betaine, abscisic acid, sucrose and potassium are involved in the cold acclimation process and have been shown to induce cold tolerance in various plant species when applied exogenously (Shi et al., 2014; Xing and Rajashekar, 2001; Zhang et al., 2006; Espevig et al., 2011; Wang et al., 2005). Calcium is an important secondary messenger which facilitates the initial plant response to cold stress through an influx of calcium from cellular storage to the cytoplasm which induces the expression of cold related genes by protein kinase activation (Janska et al., 2010; Knight et al., 1996). The exogenous application of calcium has been shown to improve chilling and freezing tolerance in bermudagrass (*Cynodon dactylon*) (Shi et al., 2014), and other plant species, such as chickpea (*Cicer arietinum*) (Nayyar et al., 2005) and alfalfa (*Medicago sativa*) (Monroy and Dhindsa, 1995). Treatment with $CaCl_2$ is reported to alleviate oxidative damages from cold stress (Shi et al. 2014). Foliar $CaCl_2$ treatments promoted plant growth by increasing photosynthetic efficiency and nitrogen assimilation under cold stress (Shi et al. 2014). In addition, calcium treatments induce the accumulation of protective compounds in amino acids, such as proline, or soluble carbohydrates like sucrose, glucose, fructose and trehalose (Nayyar et al., 2005). Exogenous calcium has been shown to provide protection to not only cold stress but to other abiotic stresses involving water deprivation, such as heat or salinity (Tan et al. 2011; Yin et al. 2015).

An increase in cold tolerance is commonly associated with the capacity to increase protective carbohydrates, such as sucrose which is reported to protect plants from freeze-induced dehydration by decreasing ice formation through increasing the intracellular solute concentration (Espevig et al. 2011). This carbohydrate may help regulate carbohydrate

metabolism and assimilate partitioning impacting carbon and nitrogen allocation which directly effects plant development and may serve roles in tolerance or recovery from stress periods (Espevig et al. 2011). In addition, sucrose has been shown to act as a source in cell signaling during stress which can induce the expression of various of genes and serves roles in metabolite transport to aid in substrate recycling to sustain metabolic processes (Espevig et al. 2011; Ruan et al., 2010). Foliar or exogenous treatments of sucrose have been reported to increase low temperature tolerance in many plants, such as Arabidopsis (Rekarte-Cowie et al., 2008), cucumber (*Cucumis sativus*) (Cao et al., 2014) and stone fruits (*Prunus cerasus*) (Barraco et al., 2012) by activating antioxidants, stress responsive genes, and other protective compounds like proline.

Abscisic acid (ABA) has been shown to accumulate in response to chilling temperatures and cold acclimation and serves many roles in plant growth and development. ABA is considered a stress-response hormone and has been thought to act as a signal to induce acclimation processes (Gilmour and Thomashow, 1991; Thomashow, 1999). Exogenous ABA treatment has been reported to induce the accumulation of protective compounds, such as non-structural carbohydrates or glycine betaine which may improve freezing or cold tolerance (Rajashekar el al., 1999; Zhang et al. 2008). Also ABA treatments have been reported to combat cold-induced dehydration by slowing intercellular freezing during freezing stress in rice cell cultures, ascertained by regrowth capability, improved TTC reduction and less electrolyte leakage (Shinkawa et al., 2013). Foliar treatment of ABA has been shown to increase cold tolerance through altering metabolic processes for the induction of stress responsive genes, osmolytes, or other protective compounds (ie. glycine betaine), as well as preventing cellular rupture or dehydration in

various plant species, such as Arabidopsis (Mantyla et al. 1995; Xing et al. 2000), bermudagrass (Zhang et al. 2006), and strawberry (*Fragaria X ananassa*) (Rajashekar el al., 1999).

Potassium, as an essential nutrient element, does not only have nutrition value providing potassium (K) fertility, but also affects various biochemical processes which regulate plant growth and stress responses (Kant et al., 2002; Wang et al., 2013). Foliar applications of K have been shown to alleviate cold injury by strengthening antioxidant systems by increasing ginsenoside (are a class of steroid glycosides)-related secondary metabolite transcripts, catalase, and ascorbate peroxidase to reduce oxidative damage, as well as protecting photosynthetic machinery to reduce the production of reactive oxygen species during excessive election transport (Devi et al., 2012; Wang et al. 2013). Potassium also may help maintain water status in the cells and has antifreeze properties by lowering the freezing point inside the cell to mitigate ice formation, shown through increased osmotic adjustment and reduced electrolyte leakage (Devi et al., 2012; Kant et al., 2002). Exogenous treatments have shown a change in gene expression for genes related to secondary metabolism or antioxidants and an increase in chilling or low temperature tolerance in ginseng (*Panax ginseng*) (Devi et al., 2012). Application of K in the fall may increase cold tolerance in cool-season grass species (Fry and Huang, 2004). Also exogenous K provides induced tolerance to stress involving water deprivation in various plants, such as maize (Abbasi et al., 2014), beans (*Phaseolus vulgaris L.*) (Singer et al., 1996) or other agricultural crops (Kant et al., 2002).

Glycine betaine (GB) is a compatible solute or osmoregulant which has been shown to play roles in maintaining water status under stress conditions such as cold stress

(Rajashekar el al., 1999), and drought stress (Burgess and Huang 2014). This osmoregulant may also protect plant cells against low temperature induced dehydration by decreasing osmotic potential for cellular water retention with beneficial downstream effects on proteins, enzymes, and membranes to prevent denaturing and maintain metabolic functions (Chen and Murata, 2011). GB is a nitrogen-rich compound that accumulates in the chloroplast, aiding in thylakoid membrane protection to maintain photosynthesis during stress periods (Ashraf and Foolad, 2007). Accumulation of this protective compound may also induce antioxidant systems in the ascorbate-glutathione cycle (Chen and Murata, 2011; Ishikawa & Shigeoka 2008). Exogenous GB treatments have been shown to increase cold tolerance in many plant species, such as Arabidopsis (Xing and Rajashekar, 2001), tomatoes (*Lycopersicon esculentum*) (Park et al. 2006), and alfalfa (*Medicago sativa L.*) (Zhao et al., 1992).

Freezing Injury and Tolerance Mechanisms

It is well established that cell membranes are the primary site of freezing injury in plants, and this damage results commonly from cellular dehydration associated with freezing (Thomashow, 1999). As temperatures decrease, ice is formed in the intercellular space first, due to intracellular space having a lower freezing point based on solute concentration. Once ice starts to form in the intercellular space there is a decrease in water potential and liquid water moves from inside the cell to the intercellular spaces, resulting in cellular dehydration (Thomashow, 1999). Also rapid freezing of cellular water can lead to membrane rupture and cell death. Along with damage to cell membranes, thylakoid membranes are sensitive to the effects of low temperature which results in reduce photosynthesis or metabolic functions. Damage to the cell also occurs through the

production of reactive oxygen species (ROS) due to disrupted electron transport and the denaturing of proteins, enzymes and membranes from cold induced dehydration (McKersie and Bowley, 1997). Therefore, cold acclimation, or the acquisition of freezing tolerance through initial exposure to low nonfreezing temperatures, is critical to stabilize plants against freezing injury. Common mechanisms for induced membrane stability during cold acclimation include, changes in lipid composition or lipid desaturation for more flexible membranes (Anchordoguy, 1987). Also, the accumulation of protective compounds or osmolytes like sugars or amino acids aid to protect membranes and other cell constituents against freezing injury, and help maintain turgor through osmotic adjustment (Chen et al., 2011). Metabolites can either aid in hydration through increasing internal solute content to maintain water status and turgor, or lowering the internal water's freezing point (Thomashow, 1999).

Multiple mechanisms are involved in initiating the cold acclimation process, like the accumulation of protective compounds and inducing cold regulated gene expression. COR genes found have been shown to play roles in cold acclimation and CRT/DRE (C-repeat/dehydration response element) sequences are stimulated by low temperature. CBF1 proteins are transcriptional activators that can activate CRT/DRE sequence containing genes which are thought to be regulators of COR gene expression, promoting freezing tolerance (Jaglo-Ottosen et al., 1998; Janska et al., 2010). CBF plant transcripts start to accumulate within 15 minutes of low temperature initiation, which is followed by accumulation of COR gene transcripts and expression (Jaglo-Ottosen et al., 1998). Although CBF and COR genes play roles in cold tolerance and acclimation, there is some crosstalk between interacting stresses. Many abiotic stresses that result in cellular

dehydration, like drought and salinity, have overlapping signaling pathways and result in similar defense responses like CBF regulation. Another commonality is found in the ROS signaling pathways with increased antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase and catalase during water stress (Janska et al., 2010). Dehydrins are common heat stable proteins that are glycine rich and play pivotal roles in membrane stability and protection of protein denaturing. These dehydrin proteins are suggested to function as chaperones, and accumulate during various stresses including cold stress (Janska et al., 2010; Allagulova et al. 2003). Also heat shock proteins have been shown to increase in abundance during low temperature, aiding in membrane stability or providing protection from protein denaturing by acting as chaperones (Janska et al., 2010; Renaut et al. 2006). The plant responses to decreasing temperatures and acclimation process are complex and effective systems to acquiring cold tolerance.

Carbon Metabolism

Photosynthesis is a process where plants capture light energy and convert it into an energy source that can be used by the plant. In C3 plants, carbon dioxide combines with ribulose bisphosphate, or rubisco, creating either two molecules of 3-phosphoglycerate, or combining with oxygen resulting in one molecule of 3-phosphoglycerate and one 2-phosphoglycolate, resulting in photorespiration or ROS production and a net loss of carbon. C4 plants concentrate carbon dioxide due to their Kranz anatomy, preventing the binding of oxygen and loss of net carbon (Gowik and Westhoff, 2011). Under stress conditions, this difference in carbon metabolism may contribute greatly to photosynthetic and nitrogen use efficiency (Gowik and Westhoff, 2011). During cold stress major changes are made to

metabolism, morphology and gene expression for C3 and C4 plants. Cellular processes like photosynthesis are altered dramatically once temperatures drop and the cold acclimation process is initiated. Chlorophyll is the pigment used to capture energy from light, and declines during low temperature, reducing photosynthetic efficiency. The degradation of chlorophyll not only reduces metabolic activity but can lead to the production of ROS by disrupted electron transport due to instable thylakoid membranes of the chloroplasts (Anchordoguy, 1987).

Carbon metabolism, accumulation and allocation are also affected by low temperature. It is important for optimal photosynthesis rates to have a balance of carbon fixation and sucrose synthesis. Excessive or inadequate sucrose synthesis can deplete phosphates needed to produce ATP, but many studies have showed a correlation with increased sugar levels and cold tolerance (Stitt and Hurry, 2002; Wanner and Junttila 1999). The dark reaction or Calvin cycle, during photosynthesis involves the fixation of carbon and regeneration of rubisco. This effectiveness of this energy production process can be reduced by low temperature due to the denaturing or dehydration of enzymes and proteins necessary for carbon fixation. Starch and sugars are primary storage carbohydrates in many plant tissues like crowns, while soluble sugars are usually cryoprotectants that contribute to the stabilization of membranes and other cell components by increasing solute concentrations in the cell and preventing freezing (Espevig et al. 2011). Cold tolerant plants or cold acclimated plants often develop large carbohydrate reserves to maintain photosynthesis, as well as increases in nitrogen rich metabolites like proline or antioxidants (Zhang et al, 2006). This alteration in metabolic pathways is important for the defense response to cold or freezing stress. Since many cell components are altered, it is difficult

to determine which modifications made actually contribute to freezing tolerance. Investigation of the metabolic changes in carbon metabolism during cold acclimation will provide a better understanding of the acquisition of cold tolerance.

Nitrogen and Amino Acid Metabolism

Nitrogen is critical for plant health due to its involvement in amino acids, proteins, chlorophyll, and many other metabolites (Stitt et al. 2001). Nitrogen metabolism interacts with many different plant processes involved in growth and development, like rubisco for light harvesting complexes during photosynthesis. Nitrogen and carbon are closely linked by the interaction of nitrogen assimilation and carbon metabolism but under periods of stress or carbon starvation, proteins and some amino acids are catabolized (Stitt et al. 2001). Amino acids are important metabolites that are crucial for the synthesis of proteins and play multiple functions in plant growth and stress response. Amino acids can be precursors for the synthesis of various metabolic factors like hormones, chlorophyll, cell wall, secondary metabolites and other cell constituents (Less and Galili, 2008). They are synthesized in many different mechanisms using their respective substrates, all requiring a source of nitrogen. Plants can readily take up nitrogen forms from the soil like ammonium, or the air to lead to the production of certain amino acids. Commonly the synthesis of one amino acid can also come from the degradation of proteins or another amino acids (Singh, 1998). These stages of amino acid metabolism can be observed during a plants life cycle. During germination, storage proteins may be broken down into amino acids, which will aid in the production of proteins necessary for growth initiation. For actively growing tissues, amino acids are upregulated to provide substrates for proteins or provide signals for the production of hormones or other metabolites. In senescing tissues, amino acids are

allocated to provide energy to stressed tissues (Singh, 1998). It is evident that free amino acid pools are constantly changing with regard to environmental sensors or plant health status. Throughout a plants life cycle or during periods of stress, there is an interplay between free amino acids, protein biosynthesis, metabolic functions and signal transduction pathways.

Adaptive mechanisms to stress allow for amino acids like proline and arginine to respond directly to environmental signals, while others are produced by protein breakdown (Less and Galili, 2008). It has been reported that stress can induce molecules or amino acids metabolism into specific compounds to alleviate the effects of stress. For example, under salt stress an accumulation of proline has been observed with less proline degradation, and the breakdown of serine into glycine betaine (Singh, 1998). During environmental stress plants undergo a bulk amino acid and protein degradation, leading to an accumulation of free amino acids to be used as alternative substrates in various processes to mitigate the effects of stress (Hildebrandt et al., 2015). Also the upregulation of nitrogen rich amino acids, like proline, supplies an energy source for not only stress periods but post stress recovery (Chen et al., 1964). This continuous process of the allocation of amino acids, proteins synthesis and degradation, is crucial for plant development as well as response to abiotic stress.

Objectives

The main goals of this research was to examine the mechanisms of cold acclimation and the physiological and metabolic changes responsible for induced freezing tolerance among cool-season and warm-season turfgrass species. This was accomplished through many inter-related projects presented in this thesis that include:

1. The evaluation of physiological traits attributing to cold tolerance in creeping bentgrass and bermudagrass, using foliar application of calcium chloride, glycine betaine, abscisic acid, sucrose and potassium phosphate. Also to screen for optimal concentrations of exogenous applied compounds.
2. The investigation of physiological and metabolic changes to cold stress and cold acclimation for induced freezing tolerance and recovery in creeping bentgrass, using foliar application of glycine betaine.
3. The investigation of physiological and metabolic changes to cold stress and cold acclimation for induced freezing tolerance and recovery in bermudagrass, using foliar application of potassium phosphate.

These projects may ultimately lead to a better understanding of the physiological and metabolic changes involved in cold acclimation for the acquisition of freezing tolerance and induced recovery in C3 and C4 turfgrass species, for the development of more tolerant cultivars or improved management practices.

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CHAPTER 2

Effects of Foliar Application of Calcium Chloride, Glycine Betaine, Abscisic Acid, Sucrose, and Potassium Phosphate on Cold Tolerance in Creeping Bentgrass and Bermudagrass

ABSTRACT

Low temperature or chilling stress limits the growth of both warm-season and cool-season turfgrass species during late fall and winter months. The objectives of this study were to determine whether exogenous application of nutrients and organic compounds may differentially enhance cold tolerance in cool and warm-season turfgrass species and to determine the optimal concentration of each compound which results in the greatest enhancement of cold tolerance in cool- and warm-season turfgrass species. Plants of bermudagrass (cv. 'Tfiway') and creeping bentgrass (cv. 'Penncross') were foliar sprayed with water (untreated control) or different concentrations of calcium chloride ($CaCl_2$), glycine betaine (GB), abscisic acid (ABA), sucrose (SC), and potassium phosphate (KH_2PO_4), and then subjected to cold stress (CS) treatments in growth chambers. Physiological analysis indicated that exogenous application of glycine betaine at 100 mM enhanced cold tolerance in both species while KH_2PO_4 at 50 mM was most effective to promote cold tolerance for bermudagrass. All other compounds resulted in either no significant effects or negative effects on turf quality and physiology under CS. These results demonstrated differential effectiveness of different compounds on cold tolerance for warm- or cool-season turfgrasses, although the underlying mechanisms of improved cold tolerance in warm- or cool-season turfgrasses by GB and KH_2PO_4 deserve further investigation.

INTRODUCTION

Low temperature or chilling stress can adversely affect turf growth and quality for both warm-season and cool-season turfgrass species during fall and winter months, although warm-season species are more sensitive to low temperature than cool-season species (Janska et al., 2009, Turgeon, 1999). Cold stress causes various unfavorable physiological responses, such as decline in membrane stability, cellular dehydration, breakdown of chlorophyll, decreases in photosynthetic activities, and the production of reactive oxygen species (Hoffman et al., 2010; 2014; Shi et al., 2014; Wanner and Junttila, 1999). There are numerous metabolic and physiological changes which occur as plants acclimate to decreasing temperatures over time and which help to protect plants from cold injury or permanent damages. For example, plants may alter their metabolic profile in different organs by accumulating various compounds, such as amino acids, nitrogen-rich compounds, and carbohydrates, some of which possess cryoprotectant functions (Janska et al., 2009). Previous studies found that calcium, glycine betaine, abscisic acid, sucrose, and potassium may serve roles in the cold acclimation process and may contribute to improved cold tolerance in various plant species (Espevig et al., 2011; Rekarte-Cowie et al., 2008; Shi et al., 2014; Wang et al., 2013; Xing and Rajashekar, 2001; Zhang et al., 2006). However, whether exogenous application of such compounds may alleviate cold stress in the absence of sufficient cold acclimation is not well documented, particularly for both warm-season and cool-season turfgrass species.

Calcium is an important secondary messenger which facilitates the initial plant response to cold stress through an influx of calcium from cellular storage to the cytoplasm which induces the expression of cold related genes by protein kinase activation (Janska et

al., 2010; Knight et al., 1996). The exogenous application of calcium has been shown to improve chilling and freezing tolerance in bermudagrass (*Cynodon dactylon*) (Shi et al., 2014), and other plant species, such as chickpea (*Cicer arietinum*) (Nayyar et al., 2005) and alfalfa (*Medicago sativa*) (Monroy and Dhindsa, 1995). Treatment with $CaCl_2$ is reported to alleviate oxidative damages from cold stress (Shi et al. 2014). Foliar $CaCl_2$ treatments promoted plant growth by increasing photosynthetic efficiency and nitrogen assimilation under cold stress (Shi et al. 2014). In addition, calcium treatments induce the accumulation of protective compounds in amino acids, such as proline, or soluble carbohydrates like sucrose, glucose, fructose and trehalose (Nayyar et al., 2005). Exogenous calcium has been shown to provide protection to not only cold stress but to other abiotic stresses involving water deprivation, such as drought or salinity (Tan et al. 2011; Yin et al. 2015).

An increase in cold tolerance is commonly associated with the capacity to increase protective carbohydrates, such as sucrose (SC) which is reported to protect plants from freeze-induced dehydration by decreasing ice formation through increasing the intracellular solute concentration (Espevig et al. 2011). This carbohydrate may help regulate carbohydrate metabolism and assimilate partitioning impacting carbon and nitrogen allocation which directly affects plant development and may serve roles in tolerance or recovery from stress periods (Espevig et al. 2011). In addition, sucrose has been shown to act as a source in cell signaling during stress which can induce the expression of various genes and serves roles in metabolite transport to aid in substrate recycling to sustain metabolic processes (Espevig et al. 2011; Ruan et al., 2010). Foliar or exogenous treatments of SC have been reported to increase low temperature tolerance in

many plants, such as Arabidopsis (Rekarte-Cowie et al., 2008), cucumber (*Cucumis sativus*) (Cao et al., 2014) and stone fruits (*Prunus cerasus*) (Barraco et al., 2012) by activating antioxidants, stress responsive genes, and other protective compounds like proline.

Abscisic acid (ABA) has been shown to accumulate in response to chilling temperatures and cold acclimation and serves many roles in plant growth and development. ABA is considered a stress-response hormone and has been thought to act as a signal to induce acclimation processes (Gilmour and Thomashow, 1991; Thomashow, 1999). Exogenous ABA treatment has been reported to induce the accumulation of protective compounds, such as non-structural carbohydrates or glycine betaine which may improve freezing or cold tolerance (Rajashekar el al., 1999; Zhang et al. 2008). Also ABA treatments have been reported to combat cold-induced dehydration by slowing intercellular freezing during freezing stress in rice cell cultures, ascertained by regrowth capability, improved TTC reduction and less electrolyte leakage (Shinkawa et al., 2013). Foliar treatment of ABA has been shown to increase cold tolerance through altering metabolic processes for the induction of stress responsive genes, osmolytes, or other protective compounds (ie. glycine betaine), as well as preventing cellular rupture or dehydration in various plant species, such as Arabidopsis (Mantyla et al. 1995; Xing et al. 2000), bermudagrass (Zhang et al. 2006), and strawberry (*Fragaria X ananassa*) (Rajashekar el al., 1999).

