ABSTRACT

CLAURE SALINAS, TITO ELMER. Evaluation of Different Laboratory-Based Methods to Assess Freezing Tolerance in St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze). (Under the direction of Dr. Susana R. Milla-Lewis).

St. Augustinegrass is a popular lawn grass in the Southeastern US due to its excellent shade tolerance and low input requirements. However, its sensitivity to low temperatures reduces its potential for adaptation further north. Counting with an efficient and accurate laboratory-based method to assess freezing tolerance in different genotypes will aid future breeding efforts in this turfgrass. The objectives of this study were to: investigate several laboratory-based freeze assays to develop a more consistent method to screen for differences among St. Augustinegrass genotypes, and examine the histology of St. Augustinegrass nodes and document how specific tissues within the node respond to freezing stress. For these purposes, six experiments were designed using four freezing methods: complete stolons in "rolls", single-node stolons (SNS) in plastic containers, SNS in sponges, and SNS in conetainers. The rolls and SNS in plastic containers methods produced much lower survival rates and were effective at detecting differences among treatments. Desiccation seems to play an important role during freezing. Thus, in methods where a media is surrounding the node (*i.e.* a sponge or soil) this media acts as a buffer retaining moisture and reducing freezing damage. Overall, results indicated that cold-acclimation has a negative effect on plant survival and in new node development. This suggests possible photoperiod effects and the need of a third step in the acclimation process in order to achieve successful cold-acclimation under laboratory conditions.

Through all experiments, it was confirmed that Raleigh is more freezing tolerant than Seville. Node position was found to be a determinant for freezing survival. Mid-region nodes consistently had higher survival rates when compared with nodes from basal and apical regions. This result will suggest structural and maybe metabolic differences that need to be determined in order to refine freezing protocols for stoloniferous grasses. Histology results showed that node death is linked to plugged vessels after freezing. Moreover, plugged vessels and disrupted tissues occur at higher frequencies in the main stem than in new shoots. This would explain why re-growth was observed to begin in new shoots, which act independently from the main stem. Data generated from all these experiments make a significant addition to the understanding of freezing processes and survival in St. Augustinegrass which should aid the development of more effective freezing survival evaluation methods.

Evaluation of Different Laboratory-Based Methods to Assess Freezing Tolerance in St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze)

by
Tito E. Claure Salinas

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Crop Science

Raleigh, North Carolina

2011

APPROVED BY:

_____	_____
Dr. Susana R. Milla-Lewis Committee Chair	Dr. David P. Livingston
_____	_____
Dr. J. Paul Murphy	Dr. Consuelo Arellano

DEDICATION

To my Lord and Savior Jesus Christ.

To my beloved wife Dutsi and my two dear sprouts, Obed and Joel.

A mi Señor y Salvador Jesucristo.

A mi amada esposa Dutsi y a mis dos queridos retoños Obed y Joel

BIOGRAPHY

Tito Elmer Claure Salinas, was born in Cochabamba, Bolivia. He studied Agricultural Engineering at San Simon University. After graduation, he participated in an International training course for wheat improvement at CIMMYT-Mexico. Later on, he worked as a wheat breeder for the Wheat National Program (PROTRIGO) in Bolivia. At the same time he participated as an associate researcher for the Durable Resistant Project in the Andean Zone (PREDUZA) in coordination with the University of Wageningen-Netherlands. After four years of working in wheat improvement, he got hired to work as a senior breeder for the Faba-bean program at the Patino Foundation, where he worked for the last five years. He also taught Statistics at San Simon University for three years, contributing in the formation of young professionals in Agriculture and NNRR. After completing his MS degree, Tito plans to go back to Bolivia and apply the knowledge acquired during his MS work on behalf of the resource–poor of the country, and into training new professionals on plant breeding subjects.

ACKNOWLEDGMENTS

First, I would like to thank Dr. Milla-Lewis and Dr. Livingston, whose mentoring was invaluable for me. Through all the ups and downs, they were always there encouraging me to continue. I am also thankful to Dr. Arellano and Dr. Murphy for their advice and support.

I am thankful to the Fulbright Foundation, whose economical support made the realization of this big goal in my life possible.

I want to express my gratitude to the Patino Foundation and especially to Dr. Gonzalo Avila, for the opportunity I got to improve my professional skills.

To my dear wife Dutsi, and my lovely sons Obed and Joel. Eventhough a paragraph is not enough to thank you; at least I want you to know that this accomplishment would never become a reality without your support, patience and affection.

To my parents Tito and Nancy; I am thankful for their love and support.

To all the relatives and friends whom always cheered me to continue. To Tan Tuong, Brian Hargett and family, Bob Page and family, Matias Menendez and family, Alfonso Tercio and family, Luis Peña, and my dear Church of First Assembly, my sincere gratitude.

Finally, I am deeply in debt to God for providing me encouragement and give me the strength to succeed. I give him the Glory.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

Literature Review

Turfgrass industry

In the U.S., the turfgrass industry is very important. In 2005, it represented around 50 million acres of managed turf (Shearman, 2006), and had an economic impact of $62.2 billion dollars with a total value added impact of $37.7 Billion (representing total personal and business net income) (Haydu et al, 2006).

In North Carolina, the economic impact of the turfgrass industry represents more than 4.7 billion dollars, having an employment effect over more than 96,465 permanent and temporary jobs (North Carolina Dept. of Agriculture and Consumer Services, 1999). According to the NCDA, turf maintenance expenses in 1999, estimated at more than 1.2 million dollars, were greater than cash receipts for any agricultural commodity. The total turf-acreage in the state, 2.1 million acres, is mainly divided among single-family dwellings, roadsides, commercial properties, golf courses, schools, churches, airports, parks, institutions and cemeteries. Family dwellings constitute, by far, the biggest section with more than 68% of the total acreage.

Apart from these economic benefits realized from turf grasses, their contributions in soil and water protection, air cleansing, cooling, recreation and aesthetics are substantial. Every 2.5 acres of golf course turf sequester about one ton of carbon from the air per year. Turfgrasses also provide safety and dust control along millions of miles of highways and thousands of airport runways, trapping more than 12 million tons of dust and dirt annually. A 250 sq. ft. lawn produces enough oxygen for a family of four, and the average-size front lawns of eight homes

have the cooling effect of about 70 tons of air conditioning (Zhang et al., 2006b; Industry Turf Initiative, 2010).

Warm-season and cool-season grasses

In general, turfgrasses can be divided in two major groups: warm- and cool-season. Located in more temperate regions with optimum growth temperatures of 27-35^{o}C, warm-season grasses are characterized for having a C_4 photosynthetic pathway and for showing better tolerance to heat and drought. Meanwhile, their tolerance to cool temperatures is very low, and they usually enter dormancy when temperatures are below 10^{o}C (Emmons, 2000; Stier and Fei, 2008). Cool-season grasses have an optimum growth temperature of 18-24^{o}C, have a C_3 photosynthetic pathway, and, opposite to warm-season grasses, they can tolerate cold-weather but have low tolerance to heat and/or drought conditions.

Regarding optimum weather characteristics for growing turfgrasses, Emmons (2000) classifies the U.S. in three major regions: cool season, warm season, and transition zones. The cool season zone consists of cool humid and cool arid regions. Located in the northern states, this zone is characterized by very cold winters and moderate summers. Meanwhile, the warm season zone is located in the southern part of the country. It includes warm humid and warm arid regions, and it is characterized by moderate winters and very hot summers. The transition zone is defined as a zone where winters could be too cold for warm-season grasses and summers too hot for cool-season grasses. It is located on the eastern United States, in between

the warm- and cool-season zones. Because of its complex characteristics, the transition zone results challenging for growing turfgrasses. Cool-season grasses can overwinter without major problems, but cannot survive hot and drought summers. Meanwhile, warm-season grasses grow comfortably during the summers, but are seriously affected by low temperatures in winter. North Carolina is considered to be in the transition zone, having three major zones for turfgrass development: the coastal plains, the piedmont, and the mountains. Warm-season grasses are recommended for the coastal plains and warmer parts of piedmont, whereas cool-season grasses are recommended for the western and piedmont regions (NC cooperative extension service, 1992).

St. Augustinegrass distribution

Characterized as a warm-season grass, St. Augustinegrass was originally found on sandy areas such as beach ridges, fringes of swamps and lagoons, water marshes, and limestone shorelines. Then, the species gradually moved inland, resulting in a tolerance to a wide range of soil types (Duble, 1989)

Although it was first discovered in South Carolina in 1788, St. Augustinegrass does not seem to be native to North America because the other six species of the genus *Stenotaphrum* are endemic and confined to shorelines from Africa to the South Pacific (Busey, 1995). Since its first record of use in the 1880s, St. Augustinegrass has been widely used as lawn and pasture grass in warm, subtropical, and tropical climate regions. It can be found along the Gulf Coast, in

Southern Mexico, throughout the Caribbean region, South America, South Africa, Western Africa, Australia and the South Pacific and Hawaiian Islands (Duble, 2010). In the US, it is found from the Carolinas to Florida, westward along the Gulf Coast to Texas, and in Southern and Central California (Green et al, 1981; Duble, 1996; Trenholm et al, 2006; Moseley et al, 2010).

The only limiting factor for its expansion further north is its poor cold tolerance. According to Maier et al (1994b), it is adapted to US Dept. of Agriculture hardiness zones 8, 9, and 10. However, severe freezing injury may occur during some winters in zones 8 and 9. In the case of North Carolina, it is recommended for the Piedmont and coastal plain regions only (NC cooperative extension service, 1992).

Taxonomy

Characterized for having panicle or raceme inflorescences, the *Stenotaphrum* genus belongs to the Paniceae tribe. Of the six species in the genus, only *Stenotaphrum secundatum* (Walt.) Kuntze, commonly known as St. Augustinegrass in the U.S. and Buffalograss in Australia, is used as a turfgrass (Emmons, 2000; Turgeon, 1999).

Kingdom: Plantae

Division: Embryophyta

Subdivision: Phanaerogama

Branch: Angiospermae

Class: Monocotyledoneae

Subclass: Glumiflorae

Order: Poales

Family: Poaceae

Subfamily: Panicoideae

Tribe: Paniceae

Genus: *Stenotaphrum*

Species: *Secundatum*

Two taxonomic races exist within the species: the Breviflorus race characterized by having a short spikelet, and the Longicaudatus race with long internodes and long leaves (Busey, 2003).

Morphology

St. Augustinegrass is a perennial coarse-textured, stoloniferous, sod forming grass that roots at the nodes (Green et al, 1981; Duble, 1996) with strong, thick stolons. The grass produces a turf of medium density (Emmons, 2000). Its round-tipped leaf blades are 5 to 14 mm wide and arranged in a distichous manner (Busey 2003). Its stems (stolons) and overlapping leaf sheaths are generally compressed. Leaf blades are generally folded, contracted at the base, and subtended by constricted collars lighter than the blade or sheath, making the leaves pseudopetiolate (Duble, 1996; Busey, 2003). The inflorescences are mostly terminal – although some might be axillary- with spike-like panicles. The branches of the inflorescences

are contracted and often reduced to single spikelets, being imbedded in one face or the sides of a corky rachis. Spikelets are lanceolate or ovate, awnless, and sesil. Glumes are membranous. The lower glume is less than half as long as the spikelet. The lower floret is staminate or neuter, and the upper floret is complete. The caryopsis which is ovate to oblong, 2.0 to 3.0 mm long, often fails to mature (Duble, 1996; Busey, 2003).