Potassium (K), as an essential nutrient element, does not only have nutrition value providing K fertility, but also affect various biochemical processes which regulate plant growth and stress responses (Kant et al., 2002; Wang et al., 2013). Foliar applications of K

have been shown to alleviate cold injury by strengthening antioxidant systems by increasing ginsenoside (are a class of steroid glycosides)-related secondary metabolite transcripts, catalase, and ascorbate peroxidase to reduce oxidative damage, as well as protecting photosynthetic machinery to reduce the production of reactive oxygen species during excessive election transport (Devi et al., 2012; Wang et al. 2013). Potassium also may help maintain water status in the cells and has antifreeze properties by lowering the freezing point inside the cell to mitigate ice formation shown through increased osmotic adjustment and reduced electrolyte leakage (Devi et al., 2012; Kant et al., 2002). Exogenous treatments have shown a change in gene expression for genes related to secondary metabolism or antioxidants and an increase in chilling or low temperature tolerance in ginseng （*Panax ginseng*) (Devi et al., 2012). Application of K in the fall may increase cold tolerance in cool-season grass species (Fry and Huang, 2004). Also exogenous K provides induced tolerance to stress involving water deprivation in various plants, such as maize (Abbasi et al., 2014), beans (*Phaseolus vulgaris L*) (Singer et al., 1996) or other agricultural crops (Kant et al., 2002). Potassium combined with forms of phosphorous, such as phosphate, have been shown to include tolerance to stress involving water deprivation such as salinity (Akram et al., 2011). Phosphates are a main component of RNA and DNA, and are involved in nucleic acid structure which regulates protein synthesis (Schachtman et al., 1998). Also it is involved in the structure of the energy form used by plants, ATP, which has critical functions in photosynthesis and metabolism. Phosphates have also been shown to induce cell division for the development or new tissues after periods of stress (Schluter et al., 2013).

Glycine betaine (GB) is a compatible solute or osmoregulant which has been shown to play roles in maintaining water status under stress conditions such as cold stress (Rajashekar el al., 1999), and drought stress (Burgess and Huang 2014). This osmoregulant may also protect plant cells against low temperature induced dehydration by decreasing osmotic potential for cellular water retention with beneficial downstream effects on proteins, enzymes, and membranes to prevent denaturing and maintain metabolic functions (Chen and Murata, 2011). GB is a nitrogen-rich compound that accumulates in the chloroplast, aiding in thylakoid membrane protection to maintain photosynthesis during stress periods (Ashraf and Foolad, 2007). Accumulation of this protective compound may also induce antioxidant systems in the ascorbate-glutathione cycle (Chen and Murata, 2011; Ishikawa & Shigeoka 2008). Exogenous GB treatments have been shown to increase cold tolerance in many plant species, such as Arabidopsis (Xing and Rajashekar, 2001), tomatoes (*Lycopersicon esculentum*) (Park et al. 2006), and alfalfa (*Medicago sativa L.*) (Zhao et al., 1992).

Despite the known physiological and biochemical effects of the various compounds promoting plant tolerance to cold stress in the model plant species and agronomic or horticultural crops, the effectiveness of those compounds for promoting cold tolerance of perennial turfgrasses is not well documented. Given the inherent physiological, metabolic, and biochemical differences between cool and warm-season plant species, it is plausible that the two plant types possess differential mechanisms contributing to their overall level of cold tolerance (Gowik and Westhoff, 2011). Moreover, the exogenous application of the aforementioned compounds may induce differential responses across cool and warm-season grasses and lend insight as to which specific metabolites induce cellular alterations

associated with improved tolerance to cold stress. Therefore, the objectives of this study were to 1) determine whether exogenous application of $CaCl_2$, GB, ABA, SC, and KH_2PO_4 differentially enhance cold tolerance in cool and warm-season grass species and 2) determine the optimal concentration of each compound which results in the greatest enhancement of cold tolerance in cool and warm-season grass species.

MATERIALS AND METHODS

Plant materials and growth conditions

Tillers of creeping bentgrass (*Agrostis stolonifera* cv. 'Penncross') and bermudagrass (*Cynodon dactylon* cv. 'Tifway') were propagated in pots (5 x 20 cm) filled with sand. Plants were established for 60 d in a greenhouse with an average temperature of 26/22 °C (day/night), 60% relative humidity, 14 h photoperiod, and 680 µmol m^{-2} s^{-1} photosynthetically active radiation from natural sunlight and supplemental lighting. During establishment, plants were irrigated daily, fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1949) twice per week, and trimmed weekly to maintain canopy height of 2 and 4 cm for creeping bentgrass and bermudagrass, respectively. Following establishment, plants were transferred to growth chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA) and allowed to acclimate for 7 d prior to chemical or cold stress treatment. At this time the creeping bentgrass were maintained at 25°C and the bermudagrass were at 30 °C with all other parameters the same as previously mentioned.

Treatments and experimental design

Creeping bentgrass or bermudagrass plants were exogenously treated with $CaCl_2$ solutions at 1, 5, 10, or 20 mM, GB at 30, 50, 75, or 100 mM, ABA at 25, 50, 100, or 150

μM, SC solutions at 2.5, 5, 7.5, or 10% , or KH_2PO_4 solutions at 5, 10, 25, or 50 mM. All compounds were applied on the day prior to CS treatment and subsequently every 14 d for a total of 3 applications during the cold treatment. Each chemical treatment had six replicates (pots). Creeping bentgrass and bermudagrass plants were each exposed to two distinct temperature regimens in temperature-controlled chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA). Creeping bentgrass plants were maintained at 20/15 °C (day/night) for 7 d and 15/10 °C (day/night) for 24 d, and 2/2°C (day/night) for 4 days in growth chambers for the cold stress treatment. Bermudagrass were maintained at 25/20°C for 32 d and 10/10°C for 3 d for the cold stress treatment. The cold treatments for creeping bentgrass and bermudagrass were conducted independently over time as two experiments, and each was replicated in four growth chambers.

Each experiment was arranged in a completely randomized design with different concentrations of different compounds randomly placed within each temperature-controlled chamber, and each compound treatment having six replicates. All plants were placed randomly within each temperature chamber and relocated between the four chambers every 3 d to avoid possible confounding effects of unique chamber conditions from occurring.

Physiological analysis

Four commonly-used parameters to evaluate plant physiological status and overall turf quality (TQ) were evaluated at 14, 21, and 35 d of CS treatment for the creeping bentgrass and 14, 28 and 35 d of CS for bermudagrass. TQ was rated on a scale of 1 to 9 with 9 being a turf plant that is healthy and green color, 1 representing a turf plant that is

brown and dead, and 6 being the minimum acceptable quality rating. Ratings were based factors such as leaf and canopy color, density, and uniformity (Beard, 1973).

Leaf relative water content (RWC) was measured to indicate leaf hydration status 14, 21, or 28 and 35 d of CS treatment. Approximately 0.2 g of fresh leaf tissue was collected from plants and measured on a mass balance for fresh weight (FW). After incubating leaf tissue in water for 12 hours at 4 °C, the leaves were blotted dry, and weighed for turgid weight (TW). Leaf tissue was then dried in an oven at 80 °C for 72 h and the dry weight (DW) was measured. RWC was calculated using the formula % = [(FW – DW) / (TW – DW)] x100 (Barrs, 1962).

Osmotic potential was determined by soaking leaves in distilled water for 24 h to reach full turgor. Turgid leaves were frozen in liquid nitrogen and stored at -20 °C until analysis. Upon analysis, leaves were slowly thawed and the leaf sap was extracted and analyzed for osmolality (mmol kg^{-1}) using a vapor pressure osmometer (Vapro Model 5520; Wescor, Logan, UT). Osmolality was converted to osmotic potential (OP) using the formula: OP = [(– C * 2.58)/1000] (Blum, 1989).

Chlorophyll content was quantified by incubating 0.1 g of fresh leaf tissue in 10 ml dimethyl sulfoxide in darkness for 3 d until all chlorophyll was extracted from the leaf tissue. The absorbance values at 663 and 645 nm were read on a spectrophotometer (Genesys 2, Spectronic Instruments, Inc., Rochester, NY), after which the leaf tissue was filtered and dried in an oven set to 80ºC for 3 d. Dry weights were measured on a mass balance and chlorophyll content was calculated using the formula by Arnon (1949).

Statistical analysis

The effects of CS and chemical treatment for each species were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Differences between treatment means and plant lines were separated by Fisher's protected least significance difference (LSD) test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Creeping bentgrass tolerance to cold stress as affected by foliar application of calcium chloride, glycine betaine, abscisic acid, sucrose, and potassium phosphate

Previous studies in other plant species found beneficial effects of $CaCl_2$, GB, ABA, SC, and KH_2PO_4, as discussed in the introduction. However, in the current study, only GB at all tested concentrations had improved cold stress tolerance in creeping bentgrass (Fig. 1), whereas all other compounds at the tested concentrations resulted in either no significant effects ($CaCl_2$, KH_2PO_4) or negative effects (ABA, SC) on turf quality and physiological factors under low temperature (Fig. 2-5). Therefore, only results for GB are further described and discussed in details.

Creeping bentgrass plants treated with GB at all concentrations had significantly higher (by 10%) TQ ratings compared to the untreated control at 21 d treatment (Fig. 1A). By 35 d of CS treatment, GB-treated plants had 12% higher TQ at 20 and 50 mM and 22% higher TQ at 75 and 100 mM compared to the untreated CS treatment. There were no significant differences in leaf RWC between the GB and untreated CS treatments on all three sampling days (Fig. 1B). Leaf OP was significantly lower (more negative, by 41-50%) for all four GB treatments compared to the untreated CS plants at 35 d of treatment and was significantly lower (more negative, by 25-31%) for the 30, 75, and 100 mM GB

treatments compared to the untreated CS plants at 21 d of treatment (Fig. 1C). All GB treatments except the lowest concentration (30 mM) resulted in a significant increase or maintenance of leaf chlorophyll content by 35 d treatment compared to the untreated CS plants (Fig. 1D). Specifically, chlorophyll content of plants treated with 50, 75, or 100 mM GB had higher chlorophyll content by 21, 49, or 36%, respectively, compared to the leaves of untreated CS plants.

Overall, exogenous application of a range of GB concentrations enhanced creeping bentgrass tolerance to extended period of CS. Extended periods of chilling temperatures may accelerate cellular dehydration and loss of leaf turgidity, techniques, such as application of osmoregulants which aim to maintain cellular hydration status are critical for sustaining metabolic functions and plant growth under suboptimal temperatures (Espevig et al., 2011; Janska et al., 2009; Wanner and Junttila, 1999). However, in this study, RWC did not decline to the water deficit level in CS treatment, and it is reasonable that GB had no effects on RWC despite of the increases in OP. The ability of exogenous GB to enhance osmotic adjustment in the absence of significant changes in cellular water content would decrease the cellular freezing point and delay or mitigate the extent of cellular damages upon prolonged periods of very low temperatures (Chen and Murata, 2011; O'Neill, 1983). Mechanisms which aim to maintain chlorophyll integrity as air temperatures decline may have beneficial effects on whole-plant carbohydrate production and subsequent effects on cold-induced dormancy and post-dormancy growth resumption (Henson et al., 2014; Chai et al., 2010; Valluru & Van den Ende 2008). In the current study, all GB-treated plants displayed improved leaf chlorophyll content compared to untreated CS control, which may have had downstream effects on photosynthesis and carbohydrate

production. The biochemical and molecular mechanisms underlying the beneficial effects of GB on creeping bentgrass tolerance to CS deserve attention in further research trials.

Bermudagrass tolerance to cold stress as affected by foliar application of calcium chloride, glycine betaine, abscisic acid, sucrose, and potassium phosphate

Warm season grass species, such as the common and hybrid bermudagrass varieties, thrive in the tropical, sub-tropical, and semi-arid climates of the world and are inherently intolerant to low temperature (Pessarakli, 2007; Turgeon, 1999). As previously noted, the current study investigated whether five nutrient and organic compounds, each of which differ with regard to their unique properties and functions within the plant system, have beneficial effects on the cold tolerance of cool or warm-season grass species. Similar to the results reported for creeping bentgrass, exogenous application of ABA, $CaCl_2$, and SC of different concentrations had no significant effects on turf quality, RWC, OP, and chlorophyll content for bermudagrass under CS treatment (Fig. 8, 9, 10). These results suggested that the addition of those three compounds could not provide beneficial effects on the adaptation of both cool-season and warm-season turfgrasses to cold stress, although they have been reported to play roles in chilling tolerance in other plant species, as cited in the introduction.

GB resulted in beneficial effects on bermudagrass tolerance to cold stress, similar to its effects on creeping bentgrass. Bermudagrass plants exogenously treated with GB at all concentrations had significantly higher (by 10-12%) TQ ratings compared to the untreated plants by 35 d of CS treatment (Fig. 6A), although GB effects on RWC or OP were not significant (Fig. 6B, C). The exogenous application of GB significantly increased

leaf chlorophyll content by 31-52% (varying with GB concentrations) at 28 and by 1.1-1.5 fold at 35 d of CS treatment (Fig. 6D). The consistent effects of GB on both creeping bentgrass and bermudagrass suggested that GB could play important roles in regulating chilling tolerance for both warm-season and cool-season turfgrasses, which would be a key regulatory pathway controlling cold tolerance in perennial turfgrasses.

The additional KH_2PO_4 at all tested concentrations resulted in significantly higher TQ than the untreated control at 35 d of treatment (Fig. 7A). There were no significant differences in bermudagrass leaf RWC or OP between the untreated CS treatments and KH_2PO_4-treated bermudagrass plants on all three sampling days (Figs. 7B, C). The exogenous application of KH_2PO_4 had significant effects on leaf chlorophyll content throughout the 35 d of CS treatment. Positive effects of KH_2PO_4 on leaf chlorophyll content were noted across all concentrations and on three sampling days, with the increases in chlorophyll content ranging from 28-44% with 10 mM at 14 and 28 d of CS treatment, 43-50% with 25 mM at 28 and 35 d, and 30-67% with 50 mM at 14, 28 and 35 d. (Fig. 7D). It is interesting to note that KH_2PO_4 enhanced TQ and chlorophyll content in bermudagrass but had no effects on creeping bentgrass tolerance to cold stress, as discussed above. The reasons for the differential effects of KH_2PO_4on warm-season and cool-season turfgrass species are unknown. Nevertheless, mechanisms of KH_2PO_4 effects on low temperature tolerance have been associated with enhanced antioxidant metabolism (Devi et al., 2012), translocation of photoassimilates (Conti and Geiger, 1982; Romheld and Kirkby, 2010), increasing or maintaining osmotic adjustment (Kant et al., 2002), and maintaining energy production (Schluter et al., 2013).

In summary, the exogenous application of GB was effective to improve cold tolerance of both warm-season and cool-season turfgrass species while KH_2PO_4 was beneficial only for warm-season bermudagrass, as manifested by the increased turf quality and chlorophyll content. However, the inherent differences between the two species in their differential responses to KH_2PO_4 deserves further investigation. All other compounds had no significant effects on either cool-season or warm-season turfgrass tolerance to cold stress, despite their reported positive effects in other non-turfgrass species. Furthermore, whether or not the physiological benefits observed during cold stress treatment translate into an enhanced capacity to recover following an extended freezing period should be explored.

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FIGURES

Figure 1: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of glycine betaine (30,50,75,100 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

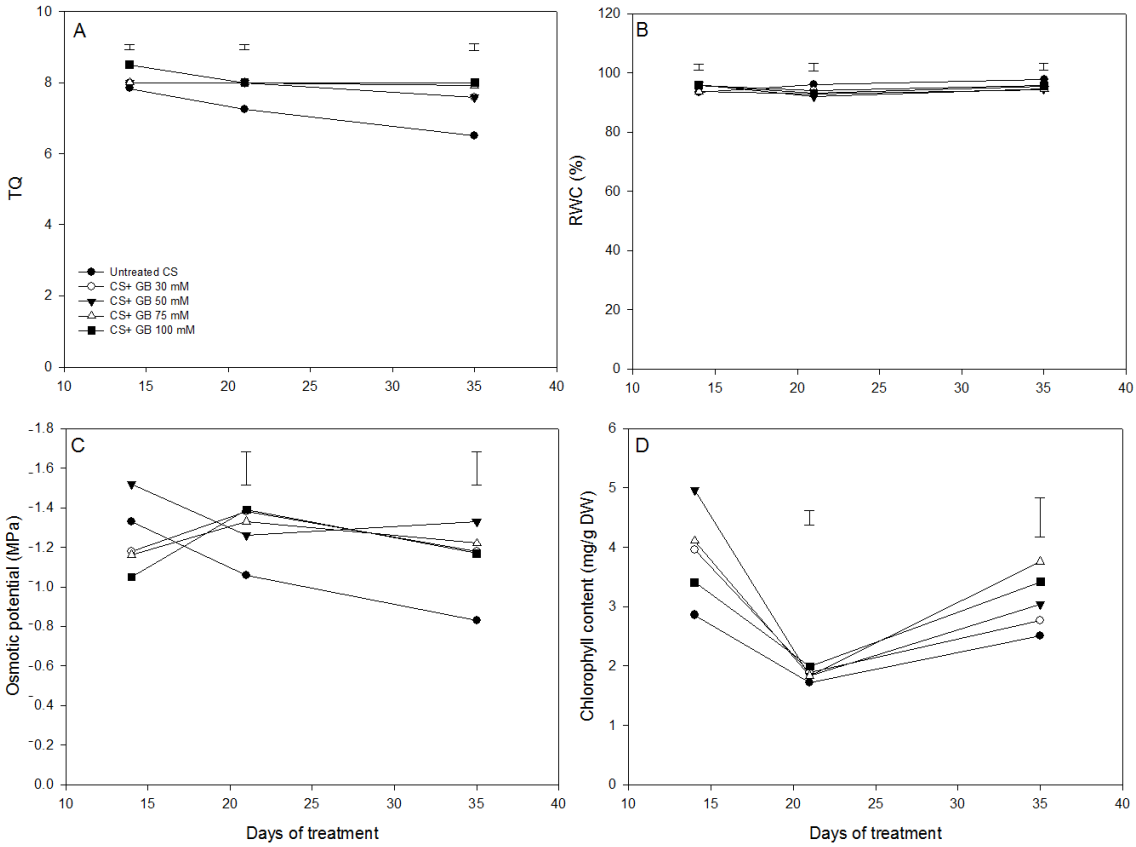


Figure 2: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of potassium phosphate (5,10,25,50 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

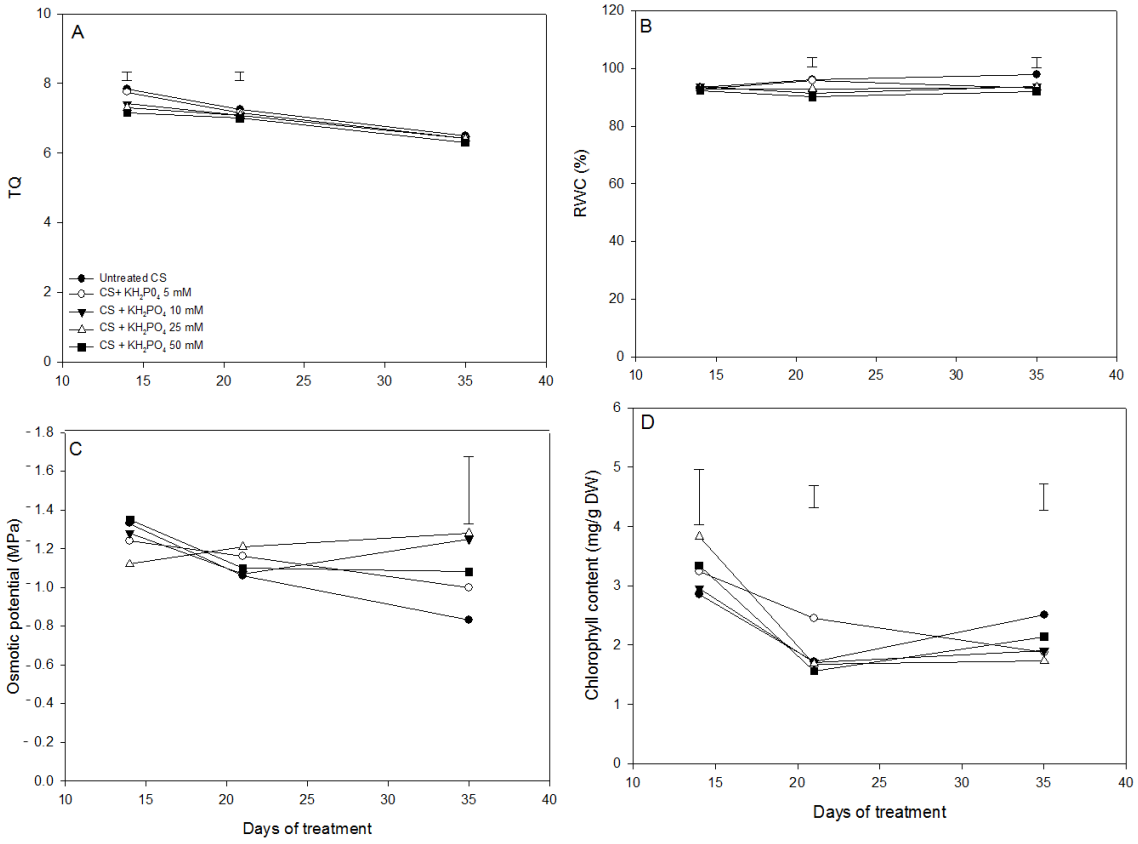


Figure 3: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of calcium chloride (1,5,10,20 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

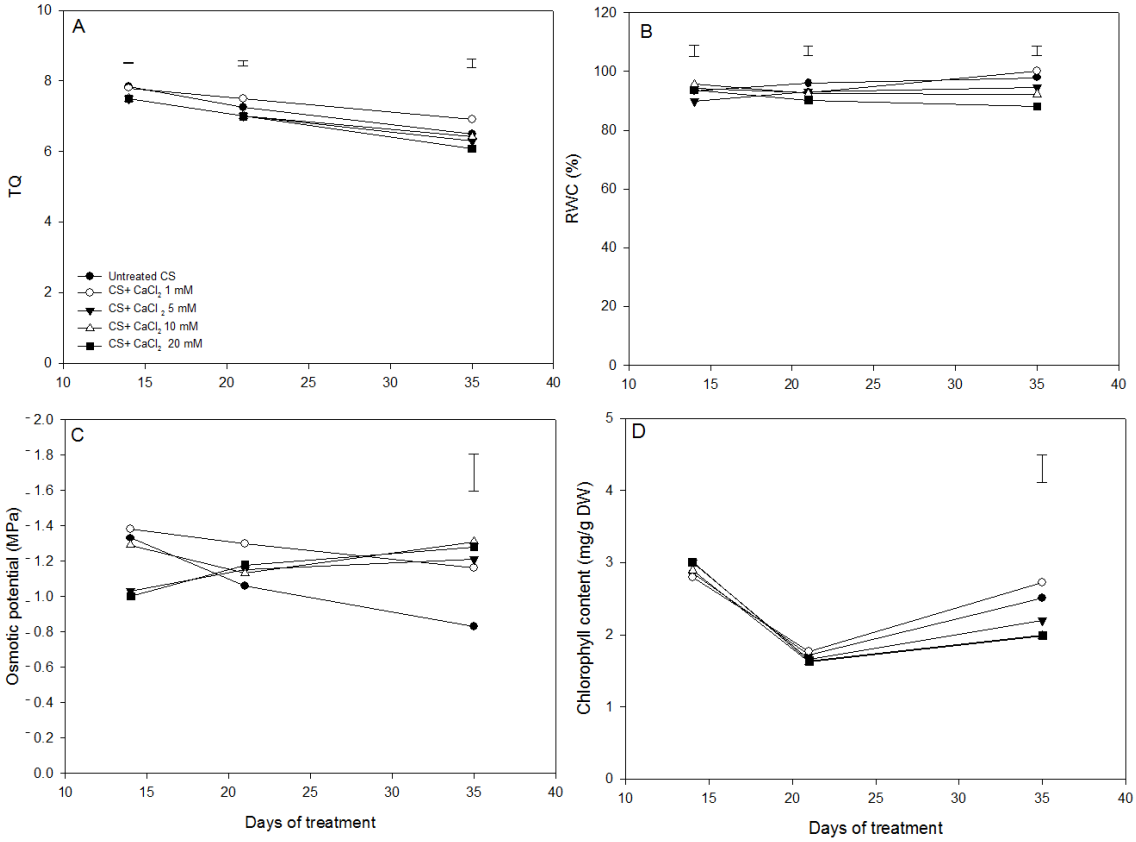


Figure 4: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of sucrose (2.5,5,7.5,10%) during 35 day of cold stress treatment, compared to untreated cold stress plants . LSD bars represent significant differences exist at $p \leq 0.05$.

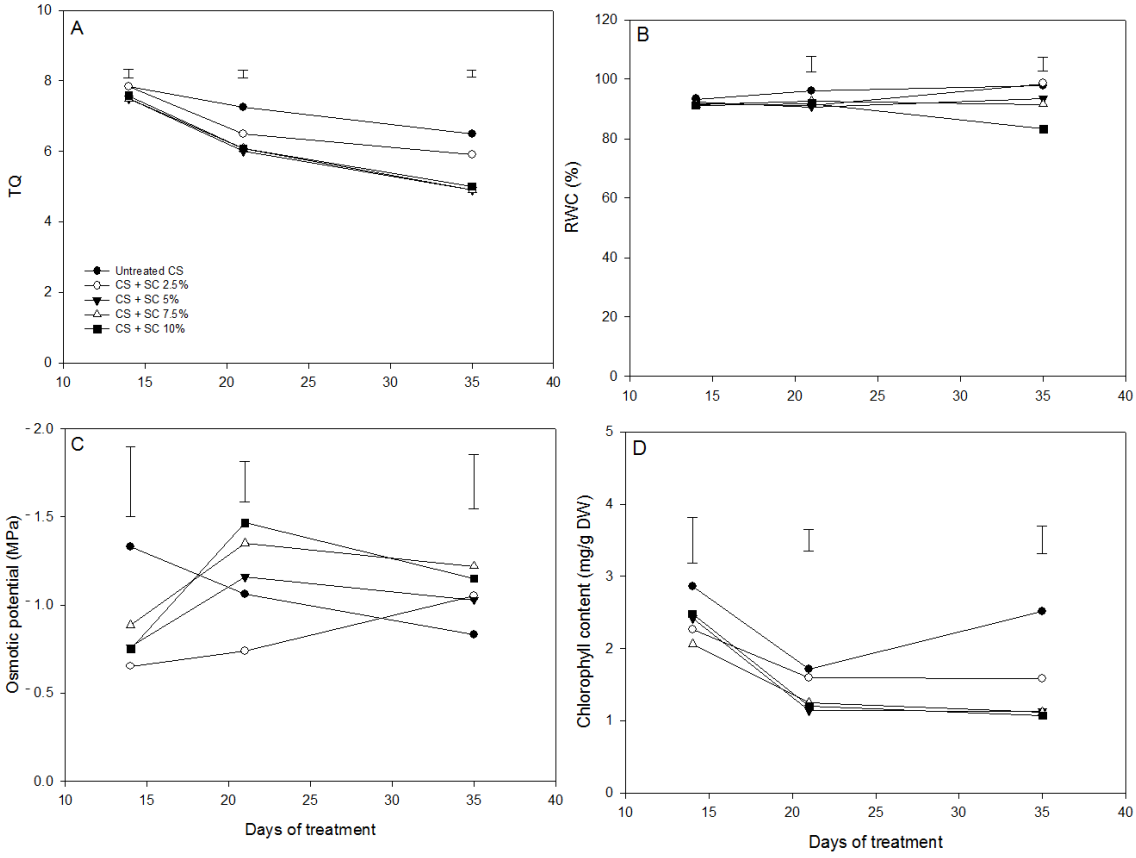


Figure 5: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of abscisic acid (25,50,75,100 µM) during 35 day of cold stress treatment, compared to untreated cold stress plants . LSD bars represent significant differences exist at $p \leq 0.05$.

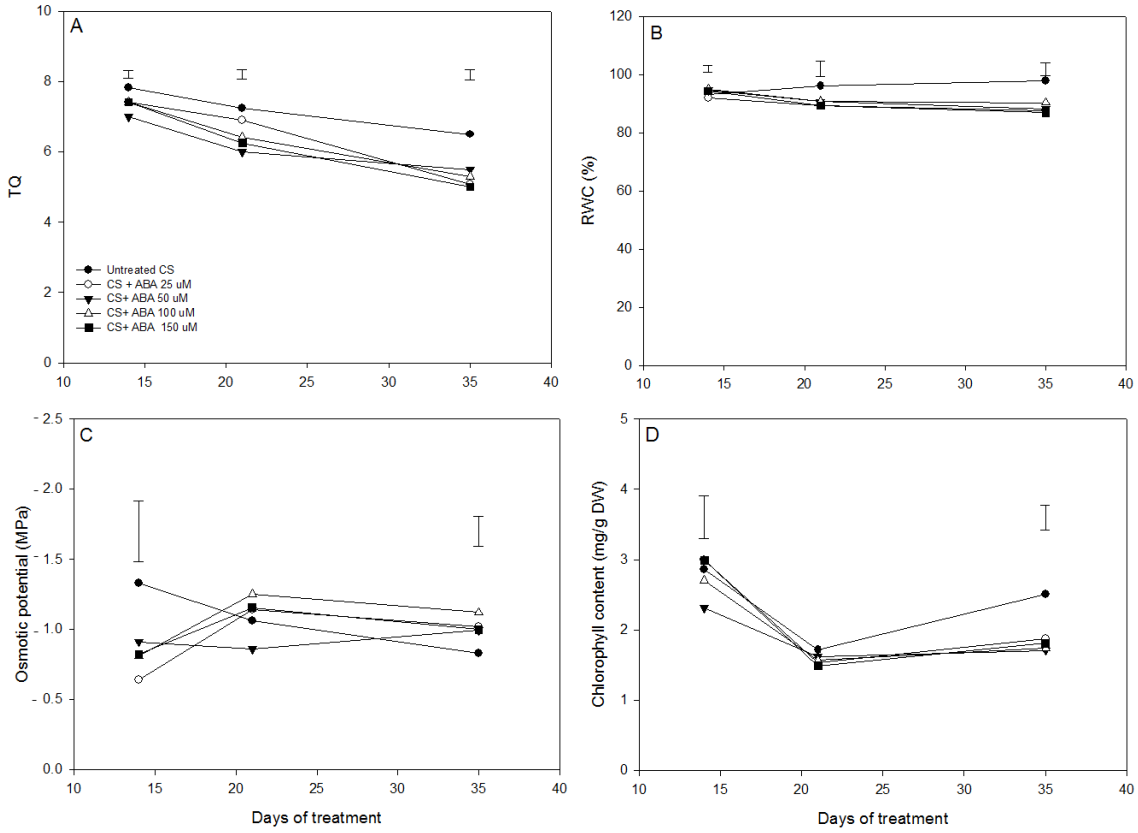


Figure 6: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of glycine betaine (30,50,75,100 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

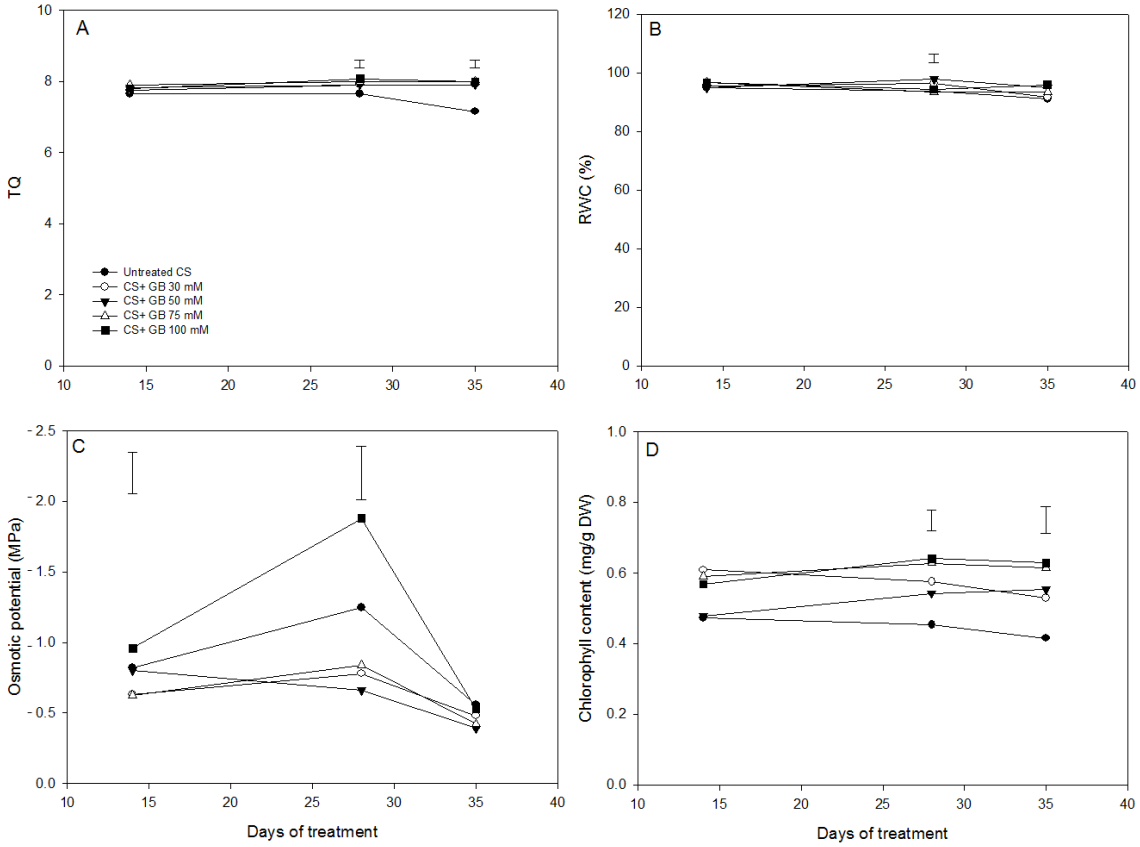


Figure 7: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of potassium phosphate (5,10,25,50 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$

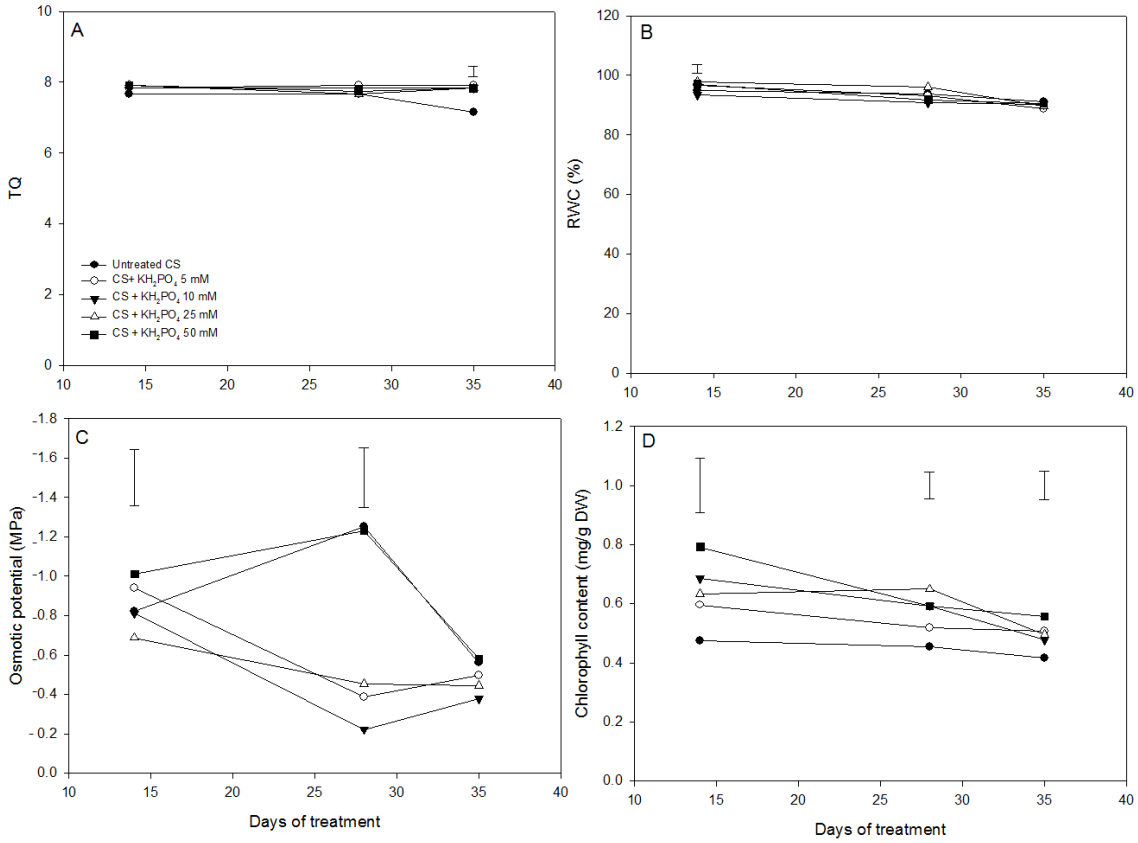


Figure 8: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of calcium chloride (1,5,10,20 mM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

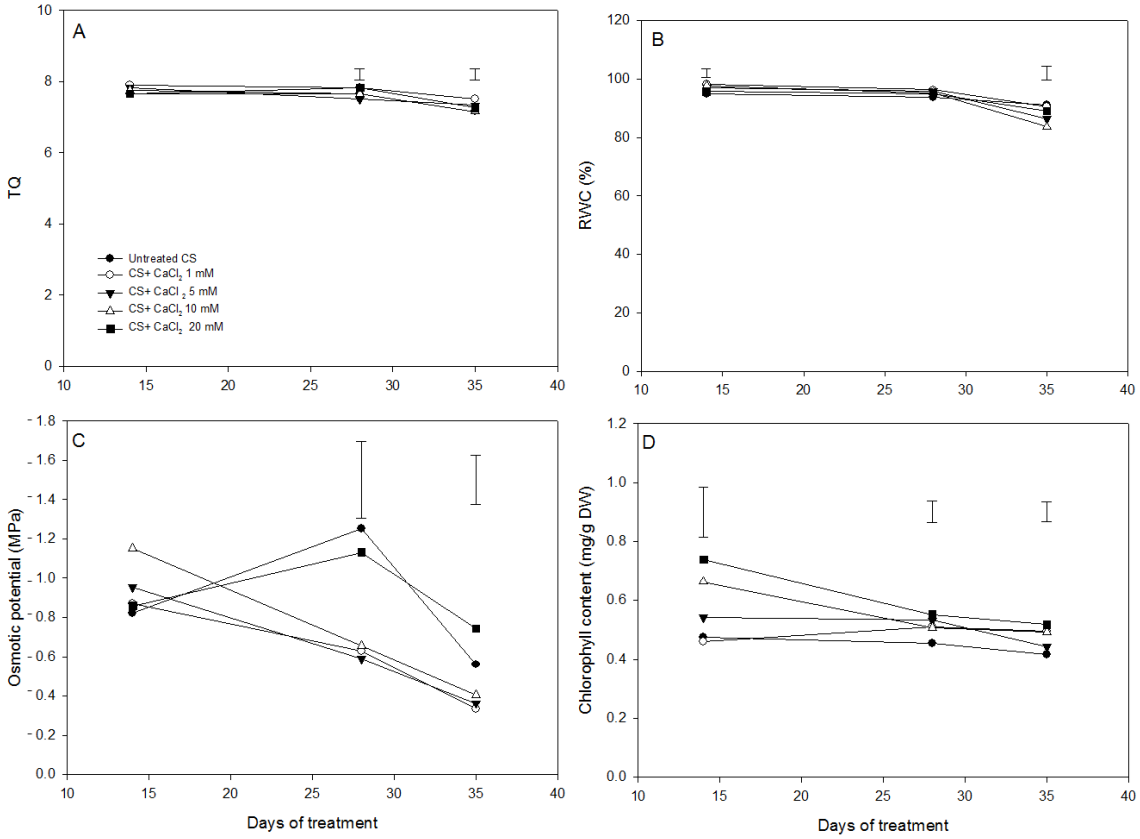


Figure 9: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) exposed to four concentrations of sucrose (2.5,5,7.5,10%) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.