As a C_4 plant, St. Augustinegrass' leaves have typical 'Kranz anatomy' (Laetsch, 1974). The inner parenchyma bundle sheath is formed by a single layer of cells with centripetal chloroplasts that facilitates compartmentalization of photosynthetic processes. Beyond this layer lays a series of elongated mesophyll cells radiating from the vascular bundles (Turgeon, 1999; Busey, 2003).

St. Augustinegrass characteristics and cultivars

St. Augustinegrass' importance as a lawn grass is due to its versatility and adaptability to different growing conditions, and its relatively low maintenance requirements in comparison to other warm-season turfgrasses (Green et al, 1980). It is a coarse textured grass with good color and establishment rates (Emmons, 2000). It is mainly used for lawns as its tolerance to traffic is only fair and not suitable for sport fields (Duble, 1989). Its propagation is mainly vegetative through sprigs, plugs or sod (Beard, 1973).

Ranked among the most shade tolerant grasses, St. Augustinegrass is highly recommended for home-lawns with shaded areas (Duble, 1989; Beard, 1973; Emmons, 2000).

This species also presents a very good salinity tolerance (Beard, 1973; Dudeck et al, 1993; Emmons, 2000). It is one of the most effective turfgrasses when looking at leachate NO_3-N concentration and cumulative N leached (Bowman et al, 2002). Therefore, it has potential for reduced environmental impact. The three main biotic problems for this species are the southern chinch bug, St. Augustinegrass decline virus (SAD), and gray leaf spot disease *Pyricularia grisea* (Cke.) Sacc. (Bussey, 2003).

Regarding St. Augustinegrass cultivars, one of the first ones mentioned in the literature is 'Texas Common' which is a strain of St. Augustinegrass grown commercially in Texas since 1920 (Busey, 2003). Other selections from Florida were available prior to 1960 such as 'Bitterblue' and 'Floratine' (White and Busey, 1987; Nutter and Allen, 1960). Following the same line, in 1972 'Floratam' was released as a SAD-resistant. All three of these cultivars are coarse textured (Horn et al., 1973). 'Floralawn', a chinch bug resistant cultivar, resulted as an improvement over Floratam (Duble, 1989; Busey, 2003). In 1980 the NC Experiment Station released 'Raleigh' as a cold tolerant SAD-resistant cultivar of medium texture (Emmons, 2000). From the private sector, O.M. Scott Company released three semi-dwarf type cultivars: 'Seville' (Riordan et al, 1980), 'Jade' and 'Delmar' (Riordan et al, 1991), all with much finer texture but more cold-sensitive (Busey, 2003). Another fine-texture cultivar is 'Palmetto', released by Mr. Tobey Wagner of Sod Solutions (Emmons, 2000; Busey, 2003).

Regarding the freezing tolerance of St. Augustinegrass, Philley (1998) found that 'Raleigh' and 'Seville' were among the varieties with the best and worst levels of freezing tolerance, respectively, measured in terms of lethal temperatures and winter survival. Others like Texas Common and Delmar were found around the average of freeze tolerance (LT_{50} > -6.2^{o}C) (Philley et al, 1995).

Duble (1996) described Seville as a SAD-resistant, fine textured variety, but with poor levels of freezing-tolerance, and Raleigh as a freeze-tolerant, SAD-resistant variety. Maier et al (1994) mentioned that 'Raleigh' had higher freezing tolerance than 'FX-332' or 'Floratam', and was a cultivar that acclimates to cold. Therefore, Raleigh is considered the industry's standard for freeze tolerance among St. Augustinegrass' cultivars. Based on this, Li et al (2010) developed an acclimation protocol based on Raleigh's response to freezing.

Low-temperature injury

As mentioned by Stier and Fei (2008), grasses may be injured by either chilling or ice formation within the plant (freezing injury). Chilling injury occurs at temperatures below 12^{o}C, and it is usually characterized by wilting, cessation of growth, loss of chlorophyll, reduction in photosynthetic rate, and presence of water-soaked leaf lesions (DiPaola and Beard, 1993; Stier and Fei, 2008). Chilling injury disrupts cell membrane activity, leading to electrolyte loss from the cytoplasm, release of vascular substances, and loss of protein activity. This process can be

acute (direct) if it occurs in a term of 24 h, or it can be chronic (indirect) if it takes several days of low temperature (Stier and Fei, 2008).

In the case of freezing injury, previous studies have determined that it is caused by ice formation rather than low temperatures per se (Fry and Huang, 2004). The process is complex because it involves the dynamics and kinetics of water as is interacts with various tissues (Livingston et al, 2006a). The place where ice formation occurs determines the kind of damage produced. If intracellular, freezing produces membrane rupture and immediate cell death. Meanwhile, extracellular freezing produces dehydration (Webb et al 1994; Guy 1999; Fry and Huang, 2004; Zhang et al, 2006a; Fujikawa et al, 1999). Other minor factors that may cause freezing damage are: cell death when limits to deep supercooling are exceeded, death of shoots due to persistent embolisms in xylem vessels, and disease when pathogens enter through lesions (Pearce, 2001).

Of all the processes referred to above, one of the most important is freezing-induced dehydration. This can be defined as a process where the ice formed in the intercellular matrix (extracellular ice) produces a gradient of chemical potential between the ice outside the cell and the unfrozen solution inside the cell. As a consequence, unfrozen water inside the cells moves out and freezes outside the cells which results in an increase of intracellular solutes (Tomashow, 1999) but, at the same time, produces extra pressure on the cell membranes and can cause permanent damage or cell death. Three processes are known to produce membrane

damage as result of freezing-induced dehydration: 1) expansion-induced-lysis (EIL), 2) lamellar- to hexagonal- II phase transitions are known as loss of osmotic responsiveness with endocytotic vesiculation and H_{II} phase (LOR-H_{II}), and 3) loss of osmotic responsiveness associated to the fracture-jump lesion (LOR-FJL) ((Tomashow, 1999; Uemura-Steponkus, 1999; Uemura et al, 2006).

Cold acclimation

To prevent or reduce the risks of freezing injury, plants have developed numerous mechanisms to be prepared for winter time, one of them being cold-acclimation (CA). This process can be defined as "an enhanced ability of plants to survive freezing temperatures by exposure to low but above freezing temperatures" (Livingston et al, 2006a). Many species are able to acclimate, showing marked seasonal differences in cold resistance (Levitt, 1980; Alberdi-Corcuera, 1991).

In general CA is an inducible and transient process (Guy, 1990) by which the plant responds to temperature decreases and photoperiod changes. There are three ways to induce CA in plants: exposure to low temperatures, photoperiod reduction, and abscisic acid (ABA) interactions. Plants exposed to cold, non-freezing temperatures become hardened or acclimated, allowing greater resistance to freezing stress (Alberdi-Corcuera, 1991; Thomashow, 1999; Fry and Huang, 2004; Stavang et al, 2008). The requirements for this process to take effect vary for different species and are a function of the season, developmental stage, how

long the low temperature persists, and the photoperiod requirements (Alberdi-Corcuera, 1991; Gray et al, 1997; Ensminger etal, 2006).

Cold acclimation can be achieved usually in two to three stages (Gusta et al, 2005). The first stage corresponds to temperatures below 10^{o}C, where a reduction in growth and up-regulation of transcriptional factors is noticeable. In the second stage, with temperatures close to 0^{o}C, cryoprotective compounds (such as proteins and sugars) are produced, and repair mechanisms developed. Finally, in the third stage, also called second-phase hardening, plants are exposed to temperatures below 0^{o}C for a very short period of time (usually days) (Livingston and Henson, 1998).

Evidence suggests that ABA can substitute the low temperature stimulus, and that maybe there are ABA-dependent and ABA-independent pathways involved in the acclimation process (Gusta et al, 2005). Even though ABA does not regulate all genes associated with acclimation, it definitely regulates many of the genes associated with an increase in freeze tolerance.

The relationship between photoperiod and CA is based on photosynthesis. It is clear that photosynthetic redox interacts with other processes like sugar-signaling pathways to regulate plant acclimation to low temperatures (Esminger et al, 2006). It is also important to consider the seasonal variations on CA. Beard (1966) found that turfgrasses reached their highest freezing tolerance in December, followed by a slight decline in January and a sharp

decline in April. As a morphological indicator of acclimation, it is common to see that plants become darker green, reduced their leaf area, and become less succulent (Ebdon et al, 2002; Ito et al., 1985). Also, the level of CA varies between the different parts of the plant; young leaves are hardier than old leaves or the leaf apex (Beard, 1973). Many types of physiological and morphological responses are correlated with the degree of CA achieved, including reduction in cell size, increase in carbohydrate level, changes in types of proteins, and reduction in cell water content (Alberdi-Corcuera, 1991; Beard, 1973).

Regarding the biochemical changes during acclimation in general, there is an increase in cytoplasmic solutes, soluble proteins, amino acids, carbohydrates and inorganic solutes. These compounds act as cryoprotectants and buffer any freezing-induced concentration of other solutes that could become toxic at high levels. This solute accumulation depresses the freezing point or ice nucleation point of cells thus increasing cell resistance to ice formation and regulating dehydration (Fry and Huang, 2004).

In turfgrasses, many studies have been carried out on the role of carbohydrates and proteins on freezing tolerance. In Annual Bluegrass, it has been determined that cold hardening induces major changes in amino acid levels in overwintering crowns, with proline, glutamine, and glutamic acid contributing the most to total amino acid accumulation after acclimation (Dionne et. al., 1991). In addition, fructose, glucose, raffinose, and stachyose were also found to exhibit clear seasonal changes, reaching their highest concentrations during midwinter. For

saltgrass, Shahba et. al. (2003) found that high molecular weight fructants (DP>6) were the most abundant carbohydrates in cold-acclimated vs. non-acclimated plants. Furthermore, sucrose levels increased at temperatures below freezing. However, variations in fructan and sucrose levels were not related to different freezing tolerance levels. In the case of Zoysagrasses, higher concentrations of total reducing sugars, glucose, and proline were positively associated with freezing tolerance, whereas higher concentrations of starch appeared detrimental to the trait (Patton et al, 2007). In Bermudagrass, cold acclimation not only induced accumulation of sugars and proline, but also of total nonstructural carbohydrates (TNC) and protein. Meanwhile, catalase (CAT), and ascorbate peroxidase (APX) activity decreased in response to cold acclimation (Zhang et al, 2006a). In St. Augustinegrass, Fry et al (1991) found that changes in the concentration of total non-structural carbohydrates or soluble sugars did not seem to influence freezing tolerance. Maier et al (1994) determined that neither starch nor sucrose -the primary storage carbohydrate in stolons- were correlated with freezing tolerance in cultivars 'Raleigh', 'Floratam' and 'FX-332'. The only significant correlation (r = - 0.80) they found was between freezing survival and stolon water content in "Raleigh". Therefore, it was concluded that water content reduction in `Raleigh' stolons during the winter months could have contributed to its freezing survival

It is generally known that warm-season grasses don't respond well to cold acclimation and may have limited capacity to cold-acclimate (Stier and Fei, 2008). However, there are

reports for Saltgrass (Shahba et al, 2003), Buffalograss (Qian et al, 2001), Zoysiagrass (Patton-Reicher, 2007) and Bermudagrass (Anderson et al, 1988; Anderson et al, 1993, Anderson et al, 2003) indicating that these warm-season grasses can cold-acclimate and exhibit good freezing tolerance. In St. Augustinegrass, it has also been observed that some cultivars show differences in freezing survival when cold acclimated (Maier et al, 1994; Li et al, 2010).

Grass anatomy and freezing survival

Regarding the relationship between plant anatomy and freezing survival, Ahring et al (1975) demonstrated that in turfgrasses internode length and rhizome depth influenced freezing tolerance. Shorter internodes and deeper rhizomes were positively correlated with more freezing resistant plants. Wood and Cohen (1984) found that in perennial ryegrass, crown height -as indicated by sub-crown internode length- was negatively correlated with freezing tolerance. These results reveal, as Livingston et al (2005) found in winter cereals, that whole plant survival results from the ability of meristematic tissues within the crown to remain intact. For turfgrasses it has being demonstrated that successful recovery from freezing stress depends upon the meristematic tissues inside crowns and nodes to survive freezing damage (Harrison, 1997; Hoffman, 2010).

Evaluation of freezing tolerance

In general, there are two ways to test freezing tolerance: field observations and laboratory-based experiments. Due to variability in environmental conditions, field

observations may vary from location to location and from year to year making it difficult to have a uniform evaluation in a short period of time. However, this is the most reliable way to asses freezing tolerance since it deals with much more factors than the few ones controlled by laboratory-based experiments.

In the case of laboratory-based experiments, freeze tolerance of grasses is evaluated by two means: measuring electrolite leakage (EL), where lethal temperatures are predicted by EL (Gusta et al, 1980; Fry et al, 1991; Maier et al, 1994a; Ebdon et al, 2002) or evaluation of tissue re-growth after freezing (Maier et al, 1994; Li et al, 2010). However, inconsistencies in the EL procedure might make re-growth tests a better option to determine freeze tolerance in grasses (Patton and Reicher, 2007).

Based on the principle that different parts of the turfgrass plant have different levels of tolerance to low temperatures (Beard, 1973), different protocols for laboratory-based freezing tests have been tried. Most of these focused on the protocol steps and freezing temperatures. Ahring and Irving (1969) froze 5-node rhizomes in Bermudagrass and evaluated response through the triphenyl tetrazolium chloride (TTC) test. Anderson (1975) used conetainers to evaluate freezing in Bermudagrass. Bush et al (2000) used single node cuttings of Carpetgrass wrapped in paper towels. Dione et al (2001a) evaluated bluegrass tillers in tubes. Quian et al (2001) used 10-node stolons wrapped in moist tissue paper for a Buffalograss shoot and root re-growth assessment. Sahba et al (2003a) measured survival and rhizome re-growth after

freezing Saltgrass' 2-node rhizomes wrapped in moist paper. Patton et al (2007a) evaluated Zoysiagrasses using single-node stolons in conetainers. In the case of St. Augustinegrass, different laboratory-based methods have been used to test freezing survival. For example, Fry et al (1991) used the electrolyte leakage (EL) method and determined an LT_{50} of -6.1 for Floratam. Philley (1995) used EL with single-node stolons, and found that Raleigh, Texas Common and Delmar had lower LT_{50} (<-6.2) than Seville, Floratam and FX-33. Other studies considered scoring survival after freezing a better way to evaluate freezing response among genotypes. For example, Maier et al (1994b) evaluated the freezing response of 4-node stolons placed in plastic bags and Li et al (2010) did the same using single-node stolons in plastic containers. Differences in technique between these experiments resulted in LT_{50}'s as high at -4^{o}C (Li et al, 2009) and as low as -6^{o}C (Maier et al, 1994b).

Furthermore, there is no uniformity in cold-acclimation response results obtained by various authors. For example, Fry et al (1991) did not observe a response to field acclimation in Floratam. In contrast, Maier et al (1994b) froze field-acclimated Raleigh at -6^{o}C and obtained superior survival (>60%) as compared to non-acclimated plants. Moreover, Li et al (2010) found higher levels of survival in laboratory-acclimated vs. non-acclimated Raleigh stolons frozen at -4^{o}C. To date, no St. Augustinegrass study has compared a wide spectrum of freezing protocols, taking also into account the acclimation process.

REFERENCES

Ahring, R.M.; W.W. Huffine, C.M. Taliaferro, and R.D. Morrison. 1975. Stand establishment of Bermudagrass from seed. Agronomy Journal 67:229-232.

Alberdi, M.; and L.J. Corcuera. 1991. Cold Acclimation in Plants. Review article 62. Phytochemistry 30(10):3177-318.

Anderson, J.A., C.M. Taliaferro, and D.L. Martin. 1993. Evaluating freeze tolerance of Bermudagrass in a controlled environment. HortScience 28:955.

Anderson, J.A.; C.M. Taliaferro, and D.L. Martin. 2003. Longer exposure durations increase freeze damage to turf Bermudagrasses. Crop Sci 43:973–977.

Beard, J.B. 1966. Direct low temperature injury of nineteen turfgrasses, Q. Bull. Michigan Agr. Exp. Stn. 48:377-383.

Beard, J.B. 1973. Turfgrass: Science and Culture. Prentice Hall, Inc., Englewood Cliffs, N.J. 658 p.

Bowman, D.C.; C.T. Cherney, and T.W. Jr. Rufty. 2002. Fate and transport of nitrogen applied to six warm-season turfgrasses. Crop Sci 42:833–841.

Busey, P. 1995. Genetic diversity and vulnerability of St. Augustinegrass. Crop Sci 35:322-327.

Busey, P. 2003. St. Augustinegrass. In: M.D. Casler and R.R. Duncan (eds). Turfgrass biology, genetics, and breeding. Wiley, Hoboken, N.J. p 309-330.

Dionne, J., Y. Castonguay, P. Nadeau, and Y. Desjardins. 2001. Amino acid and protein changes during cold acclimation of green-type Annual Bluegrass (*Poa annua* L.) Ecotypes. Crop Sci 41:1862-1870.

Duble, R.L. 1989. Southern turfgrasses: their management and use. Published by TexScape, Inc. College Station, TX. 335 p.

Duble, R.L. 1996. Turfgrasses, their management and use in the southern zone. Second Edition. Texas A&M University press.

Dudeck, A.E.; C.H. Peacock, and J.C. Wildmon. 1993. Physiological and growth responses of St. Augustinegrass cultivars to salinity. HortSci 28(1):46-48.

Ebdon, J.S.; R.A. Gagne, and R.C. Manley. 2002. Comparative cold-tolerance in diverse turf quality genotypes of Perennial Ryegrass. Crop Sci 43:973–977.

Emmons, R.D. 2000. Turfgrass Science and Management. Third edition. Delmar Thomson Learning. NY. 528 p.

Ensminger, I.; F. Busch, F.; and N.P.A. Huner. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Review. Physiologia Plantarum 126: 28–44.

Fry, J.D., N.S. Lang, and R.G.P. Clifton. 1991. Freezing resistance and carbohydrate composition of 'Floratam' St. Augustinegrass. HortSci 26:1537-1539.

Fry, J. and B. Huang. 2004. Applied turfgrass science and physiology. Hoboken, N.J. 310 p.

Fujikawa, S; Y. Jitsuyama, and K. Kuroda. 1999. Determination of the role of cold acclimation-induced diverse changes in plant cells from the viewpoint of avoidance of freezing injury. J. Plant Res. 112: 237-244.

Green, R.L., A.E. Dudeck, L.C. Hannah, and R.L., Smith. 1981. Isoenzyme polymorphism in St. Augustinegrass. Dep. of Omamental Horticulture, IFAS, Univ. of Florida, Gainesville, FL 32fi11. Published with the approval of the Director of the Florida Agric. Exp. Stn. as Journal Series Paper No. 2479

Gusta, L.V.; J.D. Butler, C. Rajashekar, and M.J. Burk. 1980. Freezing resistance of perennial turfgrasses. HortScience 15:494-496.

Gusta, L. V., R. Trischuk, and C.J. Weiser. 2005. Plant cold acclimation: the role of abscisic acid. J Plant Growth Regul 24:308–318.

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annual Review of Plant Physiology and plant Molecular Biology 41:187-223.

Gray, G.R.; L. Chauvin, F. Sarhan, and N.P.A. Hune. 1997. Cold acclimation and freezing tolerance: a complex interaction of light and temperature. Plant Physiol. 114: 467-474.

Harrison, J., C. Tonkinson, C. Eagles, and C. Foyer. 1997. Acclimation to freezing temperatures in perennial ryegrass (*Lolium perenne*). Acta Physiologiae Plantarum 19(4):505-515

Haydu, J.J.; A.W. Hodges, A.W., and Charles R. Hall. 2006. Economic impacts of the turfgrass and lawncare industry in the United States. EDIS document FE632, Food and Resource Economics Department, Florida. Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL.

Hoffman,L., M. DaCosta, J.S. Ebdon, and E. Watkins. 2010. Physiological changes during cold acclimation of Perennial Ryegrass accessions differing in freeze tolerance. Crop Sci 50:1037–1047.

Horn, G. C., A. E. Dudeck, and R. W. Toler. 1973. 'Floratam' St. Augustinegrass: A fast growing new variety for ornamental turf resistant to St. Augustine decline and chinch bugs. Fla. Agr. Exp. Stn. Circ. S-224.

Laetsch, W.M. 1974. The C4 syndrome: a structural analysis. Ann. Rev. Plant Physiol 25:27-52.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol I. Chilling, freezing, and high temperature stress. Academic, New York. p 67-290.

Li, R.; R.Qu, A.H. Bruneau; and D.P. Livingston. 2010. Selection for freezing tolerance in St. Augustinegrass through somaclonal variation and germplasm evaluation. Plant Breeding 129:417-421.

Livingston, D.P. III; and C.A. Henson. 1998. Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: responses to second-phase cold hardening. Plant Physiol. 116:403-408.

Livingston, D.P., III, S.P. Tallury, S. Owens, J.D. Livingston, and R. Premakumar. 2006a. Freezing in nonacclimated oat: thermal response and histological observations of crowns during recovery. Can. J. Bot. 84: 199–210.

Livingston, D.P., III, S.P. Tallury, R. Premakumar, S. Owens, and C.R. Olien. 2005. Changes in the histology of cold-acclimated oat crowns during recovery from freezing. Crop Sci. 45: 1545– 1558.

Maier, F. P., N. S. Lang, and J.D. Fry. 1994a. Evaluation of an electrolyte leakage technique to predict St. Augustinegrass freezing tolerance. HortScience 29(4):316–318.

Maier, F. P., N. S. Lang, and J.D. Fry. 1994b. Freezing tolerance of three St. Augustinegrass cultivars as affected by stolon carbohydrate and water content. J. Amer. Soc. Hort. Sci. 119:473-476.

Moseley, D., A. Patton, J. Trappe. 2010. Leaf and stolon characteristics of commercially available and experimental St. Augustinegrass cultivars. Arkansas Turfgrass Report 2009, Ark. Ag. Exp. Stn. Res. Ser. 579:64-68.

Nutter, G. C. and R. J. Allen, Jr. 1960. Floratine St. Augustinegrass: A new variety for ornamental turf. Fla. Agr. Exp. Stn. Circ S-123.

National Turfgrass Evaluation Program, (U.S.). 2003. "The National Turfgrass research initiative : enhancing America's beauty protecting America's natural resources ensuring the health and safety of all americans." [Beltsville, Md.] : National Turfgrass Evaluation Program, Betsville Agricultural Research Center, [2003], 2003. Agricola, EBSCOhost.

NC Cooperative Extension Service. 1992. Selecting and Managing Lawn Grasses for Shade. North Carolina Cooperative Extension Service. p 4.