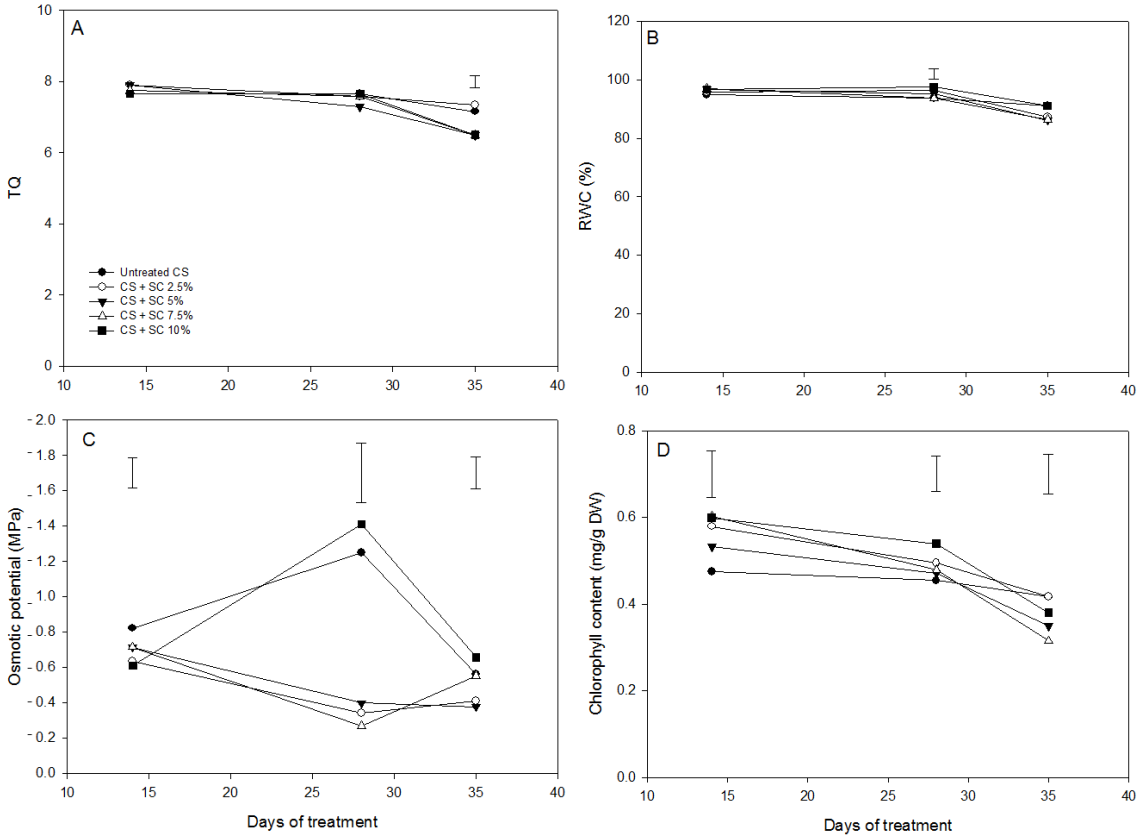
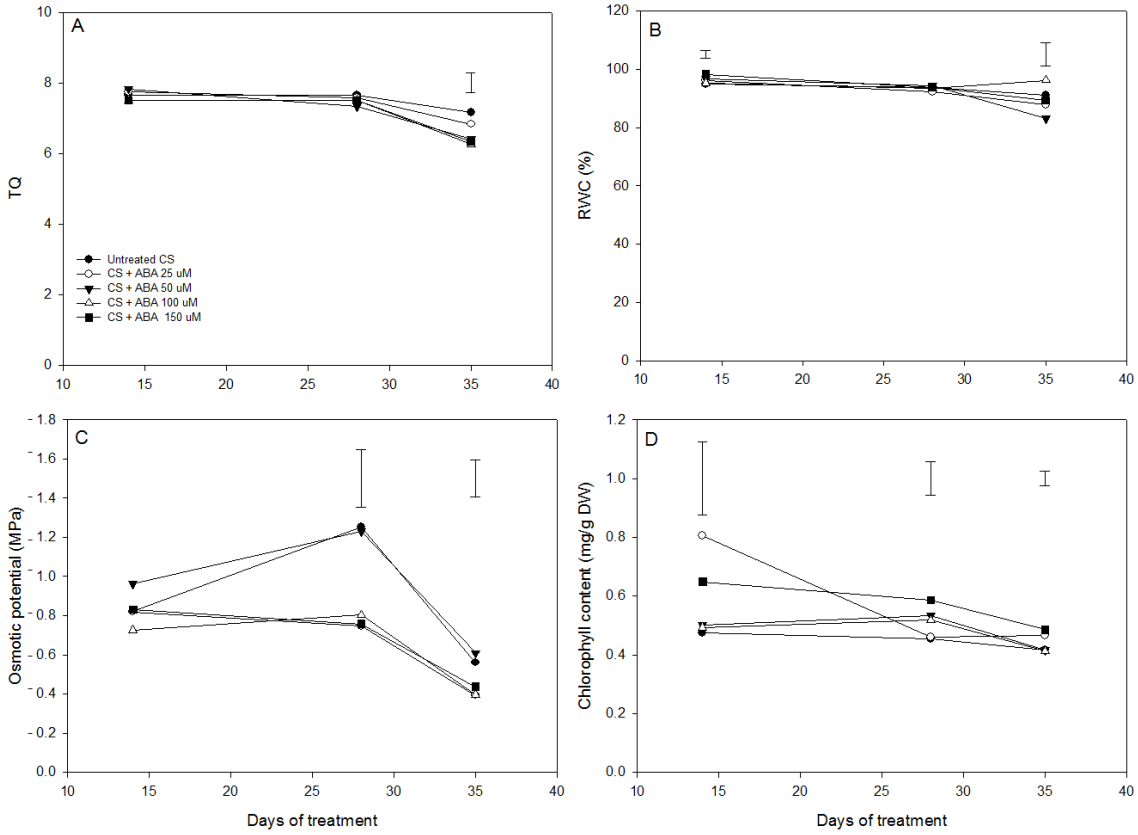


Figure 10: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of four concentrations of abscisic acid (25,50,75,100 µM) during 35 day of cold stress treatment, compared to untreated cold stress plants. LSD bars represent significant differences exist at $p \leq 0.05$.



CHAPTER 3

Physiological and Metabolic Changes Associated with Tolerance to Cold and Freezing Stress in Creeping Bentgrass as Affected by Foliar Application of Glycine Betaine

ABSTRACT

Chilling and freezing stress adversely affect the growth of cool season grass species during late fall and winter months. Exogenous application of certain naturally-occurring metabolites in plants, such as glycine betaine (GB) may promote plant tolerance to cold and freezing in cases when cold acclimation is insufficient or absent. The objective of this study was to investigate whether exogenous application of GB promotes tolerance to cold stress and post-freezing recovery of creeping bentgrass (*Agrostis stolonifera*) by inducing the accumulation of amino acids and carbohydrates in the crown tissues. Plants of creeping bentgrass (cv. 'Penncross') were foliar sprayed with water (untreated control) or 100 mM GB, and then subjected to a series of decreasing temperatures (cold stress, CS) or cold acclimation (ACL) from 15/10°C (day/night) to -2/-2°C (day/night)and freezing stress in a stepwise temperature reduction of -5°C, -10°C and -15°C in temperature-controlled chambers. Post-freezing recovery was evaluated for plants maintained at 25/15°C (day/night) following freezing stress. Physiological responses indicate that GB enhanced the tolerance of creeping bentgrass to both CS and freezing stress. GB application enhanced the accumulation of endogenous GB, amino acids (aspartic acid, asparagine, glycine, tyrosine, methionine, leucine, lysine hydrochloride), and non-structural carbohydrates (starch and fructan) in crowns exposed to cold stress. During post-freezing recovery, GB-treated plants recovered to a significantly greater extent compared to CS and ACL plants. Also, GB treatment resulted in increased soluble carbohydrates, compare to CS, and increased non-structural carbohydrates compared to CS and ACL in crowns during post-freezing recovery. This study showed that GB-induced creeping bentgrass tolerance to cold

and freezing stress was associated with the accumulation of amino acids and carbohydrates which play protective roles or serve as energy reserves for plant stress defense.

INTRODUCTION

Cold acclimation is a biochemical and physiological process required for plant survival during winter months (Janska et al., 2010). However, fluctuation in temperatures or an extended period of warm temperatures during autumn may disrupt or delay the natural cold-acclimation processes, which weakens the plants ability to survive freezing stress during the winter or recovery during spring months (Hoffman et al., 2014). Although the cold acclimation process is not completely understood, the accumulation of certain protective compounds, such as nitrogen-rich compounds, amino acids, and carbohydrates are key mechanisms of facilitating plant survival during cold and freezing stress, as well as post-freezing recovery come springtime (Janska et al., 2009; Thomashow, 1999). Glycine betaine (GB) is a nitrogen-rich compound that accumulates in the chloroplast, aiding in thylakoid membrane protection to maintain photosynthesis during stress periods (Ashraf and Foolad, 2007). Plant species that accumulate high quantity of GB naturally during cold acclimation or transgenic plants with GB-synthesizing genes with increased endogenous production of GB exhibit superior tolerance to cold and freezing stress (Holmstrom et al., 2000; Sakamoto and Murata, 2002). Exogenous application of GB also promotes plant tolerance to cold stress in various plant species, such as Arabidopsis (Xing and Rajashekar, 2001), tomatoes (*Lycopersicon esculentum*) (Park et al. 2006), alfalfa (*Medicago sativa L.*) (Zhao et al., 1992), and creeping bentgrass and bermudagrass (Keough et al., unpublished). Exogenous application of GB that can induce plant tolerance

to cold stress may provide an effective tool to protect plants from winter damages in the absence of natural cold acclimation due to warm fall seasons.

The protective roles of GB for cold tolerance has been mainly attributed to its regulation of osmotic potential protecting cells from dehydration and antioxidant systems in the ascorbate-glutathione cycle (Chen and Murata, 2011; Ishikawa & Shigeoka 2008). In addition to GB, the accumulation of amino acids and carbohydrates also occurs during cold acclimation, which play roles in protecting cells from cold or freezing injury (Kransensky and Jonak, 2012; Less and Galili, 2008). The major carbohydrates that are known to be involved in cold and freezing tolerance include sucrose (Gupta and Kaur, 2005; Shahryar and Maali-Amiri, 2016), fructose (Bogdanovic et al., 2008; Gupta and Kaur, 2005), starch (Rosa et al., 2009), and fructan (Livingston et al., 2009). Continued synthesis of amino acids under chilling is important to maintain protein synthesis and energy metabolism as substrates for the citric acid cycle (Kirma et al., 2012). Amino acids known to play roles in cold or freezing stress tolerance include methionine (Alcazar et al., 2011; Yu et al., 2001), asparagine (Azevedo et al., 2006), tyrosine (Krol et al., 2015), leucine (Ginger et al., 2000), and glycine (Kocsy et al., 2001). These are just a few amino acids that have been reported to play roles in cold and freezing tolerance, and other amino acids involved in cold adaption or acclimation and deserves further investigation.

We previously reported that exogenous treatment with 100 mM GB helped to mitigate the effects of chilling temperatures in creeping bentgrass. Increased cold tolerance was observed, as shown by increased turf quality and chlorophyll content, as well as lowering (more negative) osmotic potential. However, whether GB-induced cold tolerance in the absence of cold acclimation may be associated with changes in specific amino acid

and carbohydrate accumulation are not well understood. Therefore, the objective of this study was to investigate whether GB promotes tolerance to cold stress and enhances post-freezing recovery by inducing changes in endogenous metabolites, such as amino acids and carbohydrates, in the crowns of creeping bentgrass plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Tillers of creeping bentgrass (*Agrostis stolonifera* cv. Penncross) were propagated into pots (5 x 20 cm) filled with 100% sand. Plants were established for 60 d in a greenhouse controlled at 23/20 °C (day/night), 60% relative humidity, 14 h photoperiod, and 680 µmol m^{-2} s^{-1} photosynthetically active radiation from natural sunlight and supplemental lighting. During establishment, plants were irrigated daily, fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1949) twice per week, and trimmed weekly to maintain canopy height of 2 cm. Following establishment, plants were transferred to growth chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA) and allowed to acclimate for 7 d at 22°C prior to cold stress and chemical treatment with all environmental parameters the same as previously mentioned.

Treatments and experimental design

Creeping bentgrass plants were foliar sprayed with water (untreated control) or 100 mM glycine betaine (GB), and then exposed to cold stress by gradually decreasing temperatures at 15/10°C (day/night) for 21 d, 10/10°C (day/night) for 15 d, 4/4°C (day/night) for 3 d and -2/-2°C (day/night) for 3 d. The last two temperatures were included to prevent cold shock once the plants were introduced to freezing stress. GB was applied

on the first day of CS treatment and subsequently treated every 14 d for a total of 3 applications. Acclimated plants (ACL) were also untreated and exposed to lower temperatures to induce cold acclimation at 15/10°C (day/night) for 14 d, 2/2°C (day/night) for 14 d, and -2/-2°C (day/night) for 14 d. This temperature regime was previously determined to induce cold acclimation and freezing tolerance differences in creeping bentgrass (Hoffman et al., 2010; Espevig et al. 2014; Espevig et al., 2011). After CS or ACL periods, plants were exposed to freezing stress in a stepwise temperature reduction of -5°C, -10°C and -15°C for 2 h at each temperature in a cold chamber (SPX Thermal Product Solutions, New Columbia, Pennsylvania, USA). The range of temperature treatments was chosen based on preliminary studies in creeping bentgrass as plants could recovery from these levels of freezing stress (data not shown). Post-freezing recovery was evaluated periodically by maintaining plants at 20/15°C (day/night) for 47 d after exposure to freezing stress at -10°C or -15°C.

The experiment was arranged in a randomized complete block design with each temperature regime repeated in four cold chambers or growth chambers and GB treatment or the untreated control having 6 replicated pots, which were randomly placed inside the cold or growth chamber.

Physiological analysis

Four commonly-used parameters to evaluate plant physiological status and overall turf quality were evaluated at 14, 28, and 40 d of CS treatment. Visual evaluation of turf quality (TQ) was performed to assess overall plant performance at 14, 28, and 40 d CS treatment. TQ was rated on a scale of 1 to 9 with 9 being a turf plant that is healthy and green color, 1 representing a turf plant that is brown and dead, and 6 being the minimum

acceptable quality rating. Ratings were based factors such as leaf and canopy color, density, and uniformity (Beard, 1973).

Leaf relative water content (RWC) was measured to indicate leaf hydration status 14, 28, and 40 d CS treatment. Approximately 0.2 g of fresh leaf tissue was collected from plants and measured on a mass balance for fresh weight (FW). After incubating leaf tissue in water for 12 hours at 4 °C, the leaves were blotted dry, and weighed for turgid weight (TW). Leaf tissue was then dried in an oven at 80 °C for 72 h and the dry weight (DW) was measured. RWC was calculated using the formula % = [(FW – DW) / (TW – DW)] x100 (Barrs, 1962).

Osmotic potential was determined by soaking leaves in distilled water for 24 h to reach full turgor. Turgid leaves were frozen in liquid nitrogen and stored at -20 °C until analysis. Upon analysis, leaves were slowly thawed and the leaf sap was extracted and analyzed for osmolality (mmol kg^{-1}) using a vapor pressure osmometer (Vapro Model 5520; Wescor, Logan, UT). Osmolality was converted to osmotic potential (OP) using the formula: OP = [(– C * 2.58)/1000] (Blum, 1989).

Chlorophyll content was quantified by incubating 0.1 g of fresh leaf tissue in 10 ml dimethyl sulfoxide in darkness for 3 d until all chlorophyll was extracted from the leaf tissue. The absorbance values at 663 and 645 nm were read on a spectrophotometer (Genesys 2, Spectronic Instruments, Inc., Rochester, NY), after which the leaf tissue was filtered and dried in an oven set to 80ºC for 3 d. Dry weights were measured on a mass balance and chlorophyll content was calculated using the formula by Arnon (1949).

Recovery from freezing stress was evaluated using image analysis by SigmaScan computer software according to Karcher and Richardson (2005). Digital photographs of plant canopy were collected from a height of 0.6 m at 650 µmol $m^{-2}s^{-1}$ PAR and analyzed using SigmaScan Pro (v.5.0, SPSS, Inc., Chicago, IL) to determine canopy greenness. Red, green, and blue pixels within photographs (1127 x 813 pixels) were quantified by the software, and values for brightness (B), saturation (S), and hue (H) were assigned in order to calculate the dark green color index (DGCI). The index was measured by obtaining the amount of dark green color in an image and applying this equation: DGCI value = [(H-60)/60+(1-S)+(1-B)]/3 (Karcher and Richardson, 2005).

Metabolic analysis

High performance liquid chromatography (HPLC) analysis of free amino acids

Amino acids were extracted by grinding frozen crown material with liquid nitrogen and 4 ml of 70% ethanol. After storage overnight in 4°C, the samples were centrifuged (10500 rpm, 10 minutes) and the pellets were washed twice with 1 ml of 70% ethanol. The supernatants were collected, filtered through a membrane (0.45 um PTFE membrane) and concentrated under vacuum, and stored in -20°C (Rozan et al. 2000).

The free amino acid content of the extract was analyzed by an HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization (Khan et al. 1994). Buffer A consisting of 0.1 M ammonium acetate and buffer B consisting of 0.1 ammonium acetate, acetonitrile, and methanol (44:46:10 v/v) were used (Rozan et al. 2000).

For sample derivatization, 100 µl of the extract was removed and dried under vacuum (37°C). 20 µl of the first reagent [methanol, water, triethylamine (2:2:1 v/v)] was

added and then dried under vacuum. Then the sample was reacted with 30 µl of the PITC reagent [methanol, PITC, water, triethylamine (7:1:1:1 v/v)] at room temperature for 20 minutes before drying under vacuum. The derivatized samples were then re-dissolved in 1 ml of buffer A. A 20 µl sample was injected into the HPLC system (Waters 600 HPLC system), using a gradient system of buffer A (100-0% after 65 minutes) and buffer B (0-100% after 65 minutes) (Rozan et al. 2000). A C18 reversed phase column from Alltech (Alltima C18 5U, 250 x 4.6 mm) was used. The absorbance at 245 nm was used for the calculations. Individual standards were purchased from Sigma and prepared as above. The results were analyzed with Empower pro software (Waters). Proline and alanine could not be separated due to overlap in retention time, but since proline is accumulated more rapidly during cold stress, the majority of the content expressed in Figure 2G is thought to be proline (Hoffman et al. 2014; Zhang et al., 2011).

HPLC analysis of glycine betaine

Glycine betaine was extracted by adding 12.5 mL of water to 0.1 g of oven dried ground crown material on a shaker for 30 mins. The supernatant was filtered and the solution was transferred to solid phase cartridges (150 mg/6 mL; Poly-Sery MCX, CNW, Organomation, Germany). Then the extraction cartridges were rinsed by methanol/water (85/15, v/v) and methanol. The elution was finished by adding a mixture of ammonia water/methanol (5:95, v/v) twice. The eluent was condensed, dried and diluted with acetonitrile/water (50%, v/v) followed by passing through a 0.45 µm Millipore membrane for further analysis in HPLC (Waters 2695 HPLC system) (Waters Inc., USA). A solution of acetonitrile/water (50%, v/v) was the mobile phase for HPLC analyses. Glycine betaine was analyzed and quantified by HPLC using a Waters Atlantis HILIC Silica column (4.6

× 150mm filled with 5µm particle diameter; Milford, MA). The peak areas were integrated and compared with standard curve constructed with standard of glycine betaine purchased from Sigma.

Colorimetric analysis of water soluble sugars, fructose, glucose and sucrose

Soluble sugars were extracted by adding 5 ml of 80% ethanol to 50 mg of oven dried, ground crown material, and placed in a water bath at 30°C for 30 min. The supernatant was collected and the pellet was further extracted twice with 2.5 ml of 80% ethanol and placed in a water bath at 30°C for 30 min (Buysse et al. 1993). 1 ml of the extract solution was added to 1 ml of a 23% phenol solution, and then 5 ml of 98% sulfuric acid was added (Buysse et al. 1993). The absorbance was measured at 490 using a spectrometer (Spectronic Instruments, Inc., Rochester, NY).

Colorimetric analysis of total non-structural carbohydrates, fructan, and starch.

Total non-structural carbohydrates were extracted according to Ting (1956) by hydrolyzing 50 mg of oven dried crown material with soluble sugars removed, with 2.5 ml amylase for 24 hours in a water bath at 37°C. The following day 0.5 ml of 0.6 N hydrochloric acid was added, and samples remained in the water bath for another 18 hours. The solution pH was adjusted by adding 0.31 ml of 10N NaOH, then the volume was made up to 50 ml with distilled water. The TNC solution was filtered and 1 ml was added to 1.5 ml alkaline ferricyanide, and the mixture was boiled in a water bath for 10 minutes then quickly cooled. The solution was partially neutralized with 3 ml of 2N sulfuric acid and shaken to release gas. Finally, 1.2 ml of arsenomolybdate solution was added and the

volume was brought up to 25 ml (Ting et al., 1956). The absorbance was read at 515 nm using a spectrometer (Spectronic Instruments, Inc., Rochester, NY).

Statistical analysis

The effects of CS, ACL and GB treatment was determined by analysis of variance according to the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Differences between CS treatments with and without GB, and between CS and ACL treatments were separated by Fisher's protected least significance difference (LSD) test ($\alpha = 0.05$).

RESULTS

Physiological effects of exogenous treatment on mitigating cold injury

Turf quality declined in all treatments during CS and ACL, but the decline was less rapid in GB-treated plants. TQ of GB-treated plants was significantly higher than the untreated control plants at 40 d of CS (Fig. 1A).

Leaf RWC remained unchanged during 28 d of CS for the untreated and GB-treated plants, but declined significantly in the ACL plants (Fig. 1B). GB-treated and untreated control plants did not differ in RWC content. GB treatment and ACL resulted in significant decreases in OP compared to the untreated CS (Fig. 1C). Leaf chlorophyll content decreased under CS or ACL (Fig. 1D). Chlorophyll content was maintained 13.4% higher in GB-treated plants than the untreated control under CS at 40 d (Fig. 1D).

Amino acid accumulation as affected by GB under cold stress and during cold acclimation

A total of 18 amino acids were quantified in crowns of creeping bentgrass exposed to CS with or without GB treatment, and ACL. Out of the amino acids analyzed, 7 (serine, glutamine, histidine hydrochloride, cystine, phenylalanine, tryptophan, and proline + alanine) had significantly higher content in ACL plants, compared to CS (Fig. 2A-G). While 7 (aspartic acid, asparagine, glycine, tyrosine, methionine, leucine, lysine hydrochloride) had higher content in GB-treated plants compared to the untreated CS and ACL plants (Fig. 2H-N). The content of glutamic acid (55-64.6% lower), threonine (54-57.2% lower), valine (51.7-60% lower) and isoleucine (50-58% lower) was significantly lower in the untreated CS plants compared to GB-treated or ACL plants, although no significance in those amino acid content between GB and ACL treatments (Fig. 2P, Q, R, S). ACL resulted in higher content of serine (42.3-68% higher), glutamine (55.8-87.9%), histidine hydrochloride (27.7-86.6% higher), cystine (86.6-93% higher), phenylalanine (39.6-75.4% higher), tryptophan (15.7-47.3% higher), and proline + alanine (67.8-86.5% higher) compared to GB and untreated CS treatments (Fig. 2A-G). GB-treated plants had higher content of aspartic acid (27.3-58.9% higher), asparagine (48.5-79% higher), glycine (34.4-67.2% higher), tyrosine (36.3-80% higher), methionine (15-43% higher), leucine (22.2-52.9% higher), and lysine hydrochloride (34-57.2% higher), compared to untreated CS and ACL plants (Fig. 2H-N). Also there was a significantly higher GB content (94-97% higher) in GB-treated plants compared to untreated CS and ACL plants (Fig. 2O).

Carbohydrate accumulation as affected by GB under cold stress, during cold acclimation and after recovery from freezing stress

The untreated plants under CS had higher content of glucose and fructose (16.2-28.5% higher) and significantly higher sucrose (25.3-28% higher) compared to GB-treated

or ACL plants (Fig. 3A). After 47 d of recovery from freezing stress, ACL and GB-treated plants had a significantly higher content of soluble sugars, with GB plants having 36% higher content in all soluble carbohydrates compared to the untreated CS plants (Fig. 3B). There was no significant difference between soluble sugar content of ACL and GB plants at 47 d of freezing recovery.