Pearce, R.S. 2001. Plant Freezing and Damage. Annals of Botany 87:417-424

Patton, A.J. and Z.J. Reicher. 2007. Zoysiagrass Species and Genotypes Differ in Their Winter Injury and Freeze Tolerance. CROP SCIENCE, VOL. 47:1619-1627.

Philley, H.W., C.E. Watson Jr., J.V. Krans, J.M. Goatley Jr., and F.B. Matta. 1995. Differential thermal analysis of St. Augustinegrass. HortScience 30:1388-1389.

Philley H, Watson C, Krans J, Goatley J, Maddox V, and Tomaso-Peterson M. 1998. Inheritance of cold tolerance in St. Augustinegrass. Crop Sci 38: 451—454.

Qian, Y.L., S. Ball, Z. Tan, A.J. Koski, and S.J. Wilhelm. 2001. Freezing tolerance of six cultivars of Buffalograss. Crop Sci 41:1174–1178.

Reinert, J. A., B. D. Bruton, and R. W. Toler. 1980. Resistance of St. Augustinegrass to southern chinch bug and St. Augustine Decline Strain of Panicum Mosaic Virus. J. Econ. Entomol. 73:602-604.

Riordan, T. P., V. D. Meier, J. A. Long, and J. T. Gruis. 1980. Registration of 'Seville' St. Augustinegrass. Crop Sci. 20:824-825.

Riordan, T. P., V. D. Meier, and W.C. Mixson. 1991. Registration of 'DelMar' St. Augustinegrass. Crop Sci. 31 (2), p. 482.

Shahba, M.A., Y. L. Qian, H.G. Hughes, A. J. Koski, and D. Christensen. 2003. Relationships of soluble carbohydrates and freeze tolerance in Saltgrass. Crop Sci 43:2148-2153.

Stavang, J. , M. Hansen, and J.E. Olsen. 2008. Short term temperature drops do not enhance cold tolerance. Plant Growth Regul 55:199–206.

Stier, J.C., D. L. Filiault, M. Wisniewski, and J. P. Palta. 2003. Visualization of freezing progression in Turfgrassses using infrared video thermography. Crop Sci 43:415–420.

Stier, J.C.; and S. Fei. 2008. Cold-Stress physiology and management of turfgrasses. In: Handbook of Turfgrass Management and Physiology. Edited by Mohammad Pessarakli. CRC Press. Taylor & Francis Group, AZ. p. 473-495.

Tomashow, M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:571–99.

Trenholm,L.E., J.L. Cisar, and J.B. UnruhB. 2006. St. Augustinegrass for Florida lawns. Fact Sheet ENH5. From series of the Environmental Horticulture Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. June, 2006.

Turgeon, A.J. 1999. Turfgrass Management, Fifth Edition. Prentice Hall. Upper Saddle River, NJ. 392 p.

Uemura, M.; and P.L. Steponkus. 1999. Cold acclimation in plants: relationship between the lipid composition and the cryostability of the plasma membrane. J. Plant Res. 112:245-254.

Uemura, M., Y. Tominaga, C. Nakagawara, S. Shigematsu, A. Minami, and Y. Kawamura. 2006. Responses of the plasma membrane to low temperatures. Physiologia Plantarum 126: 81–89.

Webb,M.S.; M. Uemura, and P. Steponkus. 1994. A comparison of freezing injury in oat and rye: two cereals at the extremes of freezing tolerance. Plant Physiol. 104:467-478.

Wood, G.M. and R.P. Cohen. 1984. Predicting cold tolerance in perennial ryegrass from subcrown internode length. Agronomy Journal 76(4):516-517.

Zhang,X; E.H. Ervin, and A.J. LaBranche. 2006a. Metabolic defense responses of seeded Bermudagrass during acclimation to freezing stress. Crop Sci. 46:2598–2605.

Zhang, Y.; M.A.R. Mian, and J.H. Bouton. 2006b. Recent molecular and genomic studies on stress tolerance of forage and turf grasses. Crop Sci. 46:497–511.

Chapter II

Assessment of Freezing Tolerance in St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze)

INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a coarse-textured, stoloniferous, sod forming grass (Green et al, 1981) that produces a turf of medium density (Emmons, 2000) with strong, thick stolons. The species is popular as a lawn grass due to its versatility and adaptability to different growing conditions, its relatively low maintenance requirements in comparison to other warm-season turfgrasses (Green et al, 1981), and its superior shade tolerance. However, it is the least cold tolerant of the warm season grasses (Emmons, 2000) a characteristic that impedes the expansion of its area of adaptation further north. According to Maier et al (1994b), it is adapted to U.S. Dept. of Agriculture hardiness zones 8, 9 and 10. However, severe freezing injury may occur during some winters in zones 8 and 9. This low tolerance to cold temperatures represents a serious problem given that St. Augustinegrass is a sod-produced grass and it is not commercially multiplied by seed. While residential sites may offer some protection from cold, sod is usually produced in large open fields rendering it more susceptible to cold injury. Improvement of cold tolerance in St. Augustinegrass would increase the area of adaptation and potential use of this important turfgrass species (Philley, 1998).

Freezing injury in plants is caused by ice formation rather than low temperatures per se (Fry and Huang, 2004). The process is complex because it involves the dynamics and kinetics of water as is interacts with various tissues within meristematic regions of the plant (Livingston et

al, 2006). If freezing is intracellular, membranes are ruptured and cell death usually occurs. Extracellular freezing can cause dehydration due to osmotic differences between the cell and extracellular ice (Webb et al, 1994; Guy, 1990; Fry and Huang, 2004). Stier et al (2003) demonstrated that in grasses, freezing begins in roots, progresses into the crown, and then moves rapidly upwards into shoots and leaves. The crown contains meristematic regions from which new roots and shoots originate to produce a new functional plant. Therefore, whole plant survival depends on the ability of these meristematic regions to survive freeze injury (Livingston et al, 2005a). In some turfgrasses such as St. Augustinegrass, these meristematic regions are located in the nodes. Each node has the ability to develop leaves, roots and lateral shoots (Duble, 1996). Ahring et al (1975) demonstrated that in turfgrasses internode length and rhizome depth are unique morphological traits that influence freezing tolerance. Shorter internodes and deeper rhizomes resulted in more freezing resistant plants. Moreover, Wood and Cohen (1984) found that in perennial ryegrass, crown height –as indicated by sub-crown internode length- is negatively correlated with freezing tolerance. While these studies point to the importance of turfgrass crown and nodes in freezing survival, no information is available on the histological structure of turfgrass nodes or on how specific node regions respond to freezing stresses.

Exposure of plants to low but non-freezing temperatures enables plants to better withstand freezing conditions. This process is called cold-acclimation and it naturally occurs in

the fall prior to the onset of winter (Fry and Huang, 2004; Stavang et al, 2008). The temperature and length of time required for this process vary for different species. In general, acclimation seems to be due to changes in cell membrane properties and reductions in cell water content. In general, warm-season grasses don't respond well to cold acclimation and may have limited capacity to cold-acclimate (Stier and Fei, 2008). However, there are reports for Saltgrass (Shahba et al, 2003), Buffalograss (Qian et al, 2001), Zoysiagrass (Patton and Reicher, 2007) and Bermudagrass (Anderson et al, 1993; Anderson et al, 2003) indicating that these warm-season grasses can cold-acclimate and exhibit good freezing tolerance.

Studies on freezing tolerance in St. Augustinegrass are inconclusive and somewhat preliminary compared to those for other turfgrass species. A study on the inheritance of freezing tolerance in St. Augustinegrass (Philley et al, 1998), found that 'Raleigh' and 'Seville' were among the varieties with the best and worst levels of freezing tolerance -measured in terms of lethal temperature and winter survival- respectively. Duble (1996) confirmed these findings by describing Seville as a St. Augustine decline virus (SAD)-resistant, fine textured, freeze-sensitive variety, and Raleigh as a freeze-tolerant, SAD-resistant variety. Maier et al (1994) found that Raleigh had higher freezing tolerance than 'FX-332' or 'Floratam', and was a cultivar that acclimates to cold. The same was found by Li et al (2010), who developed an acclimation protocol based on cv. Raleigh's freezing response.

Different methods have been used to test for freezing survival in St. Augustinegrass. Fry et al (1991) used electrolyte leakage (EL), Maier et al (1994b) measured survival rates after freezing, and Li et al (2010) used a similar method as Maier, but with single nodes. Differences in technique between these experiments resulted in LT50's as high at -4^{o}C (Li et al, 2010) and as low as -6^{o}C (Maier et al, 1994a). Moreover, no information is available on how different freezing methods may result in differences in LT50.

The objectives of this study were: (1) to investigate several laboratory-based freeze assays to develop a more consistent method to screen for differences among St. Augustinegrass genotypes, and (2) to examine the histology of St. Augustinegrass nodes in order to document how specific tissues within the node respond to freezing stress.

MATERIALS AND METHODS

Plant Material

Given that St. Augustinegrass is mostly propagated asexually, all experiments were performed utilizing vegetative materials. Flats of cultivars Raleigh and Seville were maintained at the North Carolina State University Greenhouse facilities (Raleigh, NC). These were considered to be the "mother plants" (sources of vegetative material) for all experiments. Flats were grown at 25 ± 5^{o}C with regular irrigation every other day. Fertilization was performed every two months with Ironite ® 1:0:1 (Central Garden & Pet, Walnut Creek, CA) at a rate of

48.8 g m^{-2}, and every two weeks with Scotts® Starter Fertilizer (The Scotts Company LLC, Marysville, OH). Plants were mowed weekly to a 3–inch height. Terminal single stolons (apical stolon with three leaves plus the following node and internode) were collected from mother plants. Stolons were planted in flats filled with Fafard® 2 potting mix (Conrad Fafard Inc., Agawam, MA), and grown in the greenhouse during the months of May through December at 27 ± 5^{o}C. During these months, stolons were propagated every two weeks to ensure a constant supply of materials for the freezing tests. For every experiment, stolons were grown for two months without mowing, under the same watering and fertilization regimes as mother plants. No additional lighting was provided.

St. Augustinegrass stolons were divided into three regions: apical, mid and basal (Fig. 1). The apical region consisted of nodes that had young, not fully expanded leaves and no roots. The mid region was composed of nodes with well developed roots and fully expanded leaves but with no secondary shoots. The basal region consisted of the oldest nodes with fully expanded leaves, well developed roots, and usually with secondary shoots.

Figure 1. St. Augustinegrass stolon with three regions: A. basal, B. mid, and C. apical outlined.

Acclimation Treatments

Plants were acclimated by transferring flats with two month-old plants to a 13^{o}C growth chamber with a 12 h photoperiod at 300 mmol m^{-2} s^{-1} PAR (80% cool fluorescent and 20% incandescent bulb illumination) for one week. After that, flats were transferred to a 3^{o}C chamber with a 10 h photoperiod at 300 mmol m^{-2} s^{-1} for one more week. Non-acclimated plants remained under greenhouse growing conditions for the two week period.

Freezing Experiments

Experiment 1*: Comparison of Freezing Temperatures and Acclimation Treatments.*

To test the effects of cold acclimation on stolon survival, 2-month old stolons of Raleigh and Seville were acclimated and frozen. Whole stolons (with 9 to 14 nodes) were harvested from acclimated and non-acclimated flats of Raleigh and Seville. Roots and leaves were trimmed at 0.5 cm and 4 cm, respectively. Once trimmed, stolons were washed with cold water, rolled, and

placed in a 4 cup Good Sense® circular plastic container (Chelsea Industries, Inc. Peabody, MA). Four stolon-rolls (from same variety and acclimation treatment) were placed in each container, which constituted an experimental unit (Fig 2). Ice shavings were added to each container to promote nucleation and prevent supercooling. To monitor temperature, a thermocouple was inserted in the middle of a single-node stolon and placed in the middle of the container. Containers were randomly placed on the same shelf inside a modified commercial freezer. This method of using whole, rolled stolons will hereon be referred to as the "rolls method".

Figure 2. Preparation of 'rolls' for experiment 1.

Two modified commercial freezers were calibrated and pre-programmed to reach the initial temperature of -1C^{o} the night before the freezing test. The day of the test, plants were placed in freezers and remained at -1^{o}C for 4.5 h. Then, freezers were programmed to decrease their temperatures at a rate of 1^{o}C h^{-1} until reaching the target freezing temperatures of -2^{o}C

and -3^{o}C for treatments 1 and 2, respectively. Once at the target temperatures, freezers maintained them for 3 h, and then temperatures were increased to 3^{o}C at a rate of 2^{o}C h^{-1}.

Once thawed, stolons were cut into single-nodes, and planted in order according to their position (apical, mid or basal) in rows in flats filled with Fafard® 2 potting mix (Fig. 3). Stolons were allowed to recover in the greenhouse for 30 days and rated for survival. The experimental design was a randomized complete block design with two repetitions and a factorial structure for treatments (two varieties x two acclimation treatments x two freezing temperatures x three node-positions). There were four stolons within each experimental unit and the percent survival correspond to the number of cutting that survived over the total number of "cuttings" per node position in each stolon.

Figure 3. Flat with the four stolons transplanted from a plastic container after freezing.

Experiment 2: *Comparison of single-nodes vs. rolls method.*

In this experiment, the rolls method was compared to the 'Single-node method' described by Li et al (2010) in order to assess whether or not stolon integrity has an effect on freezing tolerance. Complete stolons (CS) for the rolls method were obtained as outlined for experiment 1. Single-node stolons (SNS) were obtained from the mid-region of complete stolons, and placed on trays as described by Li et al (2010). Acclimation treatments were performed as outlined for experiment 1. After the acclimation period, CS were treated as in experiment 1 while SNS were removed from trays, their roots and leaves trimmed, washed with cold water, and placed in plastic containers with air tight lids. Ten SNS were placed in each container. Ice shavings were added to both CS and SNS containers to promote nucleation and prevent supercooling. Each container represented a replicate with 10 SNS for the SNS method and with 4 rolls for the rolls method. Containers for both methods were randomized and each replication was placed on the same shelf inside the freezer. The experimental design for the combined analysis was a 2x2x2 (two varieties x two acclimation treatments x two freezing methods) factorial with 4 replications. For the CS in rolls, only mid position was considered for the combined analysis. Additionally, position effect was analyzed on the CS in rolls, following a completely randomized experimental design with a factorial structure for treatments(two varieties x two acclimation treatments x 3 node positions) with 4 replications. Freezers were programmed as in experiment 1 with target temperature of $-2^{o}C$ for all treatments.

After thawing, SNS were transplanted in flats intact, while CS were cut into single-nodes at three different node positions (apical, mid, basal) prior to transplantation. Both were allowed to recover under greenhouse conditions for 30 days and then scored for survival.

Experiment 3: *Comparison of different freezing temperatures.*

To determine the temperature that allows a better appreciation of differences between freeze-tolerant and freeze-sensitive cultivars, four freezing temperatures were tested: -1^{o}C, -2^{o}C, -3^{o}C and -4^{o}C and the experiment was run twice. Plant materials were taken from trays of Raleigh and Seville utilizing the rolls method as outlined in experiment 1. Given that each freezing temperature was assigned to a different freezer, the experimental design used was a mixed-nested design with 2 replicates, where the replicates were nested within each temperature, and the factors variety and position were crossed with temperatures (4 temperatures x 2 varieties x 3 positions x 2 replicates). Plants were maintained at -1^{o}C in the four freezers for 4.5 h, and then the temperature was decreased to -1, -2, -3 or -4^{o}C at a rate of -1^{o}C h^{-1}. After 3 h at the target temperature, temperatures were raised to 3^{o}C at a rate of 2^{o}C h^{-1}.

The stolons were thawed, cut into single-nodes, and planted in order (from apical to basal) in rows in flats filled with Fafard® 2 potting mix. The trays were then transferred to the greenhouse at 25 ± 5^{o}C where they recovered for 30 days before survival scores were taken (see data collection and analysis).

Experiment 4: *Comparison of Freezing Methods.*

The objective of this experiment was to compare different methods for assessing freezing survival in St. Augustinegrass. Three methods were evaluated: 'rolls', SNS in sponges, and SNS in conetainers (2.5 cm in diameter by 16 cm high plastic tubes with holes in the bottom). For all three methods, plant material was obtained from non-acclimated plants of Raleigh and Seville. Material for the roll method was prepared as outlined in experiment 1. For the sponge method, SNS of 4 cm in length were obtained from "mother plants" and placed in a slot cut in a moisturized sponge as described by Livingston et al (1989) and Santos et al (2006). Sponges containing the SNS were placed in plastic bags with ice shavings. Bags were sealed with a twist wire and inserted onto steel plumbing flanges to promote thermostability. Sponges were placed randomly according to position on the same shelf of the freezer. For the conetainers method, SNS were obtained as described in experiment 2. After roots and leaves were trimmed, each SNS was planted in Fafard® 4P potting-mix in an individual conetainer placed in a 12"x12" rack (100 conetainers per rack). For each replicate and variety 20 SNS were obtained from 4 plants: 2 SNS from apical region, 2 SNS from mid region and 1 SNS from basal region (5 SNS x 4 plants=20 SNS). SNS were grown under greenhouse conditions for one week to allow root development. They were then transferred to cold chambers for acclimation in the same manner as in experiment 1. The entire rack with conetainers was prepared for freezing by

placing it inside a plastic bag. Ice shavings were added to the surface of each tube, and then the bag was sealed with a twist wire and placed in the freezer.

The experimental design for analyze each method was a randomized complete block design with four repetitions and a factorial structure (2 varieties x 2 acclimation treatments). In the case of the rolls method, each variety was assigned to a different plastic container, with one container (4 rolls) per replicate. For the sponge method, each sponge contained four SNS from Raleigh and four from Seville placed alternately in the sponge. Four sponges constituted one replicate because they came from the same tray of Raleigh and Seville (acclimated and non-acclimated). For the conetainers method, SNS from each variety were randomly distributed in the grid within each replicate (20 SNS per variety and acclimation treatment for each replicate).

Due to differences in time-length to reach the freezing point, each method was assigned to a separate freezer and maintained at $-1^{o}C$ for different periods of time (4.5 h for the rolls method, 8 h for sponges, and 9h for the conetainers). After the soil/plants froze, the freezers were programmed to go to $-2^{o}C$ in one hour, stay there for 3 h, and then increase to $3^{o}C$ at $2^{o}C$ h^{-1}. After thawing, SNS from the sponges were transplanted intact into flats, rolls were cut into SNS and transplanted into flats, and SNS in conetainers, were transferred to the greenhouse without transplanting or cutting. Both flats and conetainers were grown under greenhouse conditions for 30 days and then scored for survival.

Experiment 5: *Using SNS in conetainers to compare acclimation treatments.*

In order to further evaluate the use of SNS in conetainers, an experiment was designed to compare acclimated and non-acclimated Raleigh and Seville materials. Plant material was obtained from both cultivars, and prepared as described in experiment 4. One week after transplanting, plants were transferred to acclimation chambers or the greenhouse depending on the acclimation treatment assigned to each. After two weeks of acclimation, tubes were placed in racks.

The experimental design was a 2x2x3 factorial (two genotypes x two acclimation treatments x three node regions) with four replicates. Each experimental unit consisted of six SNS in tubes representing each of the three regions (two apical, two mid and two basal). All treatments were randomized within racks (one replicate per rack). Each rack was placed in a plastic bag, ice shavings were added to each tube, and the bag was sealed with a twist wire and placed in a freezer at -1^{o}C. After 9 h at -1^{o}C, the temperature was lowered to -4^{o}C at a rate of -1^{o}C h^{-1}. After 3 h at -4^{o}C, the temperature was increased to 3^{o}C at 2^{o}C h^{-1}. Once conetainers had thawed, racks were transferred to the greenhouse where plants were allowed to recover for 30 days before scoring them for survival.

Experiment 6: *Comparison of varieties and acclimation treatments.*

To evaluate the SNS in conetainers method's ability to detect differences in freezing survival among genotypes, five cultivars were acclimated and compared for freezing-survival.

Two freeze-tolerant (Raleigh and GF2), two freeze-sensitive (Seville and Floratam), and one intermediate (Texas Common) genotypes were chosen for this experiment (Wilson et al, 1977; Maier et al, 1994b; Duble, 1996; Philley, 1998; Li et al, 2010; Moseley, 2010). These five entries were grown in flats under greenhouse conditions. Material preparation and acclimation were conducted as outlined in experiment 5. For freezing, freezers were set at -3^{o}C for 21 h, then temperature was decreased to -6^{o}C at a rate of -1^{o}C h^{-1}, this target temperature was maintained for 3h, and then freezers were warmed up to 3^{o}C at 2^{o}C h^{-1}.The experimental design was a 2x5x3 factorial with four replicates, where the two acclimation treatments, the five genotypes and the three stolon regions were the main factors. Each experimental unit consisted of five SNS in tubes representing the three stolon regions (2 apical, 2 mid and 1 basal).

Data collection and analysis

Survival ratings were determined for each individual node in each region of the stolon. A "0" rating meant the node was dead. A rating of "0.5" was assigned for a node that did not show re-growth, but was still green (still). A rating of "1" denoted a node with new shoots and with evident re-growth (survivor)(Li et al, 2010). This rating was translated to percent of survival according to the following formula:

$$\%\, Survival = \frac{(0.5 * number\ of\ nodes\ "still") + (1 * number\ of\ nodes\ "survivor")}{("still"\ nodes + "survivor"\ nodes + dead\ nodes)} * 100$$

An Analysis of Variance was conducted on all experiments using the MIXED procedure in SAS V.9.1.3 (SAS Institute, Cary, NC). Standard *F*-tests in all analyses were used to determine significance of main effects and interactions.

Histology

Nodes -including approximately 2mm of the leaf base- from St. Augustinegrass cultivars Raleigh and Seville were collected 14 days after freezing (plant material obtained from experiment 2). They were placed in a modified FAA fixative (45% methanol, 10% formaldehyde, 5% glacial acetic acid, 40% water) after trimming. Samples were subjected to fixation, dehydration, and embedding according to the protocols of Livingston et al (2009) using a laboratory microwave with integrated vacuum (Pelco Biowave Pro Tissue Processing System, Ted Pella Inc, Redding, CA). Once in paraffin blocks, samples were sectioned at 20 µm using a RM2255 rotary microtome (Leica, Wetzlar, Germany). Ribbons were floated on 0.4% Elmer's glue solution on microscope slides and placed on a 45^{o}C hot plate. After 3-5 minutes on the hot plate, excess Elmer solution was drained off and sections were dried overnight at 35^{o}C. Sections were de-paraffinized in Xylene for 30 minutes before undergoing a triple staining with Safranin, Fast Green, and Orange G (Fisher Scientific, Pittsburgh, PA) as described by Ruzin (1999). Stained sections were covered with permount and a cover-glass, and air-dried overnight.