GB-treated plants had a higher content of starch (13.1-36% higher) compared to the untreated CS and ACL plants during CS. Fructan was significantly increased (13.2% higher) compared to the untreated CS (Fig. 4A). After 47 d of recovery from freezing, GB plants had significantly higher starch (47.7-59.8%) and fructan (57-59%) compared to untreated CS and ACL plants (Fig. 4B).

Regrowth during post-freezing recovery as affected by GB under cold stress and during cold acclimation

GB treatment significantly promoted the recovery of plants from freezing stress, as expressed by green tissue percentage (Fig. 5A, B). After 32 d of plants transferred from the freezing temperatures to 10°C, the GB treatment started to show a significant increase in the green tissue percentage (by 32.5-46% higher than the untreated CS or ACL plants). By 47 d of post-freezing stress, GB plants had 58.9% and 45.5% more green tissue than the untreated CS and ACL plants, respectively (Fig. 5A). Although the recovery from -15°C was slower than that from those exposed to -10°C, regrowth also was observed in the GB plants starting at 36 d and significantly improved at 43 and 47 d. By 47 d, the GB treated plants had a 41 and 63% increase in recovery compared to ACL and the untreated CS plants (Fig. 5B).

DISCUSSION

The increased tolerance to cold stress from GB treatment was shown physiologically by increased turf quality, chlorophyll content and decreased osmotic potential. Since chlorophyll is an important molecule for photosynthesis, the increased content from GB treatment may be an indication of overall greater plant health and metabolism (Turgeon, 1999; Pavlovic et al., 2014). A more negative osmotic potential indicates a greater ability for a cell to perform osmotic adjustment, which is critical for maintaining cell integrity under cold stress for plant growth and development (Janska et al., 2009). Also the ability of exogenous GB to enhance osmotic adjustment in the absence of significant changes in cellular water content would decrease the cellular freezing point and delay or mitigate the extent of cellular damages upon prolonged periods of decreasing temperatures (Chen and Murata, 2011; O'Neill, 1983). These improved physiological responses from GB treatment may be associated with changes in carbohydrate and amino acid metabolism, as discussed below.

It is well known that protective compounds like amino acids accumulate in the response of decreasing temperatures and during the cold acclimation process to induce winter hardening (Hannah et al., 2006). Our results confirmed that ACL resulted in the accumulation of various amino acids as seen in serine, glutamine, histidine hydrochloride, cystine, phenylalanine, tryptophan, proline, and alanine. The new finding in our study was that exogenous GB application alone without cold acclimation also induced the significant accumulation of aspartic acid, asparagine, glycine, tyrosine, methionine, leucine, lysine hydrochloride, and glycine betaine. These results suggested that GB application had similar effects as cold acclimation, which could serve as a nitrogen source for the metabolism of

amino acids or activate amino acid metabolism. The accumulation of many protective free amino acids in GB-treatment plants could help explain the increased cold tolerance, as shown by improved overall quality during CS and increased recovery from freezing.

Generally amino acids are precursors for proteins and other metabolites, usually involving energy production (Less and Galili, 2008). Amino acids that were accumulated due to GB treatment, such as aspartic acid may help in continued synthesis of proteins and other free amino acids, such as lysine, threonine, methionine, isoleucine and glycine which have roles as substrates in energy metabolism or protein production (Kirma et al., 2012). GB-induced accumulation of methionine may promote the synthesis of metabolites like polyamines which are thought to stabilize lipids and proteins, and other metabolites like ethylene which has antifreeze properties and plays roles in overwintering as show in perennial ryegrass (*Lolium multiflorum*) (Alcazar et al., 2011; Yu et al., 2001). GB treatment also resulted in accumulated asparagine which has transport properties, and may provide nutrition to crucial tissues which may help maintain crown survival under cold stress (Azevedo et al., 2006). Glycine is a precursor to pyruvate in the citric acid cycle or glycolysis, to maintain energy production (Kocsy et al., 2001). Also an accumulation of glycine is necessary for glutathione synthesis, which reduces potentially toxic hydrogen peroxide concentrations (Kocsy et al., 2001). The GB accumulation of glycine may provide tolerance to cold stress through maintain metabolic activities or through detoxification properties. Also this amino acid can be methylated to produce the compatible solute glycine betaine, which has been reported to play pivotal roles in cold acclimation and hydration (Sakamoto and Murata. 2002). The accumulation of glycine and glycine betaine in the GB-treated plants could potentially be a defense response to maintain metabolic activities and

protect cells constituents from cold induced dehydration (Chen et al., 2011; Ashraf and Foolad, 2007; Naidu et al., 1991). Tyrosine is an aromatic amino acid that is commonly used for the synthesis of proteins and is a precursor for important cell components like chlorophyll and cell wall components, and may play important roles during cold stress by maintaining chlorophyll and membrane integrity (Maeda and Dudareva, 2012). Also tyrosine can lead to the production of phenolic acid compounds, which provides tolerance to stress by antioxidant effects, through the synthesis of coumaric acid and phenylalanine (Krol et al., 2015). The accumulation of tyrosine in GB treated plants may aid in detoxification or in the maintenance of chlorophyll, as shown as higher TQ and chlorophyll content in this study. GB accumulation of leucine may play roles in cold tolerance through its ability to maintain proteins synthesis or aid in transport by decreasing membrane permeability to enhance uptake of K or other important metabolites (Rai et al., 1983; Rana et al., 1996). Leucine also plays role in fatty acid and sterol synthesis, maintaining membrane fluidity under chilling, and the accumulation of leucine in this study may aid in explaining the increased cold tolerance of GB treated plants (Ginger et al., 2000). Lysine is necessary for dehydrin proteins that bind to membranes and other cell components to maintain stability, prevent protein aggregation or denaturing during stresses involving water deprivation (Hara et al., 2003). Lysine also synthesizes other crucial amino acids like threonine, methionine and isoleucine and catabolizes metabolites to release energy during stress (Azevedo and Lea, 2001). Also free amino acids contribute to the osmotic potential of the cell, since many of them are nitrogen rich or compatible solutes, which may help explain the increased cold tolerance as well as the increased (more negative) OP of the GB treated plants (Rhodes and Hanson, 1993). Studies involving salinity stress have shown

that serine, glycine, proline, aspartate (aspartic acid), leucine, lysine are main contributors to OP increases during stress (Martino et al., 2003). This study is consistent in the accumulation of many of these amino acids mentioned and may help explain the increased growth and development through maintained cell integrity during cold stress.

Soluble and non-structural carbohydrates are known to play important roles in plant tolerance to freezing stress since they serve as energy reserves among various other functions (Rosa et al., 2009). In this study, ACL plants had lower soluble sugar, starch and fructan while GB-treated plants had equivalent or lower content of those carbohydrates than the untreated control under CS; however, both ACL and GB-treated plants had improved freeing tolerance, as shown by the enhanced recovery in green leaf tissues, and both plants had significantly greater content of soluble sugars while GB also increased starch and fructan accumulation during post-freezing. These results suggest that the maintenance of greater amount of carbohydrates after freezing stress plays more important roles in promoting plant regrowth from freezing stress in creeping bentgrass, while the content of carbohydrates may not directly be correlated to cold tolerance. Although it is commonly thought that sugar content is a direct correlation to cold or freezing tolerance, studies have shown an accumulation of sugars without increasing cold tolerance, indicating that there are other important factors involved tolerance to cold stress (Koster and Lynch 1992; Sasaki et al., 1995).

Soluble sugars play roles in metabolism, structure, signaling pathways, and respond to stress (Rosa et al., 2009). Sucrose and glucose are common substrates for respiration and act in osmotic adjustment to maintain homeostasis, and fructose is involved in metabolite synthesis (Gupta and Kaur, 2005). The increase of soluble sugars for the GB treated and

ACL compared to CS plants during recovery from freezing could help explain the increased recovery from freezing stress possibly through the maintenance of metabolic activities. Also during new growth, carbohydrates are mobilized in the form of soluble sugars to tissues important for plant development (Rosa et al., 2009). The increase in soluble sugars in GB and ACL plants after freezing, may possibly contribute to the new growth of plant tissues. The stored carbohydrates, starch and fructan, are used for long-term survival under stress (Sasaki et al., 1995). The accumulation of fructan can contribute to the stabilization of membranes by binding to phosphate and choline groups of the membranes to reduce water loss (Janska et al., 2010). Accumulation of soluble and non-structural carbohydrates during recovery from freezing in GB treated plants may aid in explaining the increased tolerance to cold stress and recovery from freezing stress.

CONCLUSION

Exogenous treatment of creeping bentgrass with GB resulted in improved tolerance to cold stress, demonstrated by improved quality, more negative osmotic potential, and increased chlorophyll content under CS, and freezing tolerance, as shown by the increased green leaves during post-freezing recovery. In general, metabolic changes in the GB treatment during cold stress for induced freezing tolerance included a significant increase in various amino acids. Also metabolic changes after freezing stress of GB treatment included an increase in soluble carbohydrates and significant increase in non-structural carbohydrates during recovery from freezing stress. The enhanced cold and freezing tolerance due to GB could be associated with its regulation of amino acids and carbohydrate metabolism. For management practices, GB may be incorporated into bio-

stimulant programs, alleviating the effects of cold and freezing injury in cool-season turfgrass management.

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FIGURES

Figure 1: Creeping bentgrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of glycine betaine (100 mM) during 40 days of cold stress treatment, compared to CS and ACL plants. LSD bars represent significant differences exist at $p \leq 0.05$.

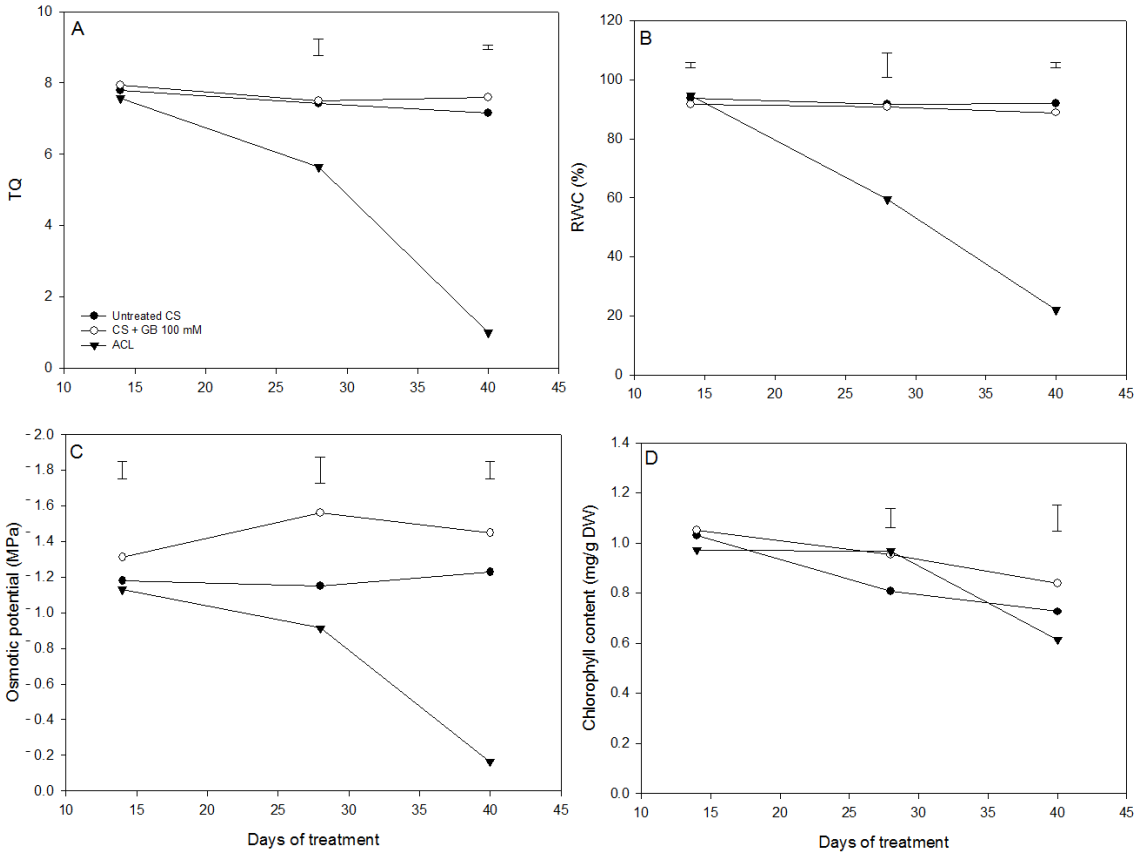
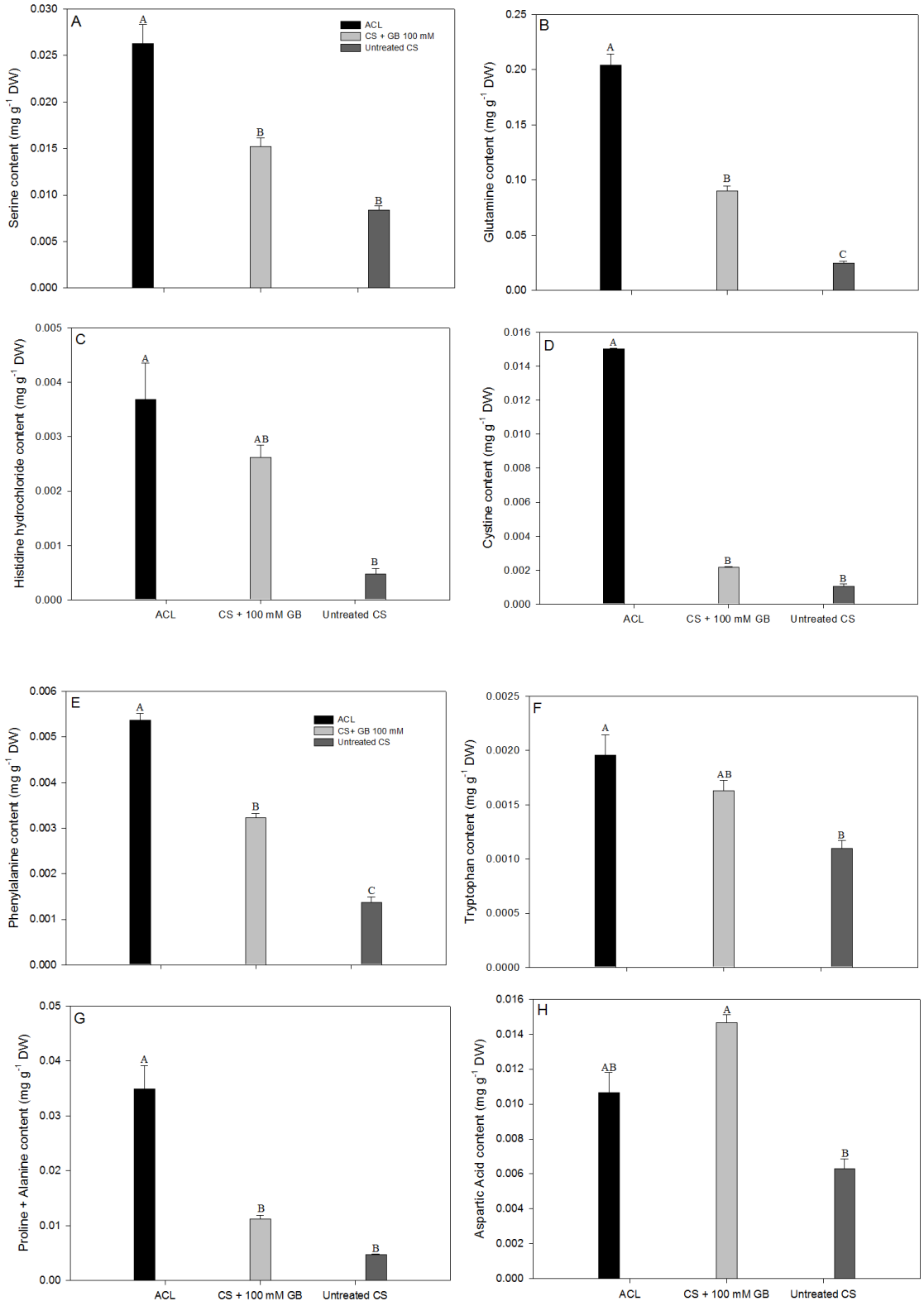
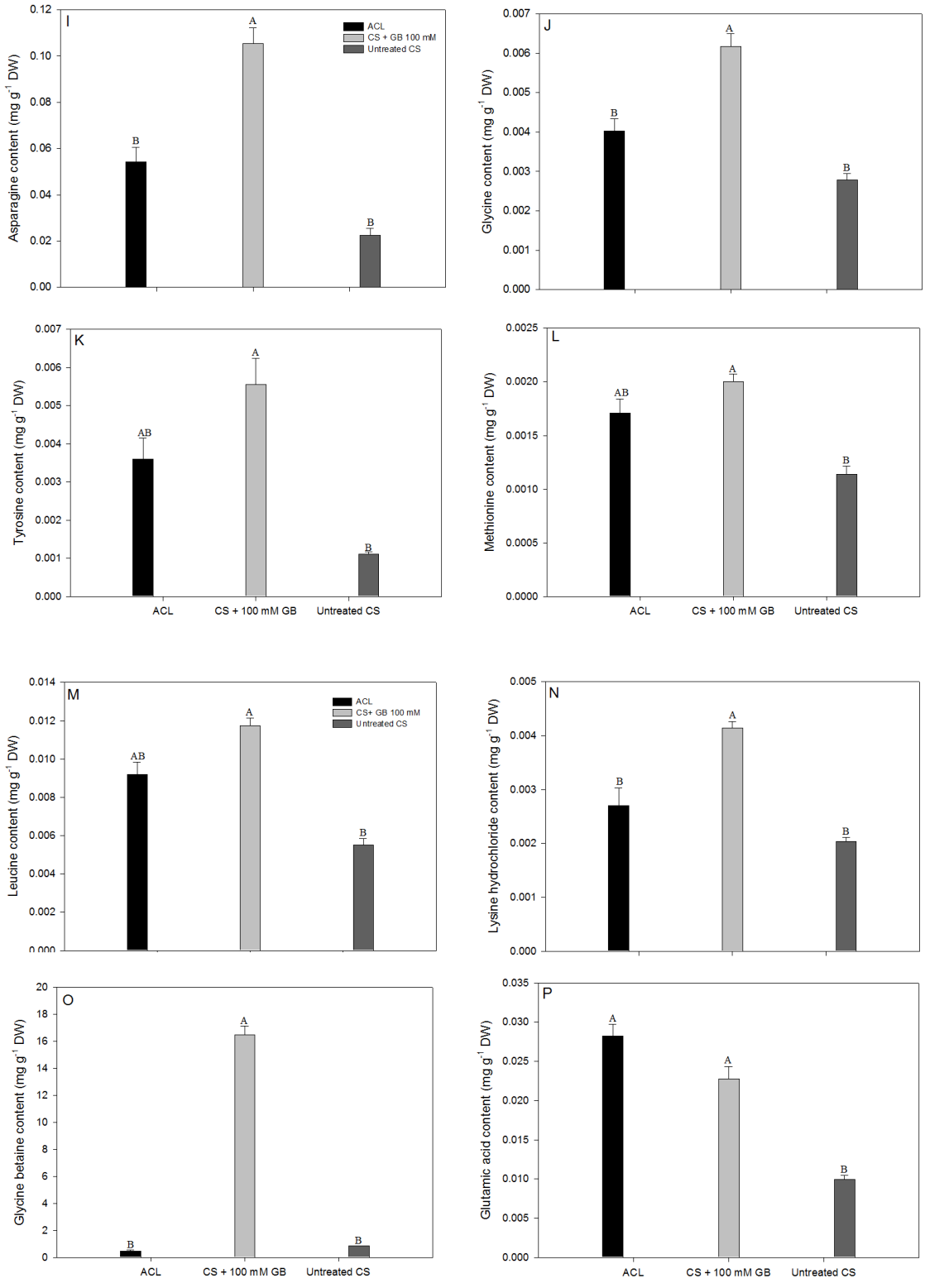


Figure 2: Creeping bentgrass crown changes in serine (A), glutamine (B), histidine hydrochloride (C), cystine (D), phenylalanine (E), tryptophan (F), proline + alanine (G), aspartic acid (H), asparagine (I), glycine (J), tyrosine (K), methionine (L), leucine (M), lysine hydrochloride (N), glycine betaine (O), glutamic acid (P), threonine (Q), valine (R), and isoleucine (S) following treatment of glycine betaine (100 mM) during 42 days of cold stress treatment, compared to CS and ACL plants. Values with similar letters are not significantly different at the $p \leq 0.05$ level.