RESULTS AND DISCUSSION

Experiment 1: *Comparison of Freezing Temperatures and Acclimation Treatments.*

Results of the ANOVA (Table 1) showed clear differences in survival rates between the two freezing temperatures ($p<0.0001$), the two varieties ($p=0.0007$), the two acclimation treatments ($p=0.0113$), the three positions of the nodes in the stolon ($p<0.0001$) and significant interaction temperature*variety*acclimation*position (Data not shown). Analyzing the pecercent of survival (Table 1), it is clear that temperature -3^{o}C almost completely killed both varieties (survival average of 1%), while -2^{o}C was less drastic, allowing a better evaluation of differences between varieties and between acclimation treatments (Fig. 4). For this reason, further analysis was performed taking into consideration only -2^{o}C freezing data.

Table 1. Average percent of survival for the interaction temperature*variety*acclimation*position.

Effect	Temperature	Variety	Acclimation	Position	Perc. of Survival
tempe*var*accl*posit	-2C	Raleigh	Accl	apical	0
tempe*var*accl*posit	-2C	Raleigh	Accl	basal	0
tempe*var*accl*posit	-2C	Raleigh	Accl	medio	0
tempe*var*accl*posit	-2C	Raleigh	Non-accl	apical	13.0208
tempe*var*accl*posit	-2C	Raleigh	Non-accl	basal	29.2857
tempe*var*accl*posit	-2C	Raleigh	Non-accl	medio	57.0833
tempe*var*accl*posit	-2C	Seville	Accl	apical	0
tempe*var*accl*posit	-2C	Seville	Accl	basal	1.7857
tempe*var*accl*posit	-2C	Seville	Accl	medio	9.3750
tempe*var*accl*posit	-2C	Seville	Non-accl	apical	5.3125
tempe*var*accl*posit	-2C	Seville	Non-accl	basal	3.1250
tempe*var*accl*posit	-2C	Seville	Non-accl	medio	15.3125
tempe*var*accl*posit	-3C	Raleigh	Accl	apical	0
tempe*var*accl*posit	-3C	Raleigh	Accl	basal	0
tempe*var*accl*posit	-3C	Raleigh	Accl	medio	0
tempe*var*accl*posit	-3C	Raleigh	Non-accl	apical	3.6458
tempe*var*accl*posit	-3C	Raleigh	Non-accl	basal	2.9762
tempe*var*accl*posit	-3C	Raleigh	Non-accl	medio	8.5417
tempe*var*accl*posit	-3C	Seville	Accl	apical	0

Table 1. Continued

Effect	Temperature	Variety	Acclimation	Position	Perc. of survival
tempe*var*accl*posit	-3C	Seville	Accl	basal	0
tempe*var*accl*posit	-3C	Seville	Accl	medio	0
tempe*var*accl*posit	-3C	Seville	Non-accl	apical	0
tempe*var*accl*posit	-3C	Seville	Non-accl	basal	0
tempe*var*accl*posit	-3C	Seville	Non-accl	medio	0

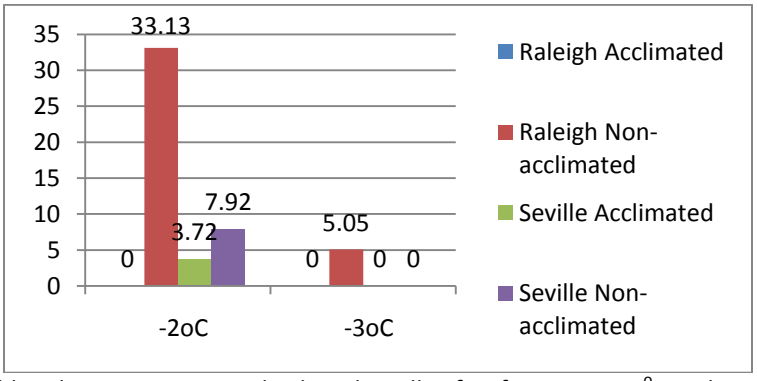

Figure 4. Effect of cold acclimation on cvs. Raleigh and Seville after freezing at -2°C and at -3°C, across the three positions.

The analysis of freezing data at -2°C (Table 2), shows borderline effect of varieties ($p<0.0554$), significant differences between acclimation treatments ($p=0.0082$) and between node-positions ($p<0.0001$). Raleigh is the hardiest cultivar with an average survival of 16%, while Seville appears to be more tender with an average survival of 6.3% (Data not shown). This difference is more marked when considering acclimation treatment. Raleigh non-acclimated had a 31.7% survival rate compared to 7.9% for non-acclimated Seville (Fig 5). These results agree with previous findings (Wilson et al, 1977; Maier et al, 1994b; Duble, 1996; Philley, 1998;

Li et al, 2010; Moseley, 2010) that indicated Raleigh is freezing-tolerant while Seville is freezing-tender.

Table 2. Analysis of variance for acclimation and node-position effects on freezing survival of St. Augustinegrass cvs. Raleigh and Seville after freezing at -2°C.

Effect	Num DF	Den DF	F Value	Pr > F
variety	1	4	7.17	0.0554
acclimation	1	4	23.74	0.0082
variety*acclimation	1	4	15.81	0.0164
position	2	8	30.75	0.0002
variety*position	2	8	4.31	0.0536
acclimation*position	2	8	16.56	0.0014
var*accl*position	2	8	13.26	0.0029

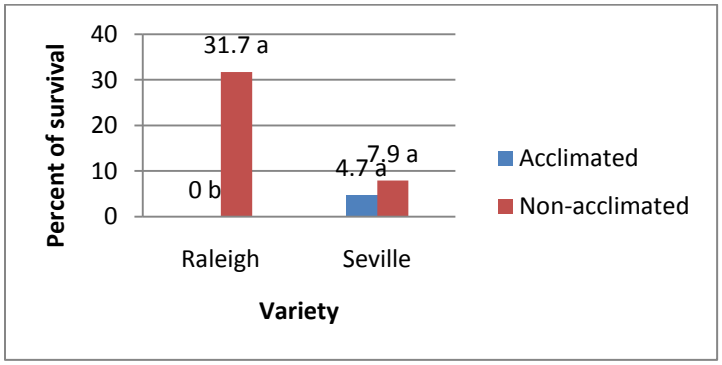

Figure 5. Effect of cold acclimation on variety survival after freezing at -2°C. Values followed by the same letter were not significantly different from each other at the 5% level.

It is evident that acclimation had a negative effect on plant survival. Non-acclimated plants showed a better response to freezing with a survival of 19.9% compared with 2.3% for acclimated plants. Considering the interaction of variety by acclimation (significant at $p<0.001$), Raleigh showed a differential response to acclimation with 31.7% and 0% survival for non-

acclimated and acclimated plants, respectively. Meanwhile, Seville's differences were minimal (Fig. 5). These results contradict previous findings by Maier et al (1994b) and Li et al (2010) that indicated Raleigh was a cold-acclimating cultivar.

These results would indicate that acclimation during the summer months somehow exposes plants to higher levels of stress. This later translates into lower freezing survival. A possible explanations to this phenomenon, can be found in the analysis comparing total number of nodes per stolon after the two weeks of acclimation (Table 3). The ANOVA results showed significant differences between the acclimation treatments ($p<0.0001$) where acclimated plants slowed their growth compared with non-acclimated ones. Non-acclimated plants developed, on average, more nodes per stolon than acclimated ones: having 1.32 more nodes for Seville and 5.37 more nodes for Raleigh (Fig. 6). However, further analysis is necessary in order to confirm these findings.

Table 3. Analysis of variance on the effect of cold acclimation on total node number for cvs. Raleigh and Seville.

Effect	Num DF	DenDF	F Value	Pr > F
variety	1	12	1.77	0.2078
acclimation	1	12	70.24	<.0001
variety*accl	1	12	25.92	0.0003

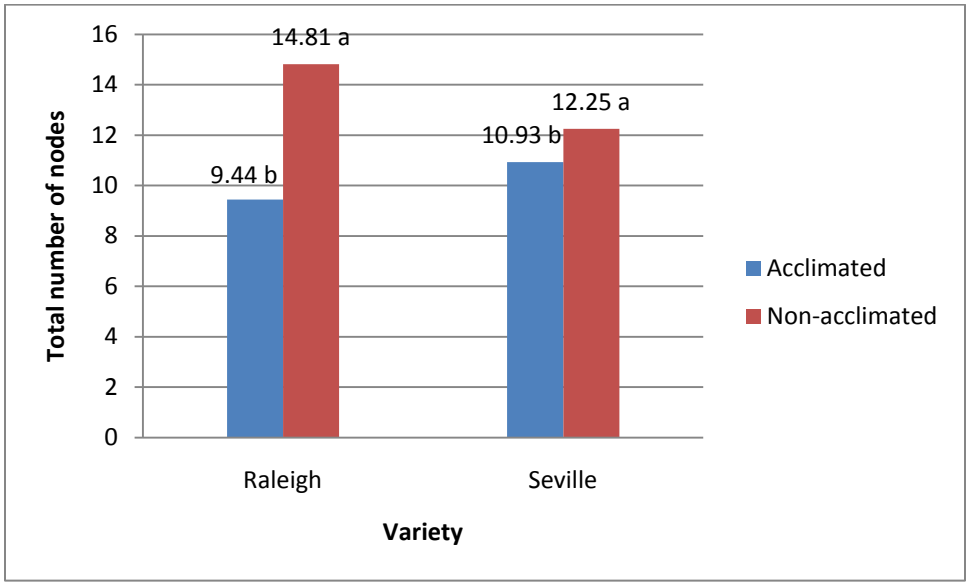

Figure 6. Effect of the interaction of cold acclimation and variety on plant growth, measured as total number of nodes per stolon after two weeks of acclimation.

Regarding node-position, results show that survival response is highly dependent on this factor. The mid region presented higher survival rates (20.4%), followed by the basal region with 8.4%, and the apical region with 4.6% (Data not shown). Considering the position effect and its interaction with variety and acclimation (Fig. 7), in all cases the mid stolon was the one with the highest survival rates, with largest differences in Raleigh No-acclimated (25.63%, 57.08%, 13.02% for basal, mid and apical). Note that Seville survival rates, for either acclimation treatment, did not show significant differences among these three positions; while Raleigh acclimated survival rate for any position was 0, showing the differential effect of acclimation on variety. These results show that differences among the three regions of the stolon follow the same trend in their response to the acclimation and varieties effects.

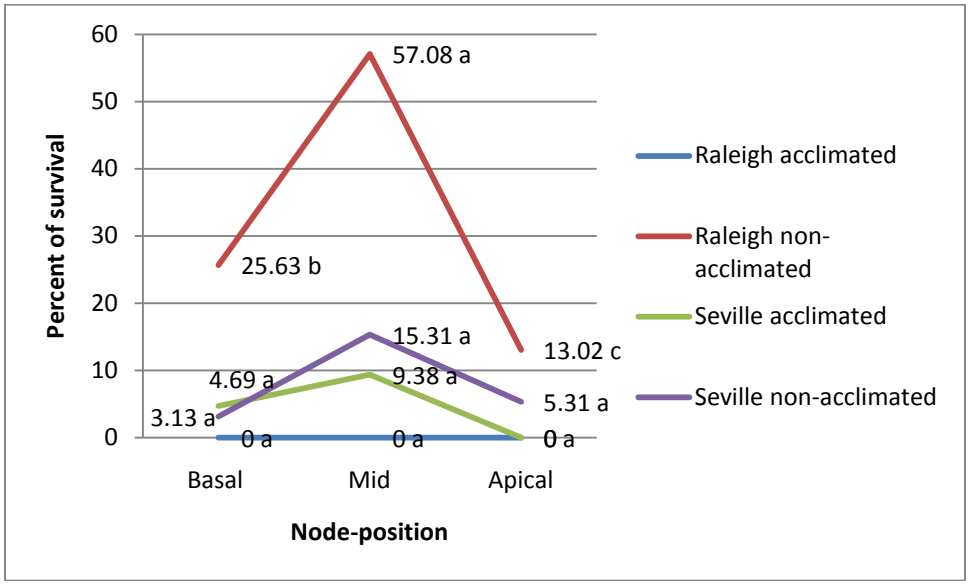

Figure 7. Percent of survival based on node-position, within the interaction of variety by acclimation treatment, after freezing at -2°C.