A
ACL
CS + GB 100 mM
Untreated CS
Serine content (mg g-1 DW)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
A
B
B
B
Glutamine content (mg g-1 DW)
0.25
0.20
0.15
0.10
0.05
0.00
A
B
C
C
Histidine hydrochloride content (mg g-1 DW)
0.005
0.004
0.003
0.002
0.001
0.000
A
AB
B
ACL
CS + 100 mM GB
Untreated CS
D
Cystine content (mg g-1 DW)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
A
B
B
ACL
CS + 100 mM GB
Untreated CS
E
ACL
CS+ GB 100 mM
Untreated CS
Phenylalanine content (mg g-1 DW)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
A
B
C
F
Tryptophan content (mg g-1 DW)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
A
AB
B
G
Proline + Alanine content (mg g-1 DW)
0.05
0.04
0.03
0.02
0.01
0.00
A
B
B
ACL
CS + 100 mM GB
Untreated CS
H
Aspartic Acid content (mg g-1 DW)
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
AB
A
B
ACL
CS + 100 mM GB
Untreated CS


I
Asparagine content (mg g-1 DW)
ACL
CS + GB 100 mM
Untreated CS
J
Glycine content (mg g-1 DW)
K
Tyrosine content (mg g-1 DW)
L
Methionine content (mg g-1 DW)
M
Leucine content (mg g-1 DW)
CS+ GB 100 mM
N
Lysine hydrochloride content (mg g-1 DW)
O
Glycine betaine content (mg g-1 DW)
P
Glutamic acid content (mg g-1 DW)
CS + 100 mM GB
Untreated CS

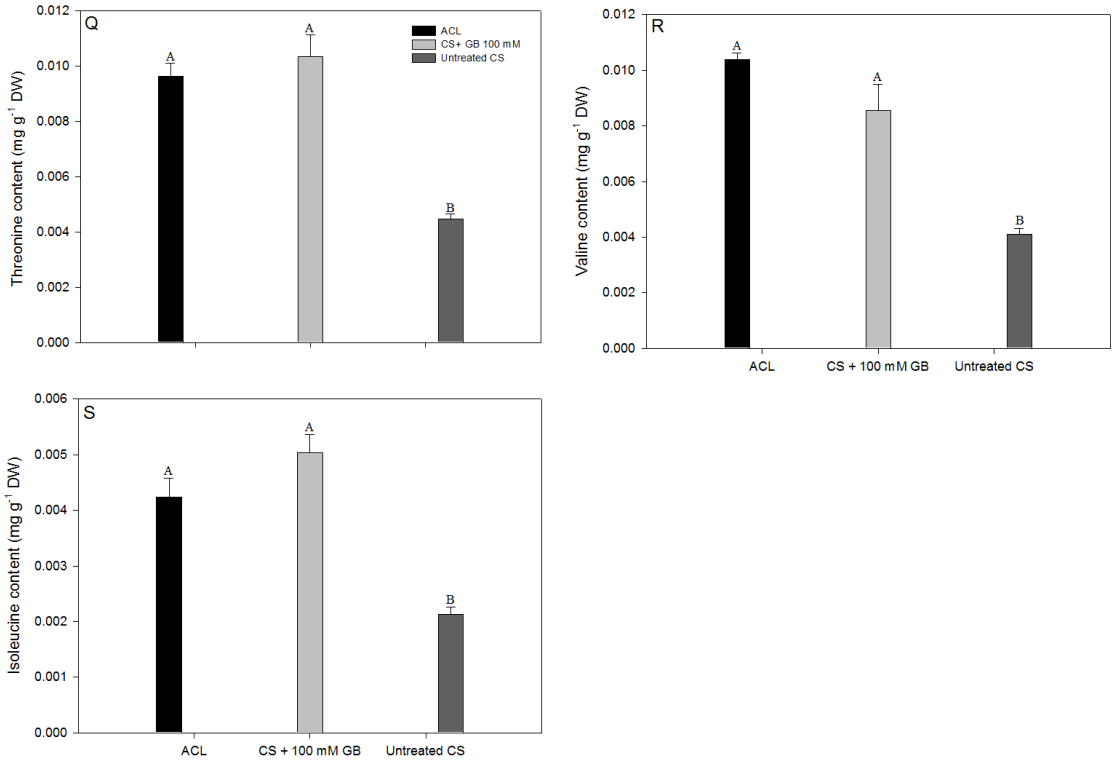

Q
ACL
CS+ GB 100 mM
Untreated CS
Threonine content (mg g-1 DW)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
A
A
B
R
Valine content (mg g-1 DW)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
A
A
B
ACL
CS + 100 mM GB
Untreated CS
S
Isoleucine content (mg g-1 DW)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
A
A
B
ACL
CS + 100 mM GB
Untreated CS

Figure 3: Creeping bentgrass crown changes in glucose, fructose, and sucrose following treatment of glycine betaine (100 mM), during 42 days of cold stress treatment (A), and after 47 days of recovery from freezing stress (B) compared to CS and ACL plants for individual carbohydrates. Values with similar letters are not significantly different at the $p \leq 0.05$ level.

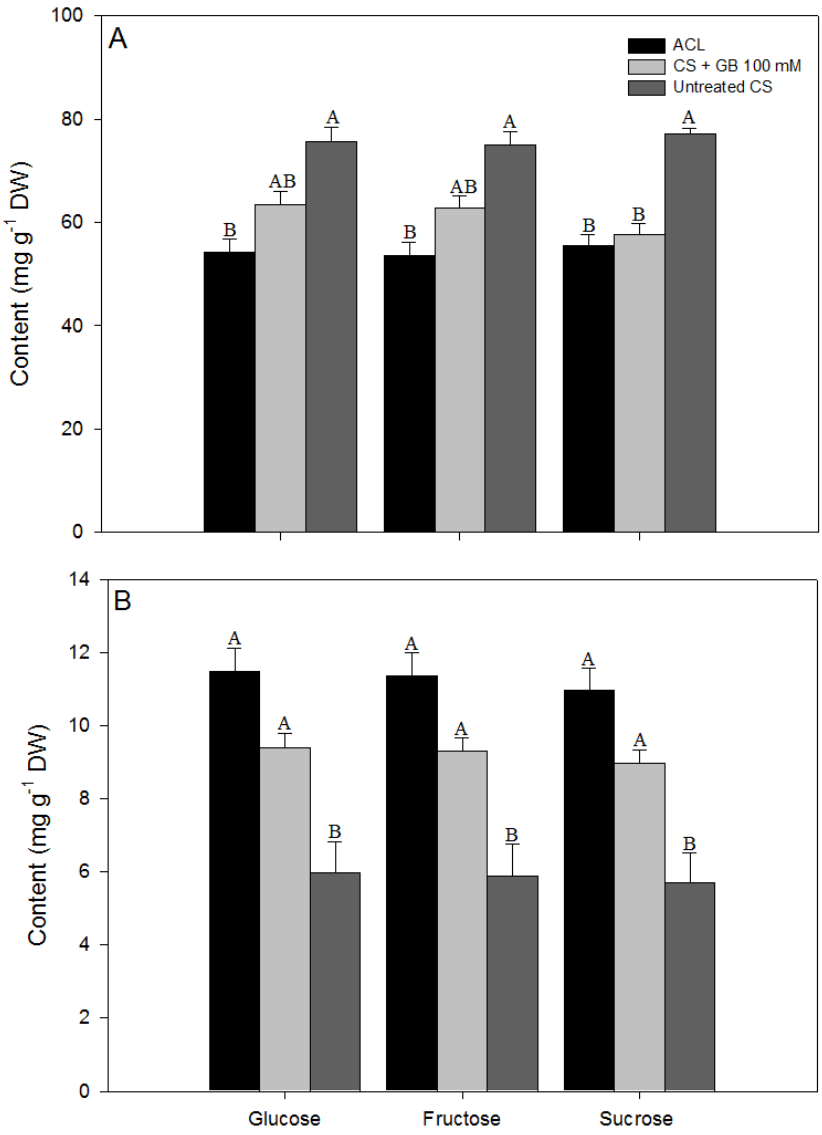


Figure 4: Creeping bentgrass crown changes in total non-structural carbohydrates, starch and fructan, following treatment of glycine betaine (100 mM), during 42 days of cold stress treatment (A), and after 47 days of recovery from freezing stress (B) compared to CS and ACL plants for individual carbohydrates. Values with similar letters are not significantly different at the $p \leq 0.05$ level.

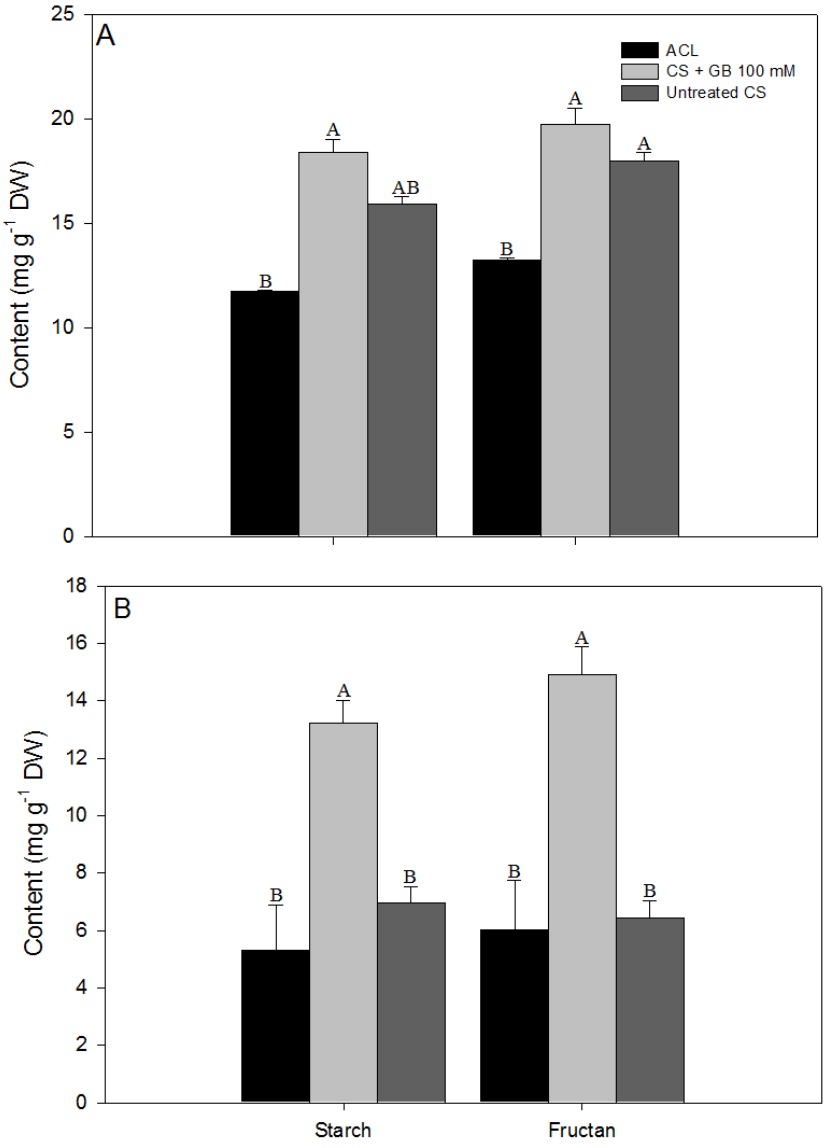
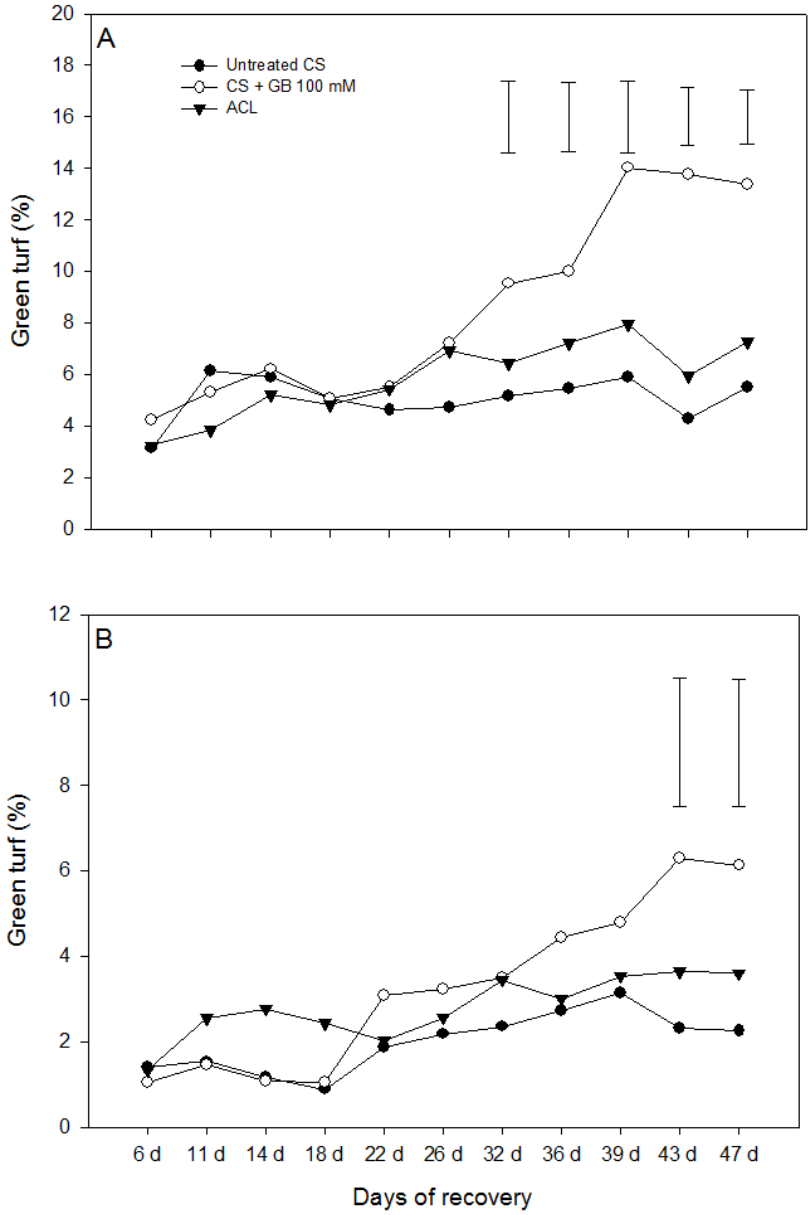


Figure 5: Creeping bentgrass recovery from freezing stress during 47-day post freezing at -10°C (A), and -15°C (B). LSD bars represent significant differences exist at $p \leq 0.05$.



CHAPTER 4

Physiological and Metabolic Changes Associated with Tolerance to Cold and Freezing Stress in Bermudagrass as Affected by Foliar Application of Potassium Phosphate.

ABSTRACT

Freezing and chilling temperature stress are major abiotic stress that influence plant growth in cool climatic regions, especially for warm-season grass species. Tolerance to low temperature has been associated with the accumulation of stress-protective metabolites. Our previous study reported exogenous treatment of plants with potassium phosphate (KH_2PO_4) promoted cold and freezing tolerance in bermudagrass (*Cynodon dactylon*). The objective of this study was to investigate whether the beneficial effects of KH_2PO_4 on cold and freezing tolerance were associated with the induction or accumulation of amino acids and carbohydrates in bermudagrass crown tissue. Bermudagrass (cv. 'Tifway') plants were foliar sprayed with water or KH_2PO_4 (50 mM), and then subjected to cold stress (CS) treatment at 25/20°C (day/night) for 32 d and 10/10°C (day/night) for 3 d or, decreasing temperatures from 25/20°C (day/night) for 7 d to 8/8°C (day/night) for 28 d in a cold chamber to induce acclimation (cold acclimation, ACL). Plants of CS with or without KH_2PO_4 and ACL plants were then subjected to freezing stress. Physiological analysis indicated that 50 mM of KH_2PO_4 induced cold tolerance in bermudagrass through improved TQ and chlorophyll content. Plants treated with KH_2PO_4 resulted in increased content of amino acids (glutamine and tryptophan) in the crowns exposed to cold stress, significantly or to a similar extent as observed in ACL, as well as the accumulation of soluble sugars (glucose, fructose and sucrose) and non-structural carbohydrates (starch and fructan) under CS. During post freezing recovery KH_2PO_4 treated plants had greater green leaf percentage compared to untreated CS and ACL plants. This study showed that KH_2PO_4 enhanced bermudagrass tolerance to cold stress and freezing tolerance associated with the accumulation of amino acids, and carbohydrates.

INTRODUCTION

Cold acclimation is a metabolic and physiological process required for plant survival during winter months and is indicated by the accumulation of key endogenous protective compounds which mediate recovery from freezing stress when temperatures increase during spring (Janska et al., 2010). Although the process of cold acclimation is not completely known, the accumulation of certain protective compounds, such as nitrogen-rich compounds, amino acids, and carbohydrates are key mechanisms of facilitating plant survival during cold and freezing stress, as well as post-freezing recovery (Janska et al., 2009; Thomashow, 1999). Exogenous application of certain compounds that typically accumulate during the cold acclimation process could be favorable to improve plant cold and freezing tolerance.

An essential nutrient element, potassium (K), does not only provide nutritional value by providing fertility, but also affects various biochemical processes which regulate growth and response to stress (Kant et al., 2002; Wang et al., 2013). Exogenous applications of K have been shown to relieve cold injury by enhancing antioxidant systems by increasing ginsenoside (a class of steroid glycosides)-related secondary metabolite transcripts, catalase, and ascorbate peroxidase to reduce oxidative damage, as well as protecting photosynthetic machinery to decrease the production of reactive oxygen species during abundant election transport (Devi et al., 2012; Wang et al. 2013). Potassium also may help maintain cellular water status and has antifreeze properties by decreasing the freezing point of the cell to alleviate ice formation shown by increased osmotic adjustment and reduced electrolyte leakage (Devi et al., 2012; Kant et al., 2002). Exogenous treatments have shown to alter gene expression for genes related to secondary metabolism or

antioxidant processes and an increase in cold tolerance as seen in ginseng(*Panax ginseng*) (Devi et al., 2012). Application of K in the autumn may also increase tolerance to decreasing temperatures in cool-season grass species (Fry and Huang, 2004). Also exogenous K provides induced tolerance to stresses involving decreased water status in various plants, such as maize (Abbasi et al., 2014), beans (*Phaseolus vulgaris L*) (Singer et al., 1996) or other agricultural crops (Kant et al., 2002). Potassium combined with phosphorous in the form of phosphate has been shown to include tolerance to stress involving water deprivation as seen in salinity stress (Akram et al., 2011). Phosphate is a main component of RNA and DNA, and is incorporated into the structure of nucleic acids which regulates enzymes and protein synthesis (Schachtman et al., 1998). Phosphates also are incorporated into the energy component used by plants, ATP, which has critical functions in photosynthesis and metabolism. Also phosphates have been shown to induce cell division for the growth of new tissues after periods of stress (Schluter et al., 2013).

The accumulation of carbohydrates and amino acids occurs during cold acclimation, which could play roles in protecting cells from cold or freezing injury (Kransensky and Jonak, 2012). The main carbohydrates that are known to be involved in cold and freezing tolerance include fructose (Bogdanovic et al., 2008; Gupta and Kaur, 2005)**,** sucrose (Gupta and Kaur, 2005; Shahryar and Maali-Amiri, 2016), starch (Rosa et al., 2009), and fructan (Livingston et al., 2009). Maintained synthesis of amino acids under cold stress is critical to maintain protein synthesis and energy metabolism as substrates for the citric acid cycle (Kirma et al., 2012). Amino acids known to play roles in cold or freezing stress tolerance include glutamine (Cai et al., 2009), methionine (Alcazar et al., 2011; Yu et al., 2001), asparagine (Azevedo et al., 2006), tyrosine (Krol et al., 2015),

tryptophan (Zhoa et al., 1998), leucine (Ginger et al., 2000), and glycine (Kocsy et al., 2001). These are just some of the amino acids that have been reported to play roles in chilling and freezing tolerance, and the complete amino acid content under cold stress deserves further investigation.

We previously reported the exogenous treatment of 50 mM of KH_2PO_4 helped to mitigate the effects of cold stress and promote recovery post freezing stress in bermudagrass species. Increased cold tolerance was observed as higher turf quality and chlorophyll content, lowered osmotic potential (more negative) and maintained relative water content. However, whether KH_2PO_4 induced cold or freezing tolerance in the absence of cold acclimation which may be associated with changes in specific amino acid and carbohydrate accumulation is not well understood. Therefore, the objective of this study was to investigate whether KH_2PO_4 promotes tolerance to cold stress and enhances post-freezing recovery by inducing changes in endogenous metabolites, such as amino acids and carbohydrates, in the crown tissue of bermudagrass plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Tillers of bermudagrass (*Cynodon dactylon* cv. Tifway) were propagated into pots (5 x 20 cm) filled with sand. Plants were established for 60 d in a greenhouse set to 26/22°C (day/night), 60% relative humidity, 14 h photoperiod, and 500 µmol m^{-2} s^{-1} photosynthetically active radiation from natural sunlight and supplemental lighting. During establishment, plants were irrigated daily, fertilized with half-strength Hoagland's solution (Hoagland and Arnon, 1949) twice per week, and trimmed weekly to maintain canopy

height 4 cm. Following establishment, plants were transferred to growth chambers (Environmental Growth Chamber, Chagrin Falls, Ohio, USA) and allowed to acclimate for 7 days at 30°C prior to chemical or cold stress treatment with all environmental parameters the same as previously mentioned.

Treatments and experimental design

Bermudagrass plants were foliar sprayed with water (untreated control) or potassium phosphate (KH_2PO_4) and then exposed to cold stress (CS) by gradually decreasing temperatures at 25/20°C (day/night) for 32 d and 10/10°C (day/night) for 3 d in cold chambers. The last temperature was implemented in order to prevent cold shock once freezing stress was applied. Foliar application was applied on the first day of CS treatment and subsequently treated every 14 d for a total of 3 applications. Acclimated plants (ACL) were also untreated and exposed to lower temperatures to induce cold acclimation at 25/20°C (day/night) for 7 d and 8/8°C (day/night) for 28 d in cold chamber. This temperature regime was previously determined to induce cold acclimation and freezing tolerance in bermudagrass (Zhang et al., 2011; Gatschet et al., 1994). After CS or ACL period, plants were exposed to freezing stress in a stepwise temperature reduction of 4, -1, -5 and -7°C, for 2 h at each temperature in a cold chamber (SPX thermal product solutions, New Columbia, Pennsylvania, USA). The range of temperature treatments was determined based on lethal temperature analysis in bermudagrass (Miller and Dickens, 1996). Post freezing recovery was evaluated periodically by maintaining plants at 28/28°C (day/night) for 35 d after exposure to freezing stress at -7°C.

The experiment was arranged in a randomized complete block design with each temperature regime repeated in four cold chambers or growth chambers and KH_2PO_4 treatment or the untreated controls having 6 replicated pots, which were randomly placed inside the cold or growth chamber

Physiological analysis

Four commonly-used parameters to evaluate plant physiological status and overall turf quality were evaluated at 14, 28, and 35 d of CS treatment. Visual evaluation of turf quality (TQ) was performed to assess overall plant performance at 14, 28, and 35 d CS treatment. TQ was rated on a scale of 1 to 9 with 9 being a turf plant that is healthy and green color, 1 representing a turf plant that is brown and dead, and 6 being the minimum acceptable quality rating. Ratings were based factors such as leaf and canopy color, density, and uniformity (Beard, 1973).

Leaf relative water content (RWC) was measured to indicate leaf hydration status 14, 28, and 35 d CS treatment. Approximately 0.2 g of fresh leaf tissue was collected from plants and measured on a mass balance for fresh weight (FW). After incubating leaf tissue in water for 12 hours at 4 °C, the leaves were blotted dry, and weighed for turgid weight (TW). Leaf tissue was then dried in an oven at 80 °C for 72 h and the dry weight (DW) was measured. RWC was calculated using the formula % = [(FW – DW) / (TW – DW)] x100 (Barrs, 1962).

Osmotic potential was determined by soaking leaves in distilled water for 24 h to reach full turgor. Turgid leaves were frozen in liquid nitrogen and stored at -20 °C until analysis. Upon analysis, leaves were slowly thawed and the leaf sap was extracted and

analyzed for osmolality (mmol kg^{-1}) using a vapor pressure osmometer (Vapro Model 5520; Wescor, Logan, UT). Osmolality was converted to osmotic potential (OP) using the formula: OP = [(– C * 2.58)/1000] (Blum, 1989).

Chlorophyll content was quantified by incubating 0.1 g of fresh leaf tissue in 10 ml dimethyl sulfoxide in darkness for 3 d until all chlorophyll was extracted from the leaf tissue. The absorbance values at 663 and 645 nm were read on a spectrophotometer (Genesys 2, Spectronic Instruments, Inc., Rochester, NY), after which the leaf tissue was filtered and dried in an oven set to 80ºC for 3 d. Dry weights were measured on a mass balance and chlorophyll content was calculated using the formula by Arnon (1949).

Recovery from freezing stress was quantified using image analysis by SigmaScan computer software according to Karcher and Richardson (2005). Digital photographs of plant canopy were collected from a height of 0.6 m at 650 µmol $m^{-2}s^{-1}$ PAR and analyzed using SigmaScan Pro (v.5.0, SPSS, Inc., Chicago, IL) to determine canopy greenness. Red, green, and blue pixels within photographs (1127 x 813 pixels) were quantified by the software, and values for brightness (B), saturation (S), and hue (H) were assigned in order to calculate the dark green color index (DGCI). The index was measured by obtaining the amount of dark green color in an image and applying this equation: DGCI value = [(H-60)/60+(1-S)+(1-B)]/3 (Karcher and Richardson, 2005).

Metabolic analysis

High performance liquid chromatography (HPLC) analysis of free amino acids

Amino acids were extracted by grinding frozen crown material with liquid nitrogen and 4 ml of 70% ethanol. After storage overnight in 4°C, the samples were centrifuged

(10500 rpm, 10 minutes) and the pellets were washed twice with 1 ml of 70% ethanol. The supernatants were collected, filtered through a membrane (0.45 um PTFE membrane) and concentrated under vacuum, and stored in -20°C (Rozan et al. 2000).

The free amino acid content of the extract was analyzed by an HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization (Khan et al. 1994). Buffer A consisting of 0.1 M ammonium acetate and buffer B consisting of 0.1 ammonium acetate, acetonitrile, and methanol (44:46:10 v/v) were used (Rozan et al. 2000).

For sample derivatization, 100 µl of the extract was removed and dried under vacuum (37°C). 20 µl of the first reagent [methanol, water, triethylamine (2:2:1 v/v)] was added and then dried under vacuum. Then the sample was reacted with 30 µl of the PITC reagent [methanol, PITC, water, triethylamine (7:1:1:1 v/v)] at room temperature for 20 minutes before drying under vacuum. The derivatized samples were then re-dissolved in 1 ml of buffer A. A 20 µl sample was injected into the HPLC system (Waters 600 HPLC system), using a gradient system of buffer A (100-0% after 65 minutes) and buffer B (0-100% after 65 minutes) (Rozan et al. 2000). A C18 reversed phase column from Alltech (Alltima C18 5U, 250 x 4.6 mm) was used. The absorbance at 245 nm was used for the calculations. Individual standards were purchased from Sigma and prepared as above. The results were analyzed with Empower pro software (Waters). Proline and alanine could not be separated due to overlap in retention time, but since proline is accumulated more rapidly during cold stress, the majority of the content expressed in Figure 2K is thought to be proline (Hoffman et al. 2014; Zhang et al., 2011).

Colorimetric analysis of water soluble sugars, fructose, glucose and sucrose

Soluble sugars were extracted by adding 5 ml of 80% ethanol to 50 mg of oven dried, ground crown material, and placed in a water bath at 30°C for 30 min. The supernatant was collected and the pellet was further extracted twice with 2.5 ml of 80% ethanol and placed in a water bath at 30°C for 30 min (Buysse et al. 1993). 1 ml of the extract solution was added to 1 ml of a 23% phenol solution, and then 5 ml of 98% sulfuric acid was added (Buysse et al. 1993). The absorbance was measured at 490 using a spectrometer (Spectronic Instruments, Inc., Rochester, NY).

Colorimetric analysis of total non-structural carbohydrates, fructan, and starch.

Total non-structural carbohydrates were extracted according to Ting (1956) by hydrolyzing 50 mg of oven dried crown material with soluble sugars removed, with 2.5 ml amylase for 24 hours in a water bath at 37°C. The following day 0.5 ml of 0.6 N hydrochloric acid was added, and samples remained in the water bath for another 18 hours. The solution pH was adjusted by adding 0.31 ml of 10N NaOH, then the volume was made up to 50 ml with distilled water. The TNC solution was filtered and 1 ml was added to 1.5 ml alkaline ferricyanide, and the mixture was boiled in a water bath for 10 minutes then quickly cooled. The solution was partially neutralized with 3 ml of 2N sulfuric acid and shaken to release gas. Finally, 1.2 ml of arsenomolybdate solution was added and the volume was brought up to 25 ml (Ting et al., 1956). The absorbance was read at 515 nm using a spectrometer (Spectronic Instruments, Inc., Rochester, NY)

Statistical analysis

The effects of CS, ACL and KH_2PO_4 treatment were determined by analysis of variance according to the general linear model procedure of SAS (version 9.2, SAS

Institute, Cary, NC). Differences between CS treatments with and without KH_2PO_4, and between CS and ACL treatments were separated by Fisher's protected least significance difference (LSD) test ($\alpha = 0.05$).

RESULTS

Physiological effects of exogenous treatment on mitigating cold injury

Turf quality declined in all treatments during CS and ACL, but the decline was less rapid in plants treated with KH_2PO_4. TQ of KH_2PO_4 was significantly higher than the untreated control plants at 35 d of CS (Fig. 1A).

Leaf RWC remained unchanged during 35 d of cold stress for the untreated and KH_2PO_4, but declined significantly in the ACL plants (Fig. 1B). KH_2PO_4 treated and untreated control did not differ in RWC content at 35 d. There was no significant effect on osmotic potentials due to treatments at 14 d, but by 28 d ACL had a significantly lower negative osmotic potential (by 14.8-20.2%) compared to CS and KH_2PO_4 (Fig. 1C). OP at 35 d did not differ significantly between treatments (Fig. 1C). Leaf chlorophyll content decreased under CS or ACL (Fig. 1D). Chlorophyll content by 35 d of cold stress was 23.7% higher in KH_2PO_4 treated plants than the untreated control under CS (Fig. 1D).

Amino acid accumulation as affected by KH_2PO_4 under cold stress and during cold acclimation

A total of 14 amino acids were quantified in crowns of bermudagrass exposed to CS with or without KH_2PO_4 treatment, and ACL. Out of the amino acids analyzed 12 (serine, asparagine, glycine, threonine, tyrosine, methionine, cysteine, isoleucine, leucine, lysine hydrochloride, and proline + alanine and valine) had significant accumulation in

ACL plants, compared to CS (Fig. 2A-L). ACL and KH_2PO_4 treatment resulted in significant accumulation of glutamine, although there was no difference between ACL and KH_2PO_4 plants (Fig. 2M). KH_2PO_4 treatment resulted in significant accumulation of tryptophan compared to ACL and CS plants (Fig. 2N). ACL resulted in significantly higher content of serine (by 68-77.6%), asparagine (by 46.3-74.9%), glycine (by 71.9-79.2%), threonine (by 84.3-93%), tyrosine (by 34.8-63.5%), methionine (by 52.5-60.3%), cysteine (by 44.1-61.7%), isoleucine (by 54.2-63%), leucine (by 16.7-53.1%), lysine hydrochloride (by 24.7-57.1%), proline + alanine (by 45.6-67%), and valine (by 33.7-41.4%) compared to CS (Fig. 2A-L). Glutamine was 50.1-60.4% significantly higher in ACL and KH_2PO_4 treated plants compared to CS (Fig. 2M). Tryptophan was 27.5-46.7% higher in KH_2PO_4 treated plants compared to CS and ACL (Fig. 2N).

Carbohydrate accumulation as affected by KH_2PO_4 under cold stress, during cold acclimation and after recovery from freezing stress

There was no significant difference in soluble sugar content between KH_2PO_4 and CS, while soluble sugar content in ACL plants was significantly lower than the untreated and KH_2PO_4 treated CS plants (Fig. 3A). After 35 d of recovery from freezing the content of all soluble sugars in KH_2PO_4 -treated and ACL plants were significantly higher than in the untreated CS control (Fig. 3B). There was no significant difference between soluble sugars of KH_2PO_4 treatment and ACL plants, and CS plants had significantly lower content compared to all treatments at 35 d of freezing recovery (Fig. 3B).

KH_2PO_4 treated plants had a significantly higher content of starch and fructan compared to the CS (36-43% higher), ACL (38-45% higher) during cold stress (Fig. 4A). After 35 d of recovery from freezing KH_2PO_4 treated and ACL plants had significantly

higher content of starch and fructan compared to untreated CS (36.4-47.8% higher) (Fig. 4B).

Regrowth during post-freezing recovery as affected by KH_2PO_4 under cold stress and during cold acclimation

Untreated CS plants did not show recovery from freezing stress (Fig. 5). KH_2PO_4 treatment significantly promoted the recovery of plants from freezing stress, as expressed by the greater green tissue percentage compared to ACL and the untreated CS control (Fig. 5). KH_2PO_4 treatment showed significantly increased recovery starting at 5 d of recovery from freezing (69-77.8% increased) compared to ACL, the untreated CS and the treatment effects continued until the end of the experiment (35 d).

DISCUSSION

Leaves of warm-season turfgrass species, such as bermudagrass typically turn brown under low temperature stress, which loss the functions of photosynthesis and plants may suffer from carbohydrate depletion (Fry and Huang, 2004). Bermudagrass lost turf quality and became brown during CS and ACL. The exogenous application of KH_2PO_4 enhanced cold tolerance of bermudagrass, as shown by the increased turf quality and chlorophyll content, although it has no effects on plant water relations. The treatment also significantly improved bermudagrass tolerance to freezing stress, as manifested by the increased green leaf percentage during post-freeing recovery. The ACL plants recovered to some extent following freezing stress, but to the recovery was to a lesser extent compared to the KH_2PO_4 treatment. Since chlorophyll is an important molecule for photosynthesis, the increased chlorophyll content and the amount of green leaves regenerated post-freeing

stress due to KH_2PO_4 treatment suggested that potassium could have provided protection from leaves and crowns from cold and freezing damages. Also phosphates may have aided in maintaining metabolic processes for continued energy production.

It is well known that compounds such as amino acids are protective and accumulate in the response of decreasing temperatures and during the cold acclimation process to induce winter hardening (Hannah et al., 2006). Consequentially, it is not surprising that ACL resulted in the induction of various amino acids such as serine, asparagine, glycine, threonine, tyrosine, methionine, cysteine, isoleucine, leucine, lysine hydrochloride, proline +alanine, and valine, which could at least partially contribute to the beneficial effects of freezing tolerance acquired through cold acclimation. Serine, threonine and alanine have been shown to be involved in cold stress response and increase cold tolerance possibly by inducing cold responsive genes or preventing denaturing of cell components (Miura et al., 2011). Proline commonly increases during decreasing temperatures and is a compatible solute, possibly mediating cold stress through maintain hydration of cell constituents for continued metabolic process (Thomashow. 1999). Methionine may promote the synthesis of secondary metabolites like polyamines that are thought to stabilize proteins and lipids, and other compounds like ethylene which has antifreeze properties and plays roles in overwintering as show in perennial ryegrass (Lolium multiflorum) (Alcazar et al., 2011; Yu et al., 2001). Asparagine has transport properties, and may provide nutrition to crucial tissues which could help maintain crown survival under chilling stress (Azevedo et al., 2006). Glycine is a precursor to pyruvate in the citric acid cycle or glycolysis, to maintain energy production (Kocsy et al., 2001). Glycine is also necessary for glutathione synthesis to reduces potentially toxic hydrogen peroxide concentrations (Kocsy et al., 2001). Also

this amino acid can be methylated to produce the compatible solute glycine betaine, which has been reported to play pivotal roles in cold acclimation and hydration (Sakamoto and Murata. 2002). Tyrosine is an aromatic amino acid that is used for the synthesis of proteins and is a precursor for important cell components like cell wall components and chlorophyll which may play important roles during chilling stress by maintaining chlorophyll and membrane integrity (Maeda and Dudareva, 2012). Also tyrosine can lead to the synthesis of phenolic acid compounds, which provides tolerance to stress by antioxidant properties, through the synthesis of coumaric acid and phenylalanine (Krol et al., 2015). Cysteine has many roles in defense response acting as a precursor to antioxidants, vitamins or other immune or stress responses (Alvarez et al., 2011). Lysine is incorporated into dehydrin proteins that bind to membranes and proteins to maintain stability, prevent protein aggregation or denaturing during environmental stresses usually involving water deprivation (Hara et al., 2003). Also lysine synthesizes other important amino acids like threonine, methionine and isoleucine and catabolizes metabolites in order to release energy during stress (Azevedo and Lea, 2001). Leucine has been shown to play roles in cold tolerance through its ability to maintain proteins synthesis or aid in transport by decreasing membrane permeability to enhance uptake of K or other important metabolites (Rai et al., 1983; Rana et al., 1996). Leucine also plays role in fatty acid and sterol synthesis, maintaining membrane fluidity under chilling (Ginger et al., 2000). Isoleucine and valine have also been shown to play roles in fatty acid synthesis to maintain membrane fluidity (Zhu et al., 2005). Accumulation of many of these protective amino acids may contribute to the increased recovery from freezing stress found in ACL plants.

Glutamine is a product of nitrogen metabolism from ammonium by glutamine synthase, which then provides nutrition and substrates for continued amino acid production or other cell components requiring nitrogen like chlorophyll (Cai et al., 2009). Glutamine is involved in the remobilization of nitrogen for energy production during photorespiration, and acts as a signaling molecule during metabolic reactions and stress, possibly due to its roles in nitrogen translocation (Kan et al., 2015). Accumulation of glutamine due to KH_2PO_4 treatment may have roles in the increased chlorophyll content and quality found in this study, potentially by facilitating energy production and nitrogen translocation for KH_2PO_4 treatments. Tryptophan production pathways are sensitive to stress and have been shown in Arabidopsis to lead to the production of auxin or the synthesis of secondary metabolites from indole or indole-3-glycerol phosphate (Zhoa et al., 1998; Ke et al., 2015). Also metabolites like serotonin and melatonin are synthesized from tryptophan which play roles in stress response through regulating auxin and plant development, or promoting, ROS scavenging (Kaur et. al 2015). The accumulation of tryptophan found in KH_2PO_4 treated plants may result in the induction or synthesis of secondary metabolites for a defense response to promote tolerance during cold stress. Also, increased quantities of tryptophan under cold stress could aid in the production of growth hormones necessary for cell division, cell development, apical dominance, and overall plant growth and development (Zhoa et al., 1998).

Potassium has been shown to trigger various biochemical and physical processes related to plant growth and metabolism, having effects on solute accumulation, enzyme activity and protein synthesis which can alter metabolite concentrations impacting sugars, carbohydrates, amino acids and other cellular compounds (Kant et al., 2002; Wang et al.,

2013). When there is adequate potassium in an environment it has been reported that protein and starch synthesis is maintained while amino acid and soluble sugars are depleted, indicating that potassium has effects on amino acid and carbon metabolism which can potentially help explain the lack of accumulation of amino acids from KH_2PO_4 treatment in this study (Marschner, 2012). In this study, exogenous treatment of KH_2PO_4 had only significant effects on amino acids tryptophan despite its promotive effects on both cold and freezing tolerance in bermudagrass.

There is also an increase in TNC starch and fructan during cold stress for KH_2PO_4 treatments compared to ACL which may help explain the increased tolerance due to greater carbohydrate reserves for increased survival under cold stress. Also fructan synthesizes other cyroprotectants and contributes to the stabilization of membranes by reducing water loss (Janska et al., 2009; Valluru et al. 2008). During new growth, carbohydrates are mobilized in the form of soluble sugars to tissues important for plant development (Rosa et al., 2009), and again it has been shown that cold tolerance is not always correlated to sugar accumulation (Koster and Lynch 1992; Sasaki et al., 1995). Since the accumulation of soluble sugars and TNC does not seem to be significant in the KH_2PO_4 treatment compared to ACL after 35 d of recovery, the increased recovery could indicate that other factors are necessary for cold tolerance or recovery from freezing. Also the increased recovery from the KH_2PO_4 treatment can potentially be due to the incorporation of soluble sugars and TNC into energy for regrowth. It has been shown that carbohydrates are constantly changing in regard to plant stage of development, and can potentially help explain the lack of significance in carbohydrate content of KH_2PO_4 treatments after 35 d of recovery from freezing in this study (Yee and Tissue, 2005). Also potassium plays

critical roles in translocation of carbon and aids in the conversion of soluble metabolites to other forms, as well as exportation of photosynthetic products (Conti and Geiger, 1982). It has been proposed that potassium's role in importation of sugars to sink tissues is short term while translocation is more gradual, which can potentially explain the absence of significance in soluble sugars and TNC content 35 d after freezing stress in KH_2PO_4 treated plants compared to ACL (Conti and Geiger, 1982). Based on the information from this study KH_2PO_4 may play roles in carbon metabolism and continued metabolite synthesis during cold stress for induced tolerance to freezing stress.

CONCLUSION

Exogenous treatment of bermudagrass with KH_2PO_4 resulted in improved cold tolerance compared to CS and ACL controls, demonstrated as improved chlorophyll content and overall turf quality as well as mitigate the effects of freezing stress and induce recovery after freezing. In general, metabolic changes during cold stress for the KH_2PO_4 treatment, included an increase in soluble sugars, TNC, and an accumulation of amino acids glutamine, and tryptophan, which may aid in cyroprotection, through osmoregulation and improved carbon metabolism. Information from this study will provide further insights into the metabolic factors controlling cold acclimation and induced freezing tolerance in warm season grass species. For management practices, KH_2PO_4 may be incorporated into bio-stimulant programs, alleviating the effects of cold and freezing injury in warm-season turfgrass management.

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FIGURES

Figure 1: Bermudagrass turf quality (A), relative water content (B), osmotic potential (C) and chlorophyll content (D) following treatment of potassium phosphate (50 mM) during 35 days of cold stress, compared to untreated cold stress and acclimated plants. LSD bars represent significant differences exist at $p \leq 0.05$.