This is the first report of stolon differences on any stoloniferous turfgrass. In grasses, the different developmental stages of certain parts of the plant produce different results in terms of freezing tolerance with new leaves having higher survival rates than apical and old leaves (Beard, 1973; DiPaola and Beard, 1992; Stier and Fei, 2008). Our results indicate that this concept can be extended to the node-regions. Therefore, new, active nodes (mid region) have better freezing survival than immature (apical-region) and old (basal-region) nodes. The mid-region is characterized by new, but well-developed nodes. Meanwhile, the apical region is characterized by tender nodes without roots and with leaves that are just-emerging.

Up to this point, all considerations were made based on the response of individual nodes, but some reports in the literature also mention multi-node stolon assessments as a method to record freezing survival (Ahring and Irving, 1969; Maier et al, 1994a; Quian et al,

2001; Sahba et al, 2003). In order to test if there are any differences between the two approaches, an analysis was done considering whole stolon survival for each individual. The survival rate for each stolon was calculated without separating by regions and scoring the stolon as a unit. The ANOVA for this analysis (Table 4) shows a borderline effect of variety (p=0.0414) and a significant effect of acclimation (p=0.0067). Raleigh has a higher survival (16.3%) than Seville (6.3%), and non-acclimated plants survive 17% more than acclimated ones (data not shown). Analyzing the interaction of the two factors (variety and acclimation) (Fig. 8), it is evident that non-acclimated Raleigh has the highest survival rate (32.7%) while acclimated Raleigh has the lowest (0%). Meanwhile, Seville shows a minimum difference between its acclimation treatments. As can be seen, analyzing the stolon as a whole gave a similar result as doing the analysis as considering the three regions separately. Therefore it can be assumed that the single-node approach is similar to the multi-node one (whole-stolon).

Table 4. Analysis of variance for the effect of cold acclimation on whole stolon survival (without considering node position) of St. Augustinegrass cvs. Raleigh and Seville.

Effect	Num DF	Den DF	F Value	Pr > F
variety	1	4	8.78	0.0414
acclimation	1	4	26.67	0.0067
variety*accl	1	4	20.09	0.0110

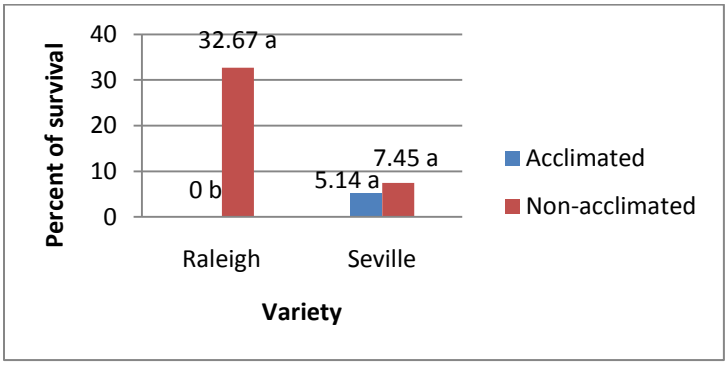

Figure 8. Effect of cold acclimation on freezing survival of St. Augustinegrass cvs. Raleigh and Seville considering whole stolon performance.

Considering all the information presented above, it is evident that: (i) -2°C is a more suitable temperature than -3°C to assess freezing survival differences between treatments, (ii) at -2°C, Raleigh has a higher freezing survival rate than Seville, (iii) cold-acclimation seems to slow new node development and have a detrimental effect on freezing survival on both varieties, and (iv) there are significant differences in freezing survival among node-positions, being the mid-region the one with the highest survival for all treatments.

Experiment 2: *Comparison of single-nodes vs. rolls method*

To compare the 'rolls' method and the single-node stolon method (SNS), a combined analysis was carried out for variety and acclimation effects (considering only the mid region)(Table 5). The ANOVA shows that there is no statistical difference between the two methods ($p=0.6670$), which means that both methods report similar results. Variety shows a very significant effect ($p<0.0001$) with Raleigh's survival (57.2%) being much higher than that of Seville's (22.7%). For acclimation, the results demonstrate that acclimated plants have a lower

freezing survival (30%) compared with the non-acclimated ones (50%) (data not shown). The only significant interaction was method by variety (p=0.0124) where bigger differences were found for the rolls method than for the SNS method, even though both followed the same trend showing Raleigh hardier than Seville (Fig. 9).

Table 5. Combined analysis of variance of SNS vs. rolls methods for measuring cold acclimation effects on two varieties of St. Augustinegrass[1].

Effect	Num DF	Den DF	F Value	Pr > F
method	1	6	0.20	0.6670
variety	1	18	67.49	<.0001
method*variety	1	18	7.72	0.0124
acclimation	1	18	25.94	<.0001
method*acclimation	1	18	0.38	0.5433
variety*acclimation	1	18	2.49	0.1317
method*variety*accl	1	18	0.57	0.4616

[1]Data was transformed for the ANOVA, using square root of original value

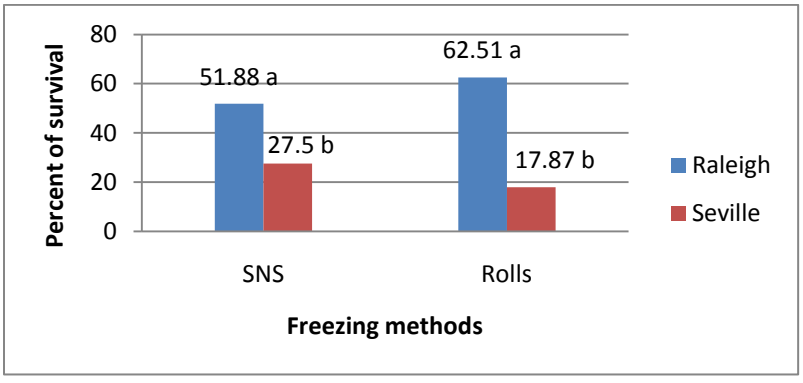

Figure 9. Effect of freezing method over varieties for survival response after freezing at -2°C.

Analysis of the effect of position on the rolls method separately was done as a confirmation run for experiment 1. The ANOVA results (Table 6) showed a strong effect of varieties ($p=0.0012$), a borderline effect of acclimation ($p=0.0650$), a strong effect of node-position ($p<0.0001$), and a highly strong variety by position interaction effect ($p<0.0001$) and a significant acclimation by position effect ($p=0.0216$). For varieties, Raleigh continues being the most freeze-tolerant with a survival of 38.4%, compared to 10.7% for Seville (data not shown).

Table 6. Analysis of variance for the effect of cold acclimation on freezing survival of St. Augustinegrass cvs. Raleigh and Seville considering node position using the rolls method.

Effect	Num DF	Den DF	F Value	Pr > F
variety	1	12	17.62	0.0012
acclimation	1	12	4.12	0.0650
variety*acclimation	1	12	0.01	0.9300
position	2	120	46.18	<.0001
variety*position	2	120	12.29	<.0001
acclimation*position	2	120	3.96	0.0216
variety*accl*position	2	120	0.92	0.3997

In the case of acclimation treatments, results indicate that non-acclimated plants have a higher survival rate (31.2%) than acclimated ones (17.8%) (data not shown), as seen also in the interactions with node-position where non-acclimated positions have higher survival rates (14.71, 50.19 and 28.74% for apical, mid and basal regions respectively) than their corresponding acclimated ones (8.58, 28.08 and 16.73%) (Fig. 10).

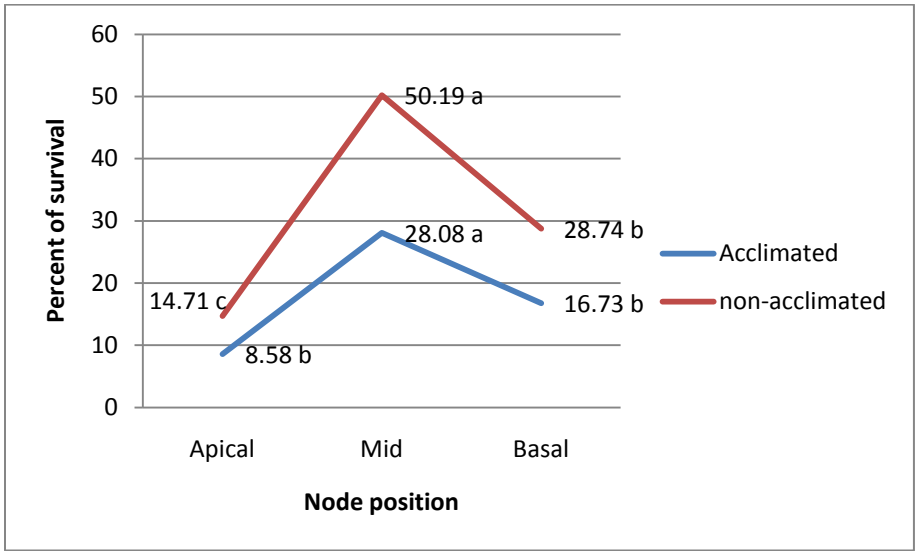

Figure 10. Effect of cold acclimation over node-position for survival response after freezing at -2°C.

For comparing total number of nodes per stolon for each variety after two weeks of acclimation, seven stolons were selected for each treatment. The ANOVA (Table 7) shows differences between the acclimation treatments ($p<0.001$). Non-acclimated plants developed, on average, 3.5 new nodes while acclimated plants only developed 0.5 (Fig. 11). These results confirm our observation from the first experiment that cold acclimation produces a reduction in plant growth in terms of new node formation.

Table 7. Effect of acclimation treatment and variety on node development after two weeks of cold acclimation.

Effect	Num DF	Den DF	F Value	Pr > F
variety	1	24	2.68	0.1148
acclimation	1	24	198.11	<.0001
variety*acclimation	1	24	0.11	0.7463

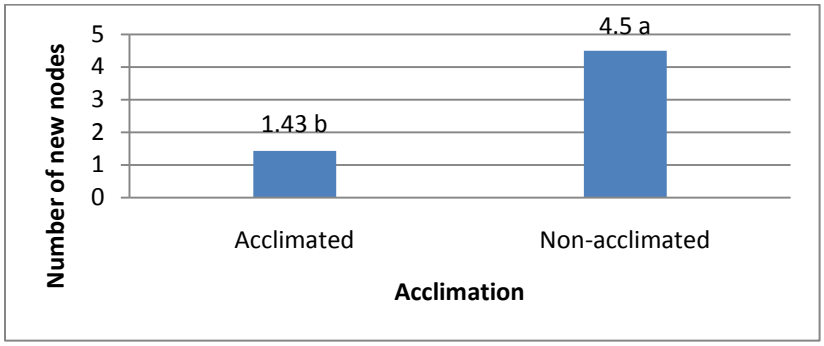

Figure 11. Effect of cold acclimation over node development across varieties after the two weeks of cold acclimation. Values followed by the same letter were not significantly different from each other at the 5% level.