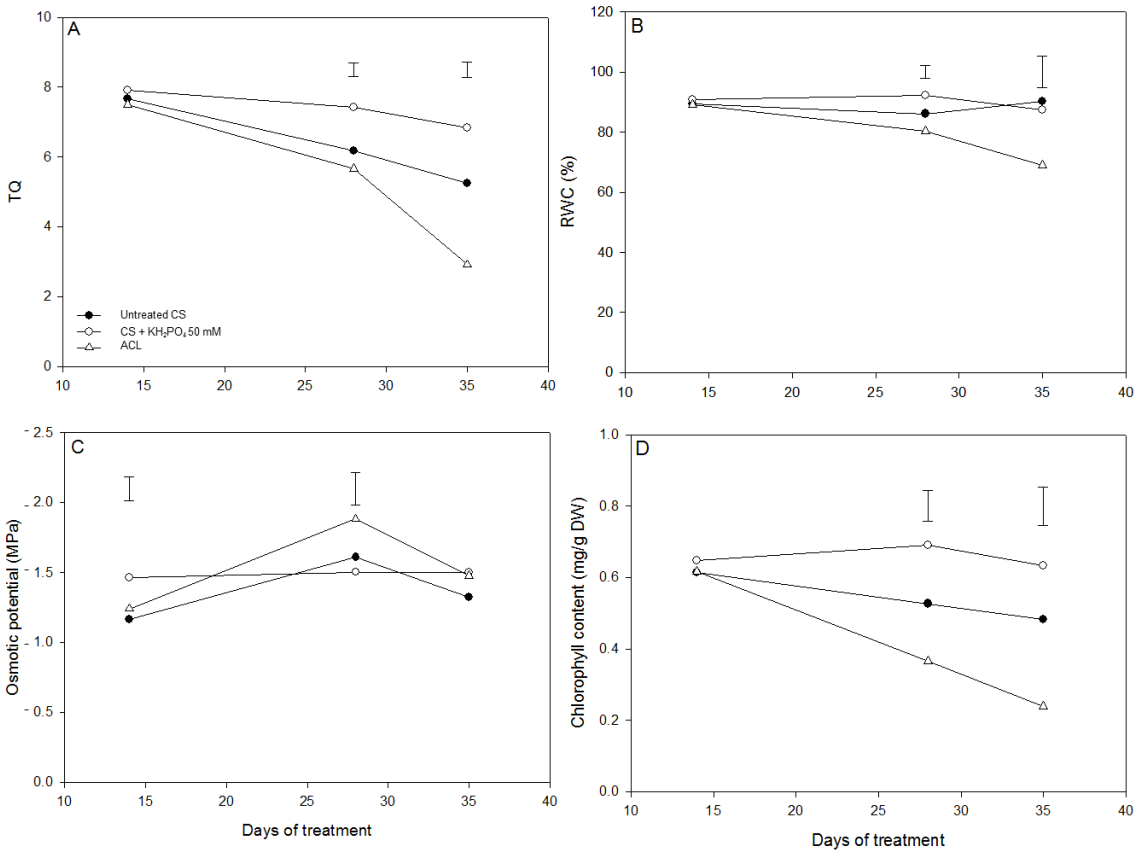
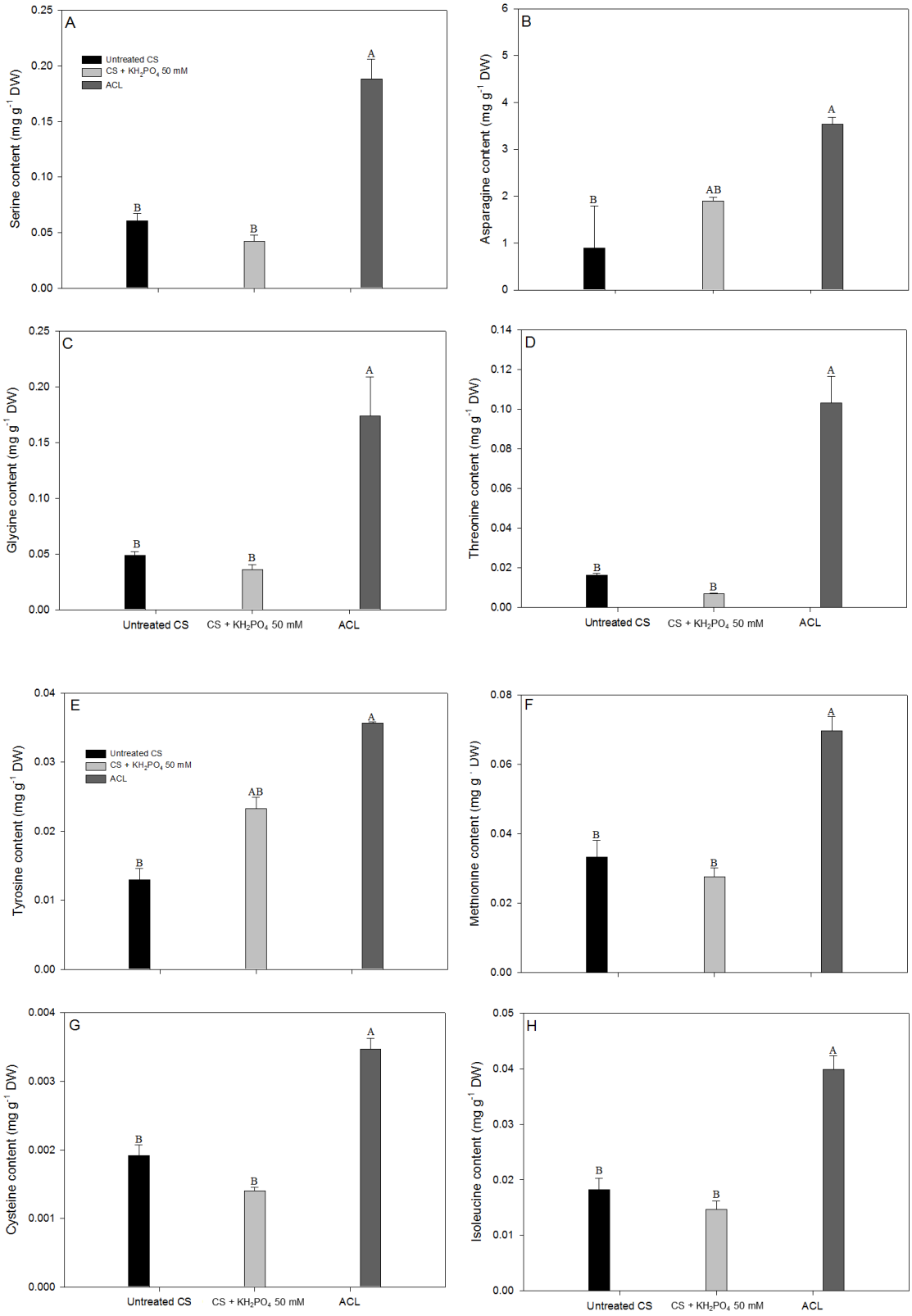


Figure 2: Bermudagrass crown changes in serine (A), asparagine (B), glycine (C), threonine (D), tyrosine (E), methionine (F), cysteine (G), isoleucine (H), leucine (I), lysine hydrochloride (J), proline + alanine (K), valine (L), glutamine (M), tryptophan (N) following treatment of potassium phosphate (50 mM) during 35 days of cold stress, compared to untreated cold stress and acclimated plants. Values with similar letters are not significantly different at the $p \leq 0.05$ level.


A
Untreated CS
CS + KH2PO4 50 mM
ACL
Serine content (mg g-1 DW)
B
Asparagine content (mg g-1 DW)
C
Glycine content (mg g-1 DW)
D
Threonine content (mg g-1 DW)
E
Tyrosine content (mg g-1 DW)
F
Methionine content (mg g-1 DW)
G
Cysteine content (mg g-1 DW)
H
Isoleucine content (mg g-1 DW)
Untreated CS
CS + KH2PO4 50 mM
ACL

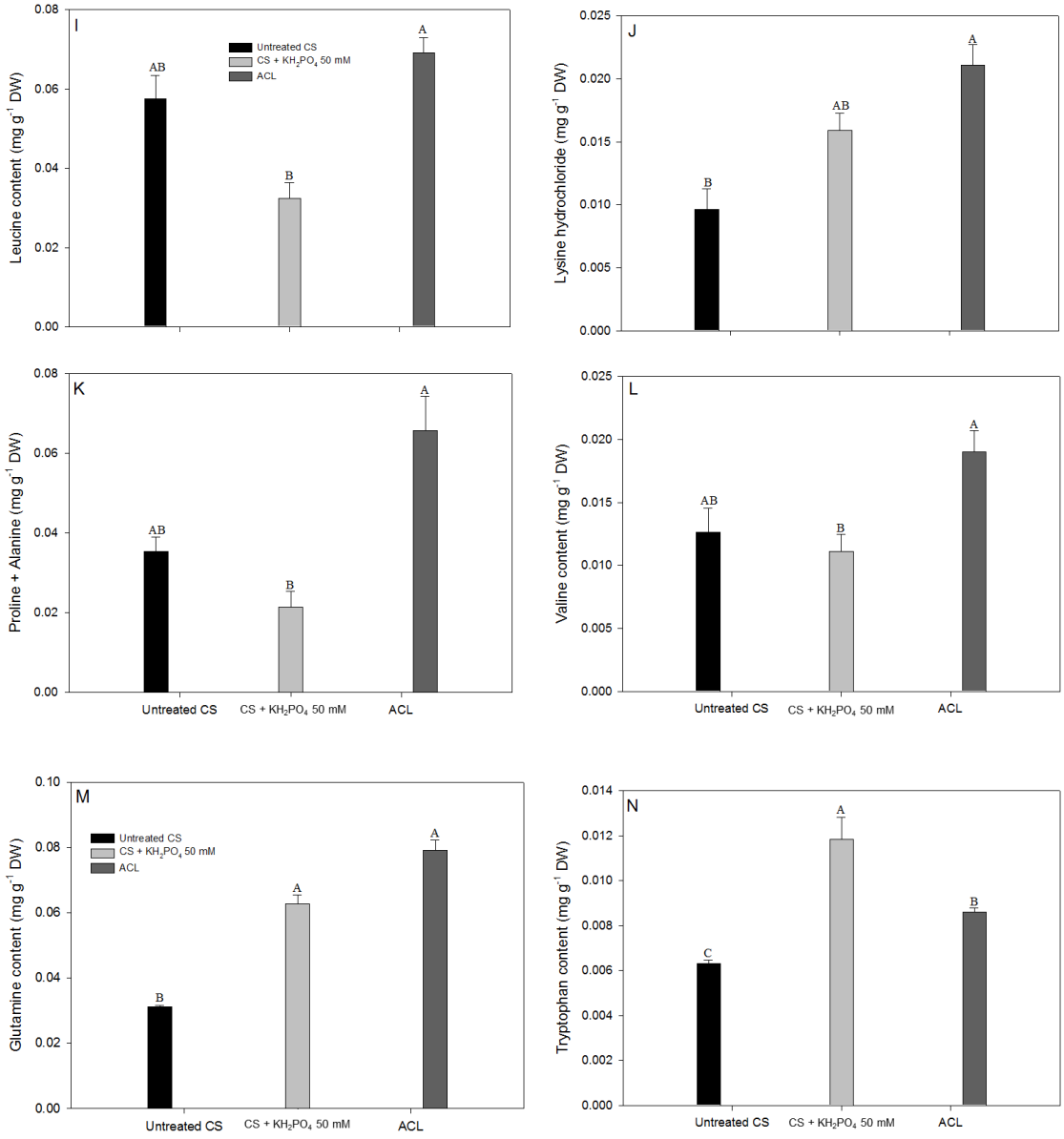

I
Untreated CS
CS + KH2PO4 50 mM
ACL
Leucine content (mg g-1 DW)
AB
B
A
J
Lysine hydrochloride (mg g-1 DW)
B
AB
A
K
Proline + Alanine (mg g-1 DW)
AB
B
A
L
Valine content (mg g-1 DW)
AB
B
A
M
Glutamine content (mg g-1 DW)
B
A
A
N
Tryptophan content (mg g-1 DW)
C
A
B
Untreated CS
CS + KH2PO4 50 mM
ACL

Figure 3: Bermudagrass crown changes in glucose, fructose, and sucrose following treatment of potassium phosphate (50 mM) during 35 days of cold stress (A), and after 35 days of recovery from freezing stress (B) compared to untreated cold stress and acclimated plants for individual carbohydrates. Values with similar letters are not significantly different at the $p \leq 0.05$ level.

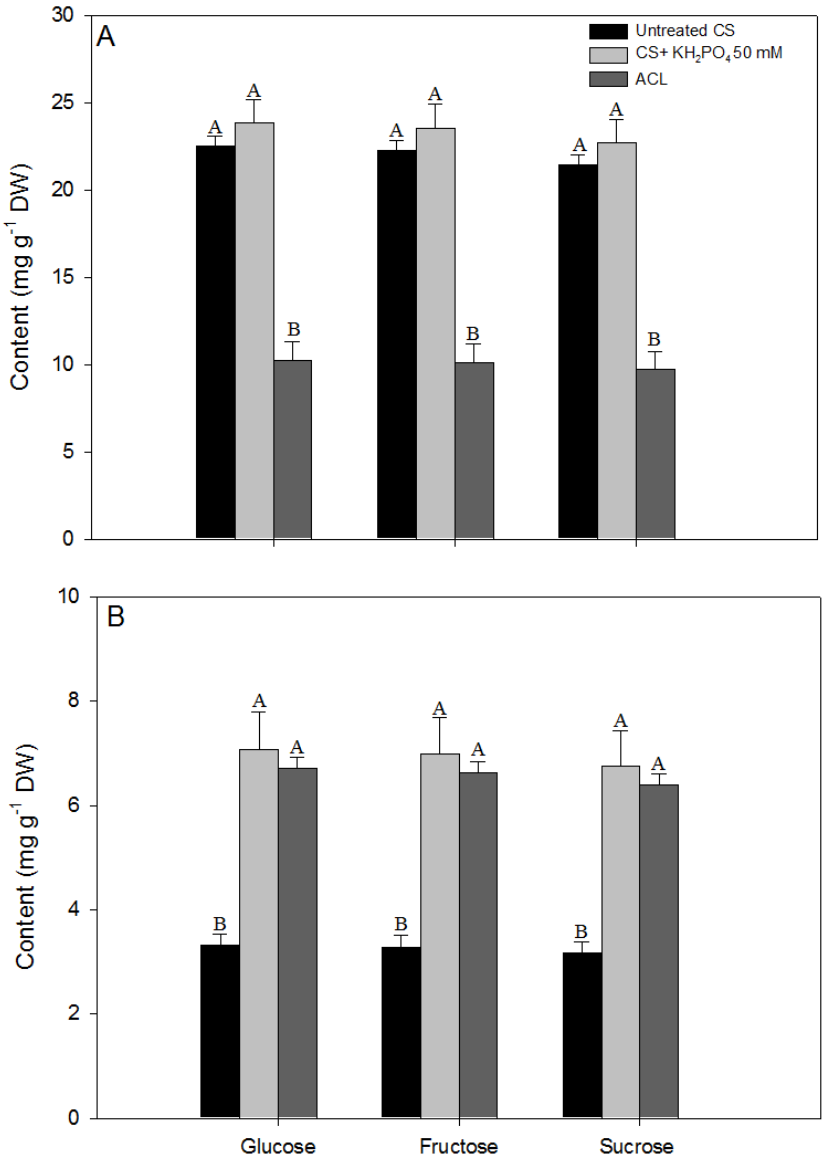


Figure 4: Bermudagrass crown changes in total non-structural carbohydrates, starch and fructan, following treatment of potassium phosphate (50 mM) during 35 days of cold stress (A), and after 35 days of recovery from freezing stress (B) compared to untreated cold stress and acclimated plants for individual carbohydrates. Values with similar letters are not significantly different at the $p \leq 0.05$ level.

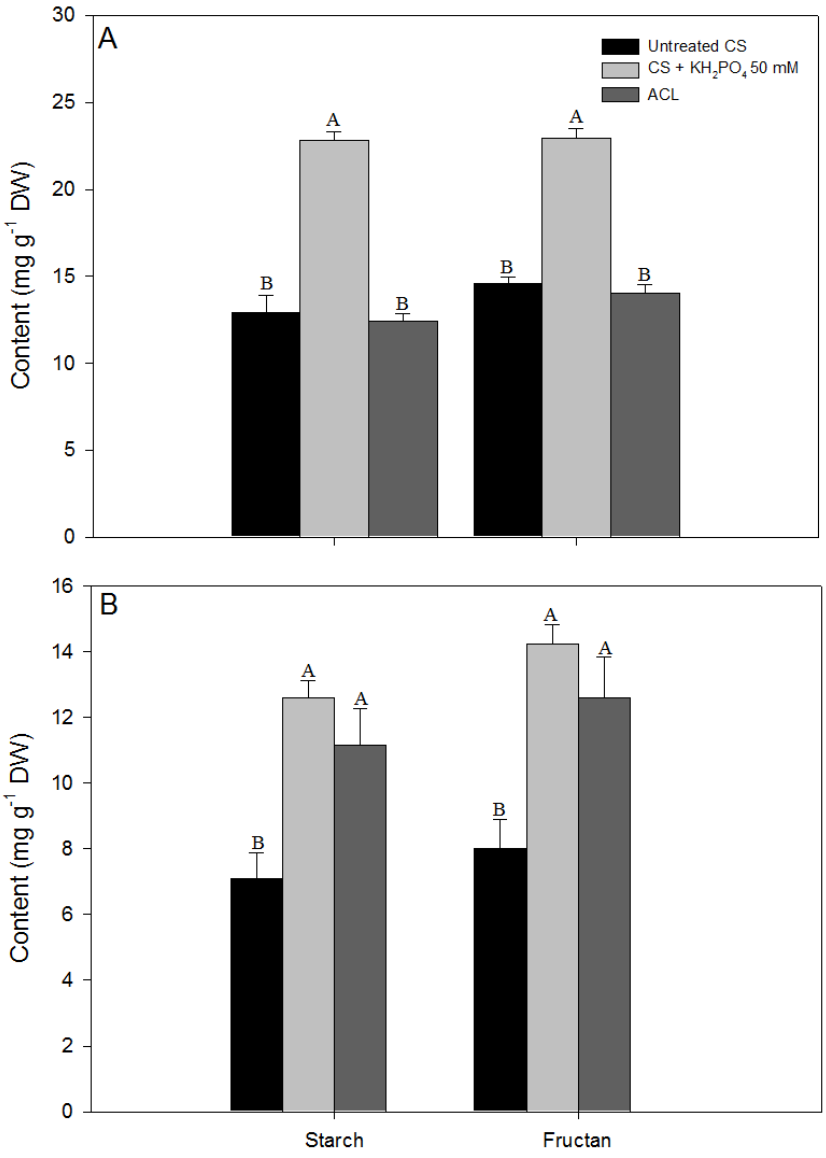
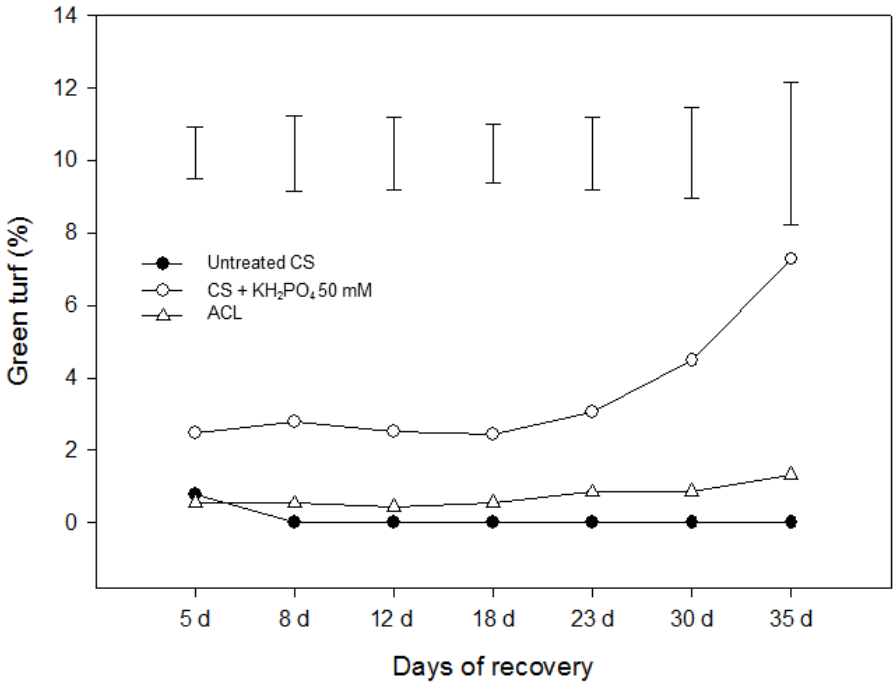


Figure 5: Bermudagrass recovery from freezing stress during 35-day post freezing at -7°C (A) following treatment of potassium phosphate (50 mM). LSD bars represent significant differences exist at $p \leq 0.05$ level.



THESIS CONCLUSION

The goal of this thesis research was to investigate the physiological, and metabolic factors associated with cold acclimation, cold and freezing tolerance in *Agrostis* and *Cynodon* species. The main approach to explore this goal was to compare the effects of exogenous calcium dichloride, glycine betaine, abscisic acid, potassium phosphate and sucrose, on cold tolerance and freezing stress recovery in C3 and C4 grasses. The initial screening study found that C3 grasses have beneficial physiological effects during cold stress when applied with glycine betaine, while C4 grasses benefited from glycine betaine and potassium phosphate treatments. Recovery from freezing stress for C3 grasses was also promoted by glycine betaine treatment and C4 grasses showed induced recovery due to potassium phosphate treatments.

The improved physiological responses under cold stress due to exogenous treatments manifested as increased quality, chlorophyll content or more negative osmotic potential, were further examined metabolically. Metabolic analysis of amino acids, soluble and nonstructural carbohydrates found that in creeping bentgrass the glycine betaine treatment induced the accumulation of various amino acids and nonstructural carbohydrates before freezing stress, with significantly increased nonstructural carbohydrate reserves after recovery from freezing stress. Bermudagrass potassium treatment resulted in an increase in soluble and nonstructural carbohydrates after cold and freezing stress, indicating changes in metabolism. This research portrays that there is a differential response in cold tolerance and recovery from freezing stress due to certain exogenous treatments in C3 and C4 species, and can provide insight on the different mechanisms of cold acclimation that warm season or cool season grass species undergo.

Future research may further investigate the differential roles these naturally occurring metabolites have in the cold acclimation process in C3 and C4 grasses. Increased knowledge of the cold acclimation process will help better our understanding of low temperature tolerance, for the future development of more cold tolerant species or better management practices to maintain higher quality and plant productivity during chilling or freezing stress.