As seen in the first experiment, cold acclimation affects plant response in a negative way. Comparing our protocols with those used by Maier et al (1994b) and Li et al (2010), the only difference found is the season where the studies were done. In the case of Maier et al, they froze field-acclimated Raleigh, and Li et al (2010) performed their laboratory-based acclimation during the fall-winter. On the other hand, our experiment was carried out during the summer. This agrees with previous findings (Beard, 1966) that strong seasonal variations in cold acclimation can be observed in turfgrasses and would indicate a possible role of photoperiod in St. Augustinegrass acclimation, as was demonstrated for other cereals (Gray et al, 1997). A third step might be needed in order to successfully cold-acclimate plants under laboratory conditions. However, further studies are needed to verify these assumptions.

Regarding node-position, it is evident that there are differences in survival between the different regions (Table 6). The mid region's survival of 39.1% is far superior to that of the apical and basal regions with 11.6 and 22.7%, respectively (Fig. 12). Moreover, looking at the

interaction between position and variety, it can be observed that the mid-region is the one that has the highest survival with values of 61 and 17% for Raleigh and Seville, respectively. This compared with the apical and basal with values of below 30% for Raleigh and below 12% for Seville (Fig. 13). These differences are also observed for acclimation treatments. The mid region reports the highest survival rates (data not shown). These results support those obtained in the first experiment, indicating that the mid region is the hardiest one (Fig. 14).

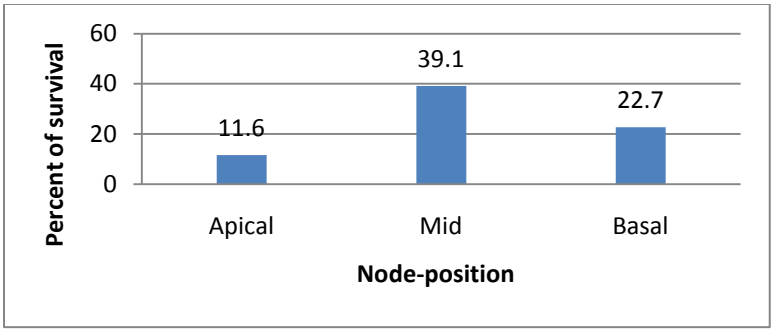

Figure 12. Node-position effect on plant survival across varieties and acclimation treatments after freezing at -2°C.

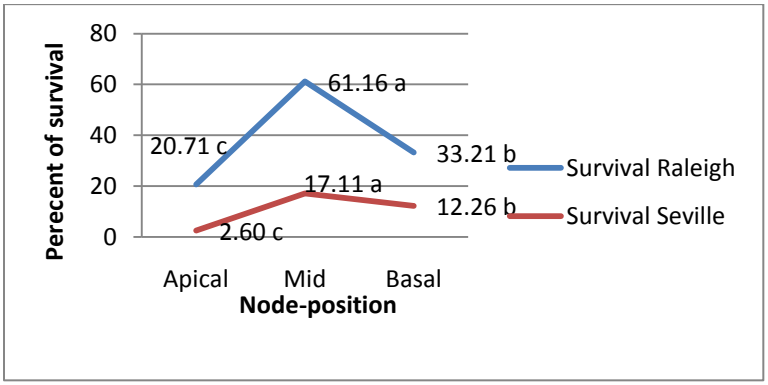

Figure 13. Effect of node-position on plant survival for cvs. Raleigh and Seville after freezing at -2°C.

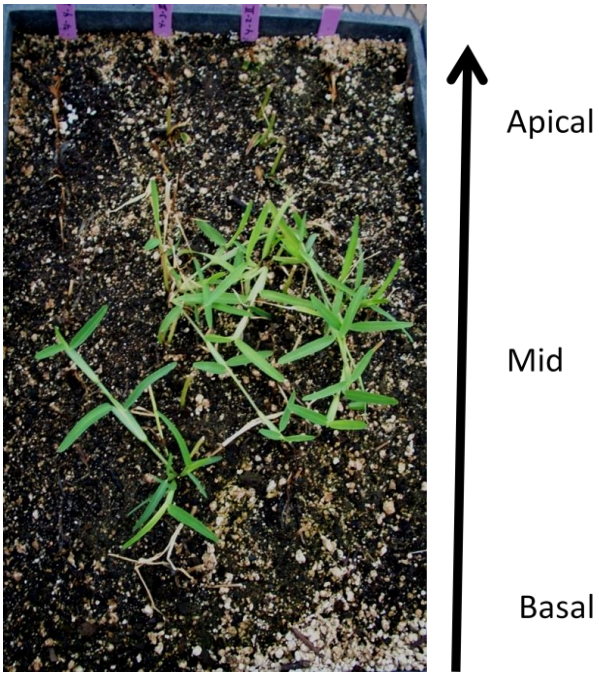

Figure 14. Cultivar Seville showing differences in survival among the three stolon regions after freezing at -2°C.

Results obtained in experiment 2 indicate that: (i) there are no differences between the rolls and the SNS methods, (ii) cold-acclimation shows a negative effect on freezing survival, indicating the possible need of considering photoperiod adjustments and adding a third step, (iii) cold-acclimation slows plant growth in terms of new node formation, and (iv) node position clearly defines freezing survival response.

Experiment 3: *Comparison of different freezing temperatures*

Looking the effect of different freezing temperatures on node survival of Raleigh and Seville, the ANOVA (Table 8) shows that there is no difference between the two runs performed

for this experiment (p=0.0809). In addition, there is a significant effect of freezing temperature (p<0.0001), variety (p=0.0088), and node-position (p<0.0001).

Table 8. Analysis of variance for the effect of freezing temperature, cold acclimation, variety, and node-position on the freezing survival of St. Augustinegrass.

Effect	Num DF	Den DF	F Value	Pr > F
run	1	16	3.47	0.0809
temperature	3	16	471.91	<.0001
run*temperature	3	16	0.38	0.7687
variety	1	16	8.88	0.0088
run*variety	1	16	0.21	0.6524
variety*temp	3	16	0.19	0.8985
run*variety*temp	3	16	0.56	0.6487
pos	2	221	127.31	<.0001
run*pos	2	221	11.09	<.0001
temp*pos	6	221	41.60	<.0001
run*temp*pos	6	221	3.24	0.0045
variety*pos	2	221	5.76	0.0037
run*variety*pos	2	221	1.75	0.1770
variety*temp*pos	6	221	2.48	0.0245
run*variety*temp*pos	6	221	2.98	0.0080

Considering temperature, it was observed that freezing temperatures of -3^{o}C and -4^{o}C produced the lowest survival (2.0 and 2.5%, respectively). Meanwhile, -1^{o}C and -2^{o}C presented higher freezing survival rates (83.8 and 59.6%, respectively) (Figs. 15 and 16). Moreover, -2^{o}C showed a greater power of discrimination between treatments for freezing survival rates. For example, when analyzing the interactions of temperature with variety and position, the most marked differences among treatments are obtained at -2^{o}C (Fig.17). In this sense, this temperature seems to be the most suitable one for evaluating freezing survival in St.

Augustinegrass using the rolls method. However, previous reports found 60% survival when freezing Raleigh at -6°C (Maier et al, 1994b) and 50% survival when freezing Raleigh at -4°C (Li et al, 2010) compared to overall average for Raleigh of 63% observed in this study at -2°C. This difference in target temperatures might stem from differences in freezing regimes between the different methods. First, Li et al (2010) reduced the temperature directly from 3°C to -4°C at a rate of 1°C h^{-1} and Maier et al (1994b) reduced it from 1°C to -6 at a rate of 2°C h^{-1}. Meanwhile, our protocol placed the stolons at -1°C for 5 h to stabilize the temperature, and then reduced the temperature to the target freezing temperature at a rate of 1°C h^{-1}. Moreover, in our experiments, temperature was monitored trough thermocouples inserted directly into nodes. In the other studies, thermocouples were placed so that they were measuring the temperature surrounding of the surrounding media (plastic bag or container) rather than the node's temperature itself. These results might indicate that node temperature reached by other protocols probably were higher than -2°C. So this remark the relevance of the stabilization step as an important factor to consider when developing freezing methods for St. Augustinegrass.

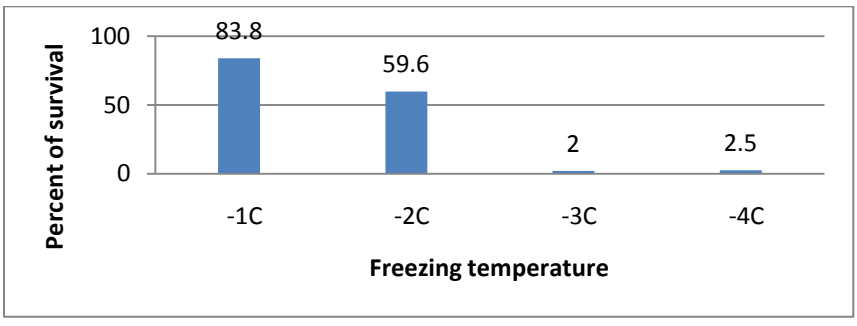

Figure 15. Effect of freezing temperature on node survival on St. Augustinegrass across varieties, temperatures, and node positions.

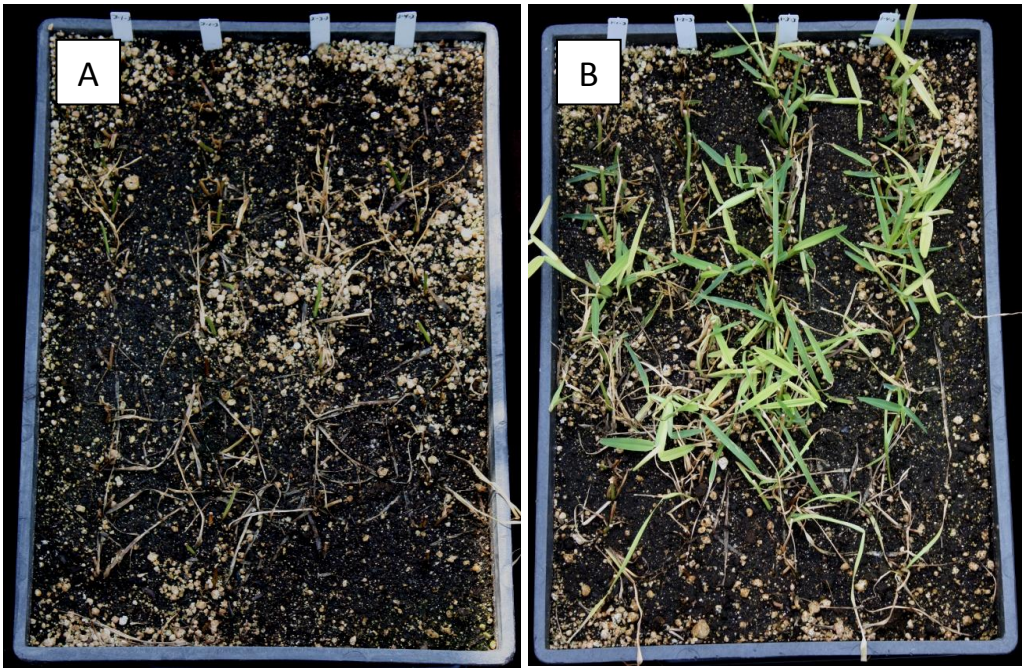

Figure 16. Differences in survival for cv. Raleigh frozen at A) -4°C, and B) -2°C.

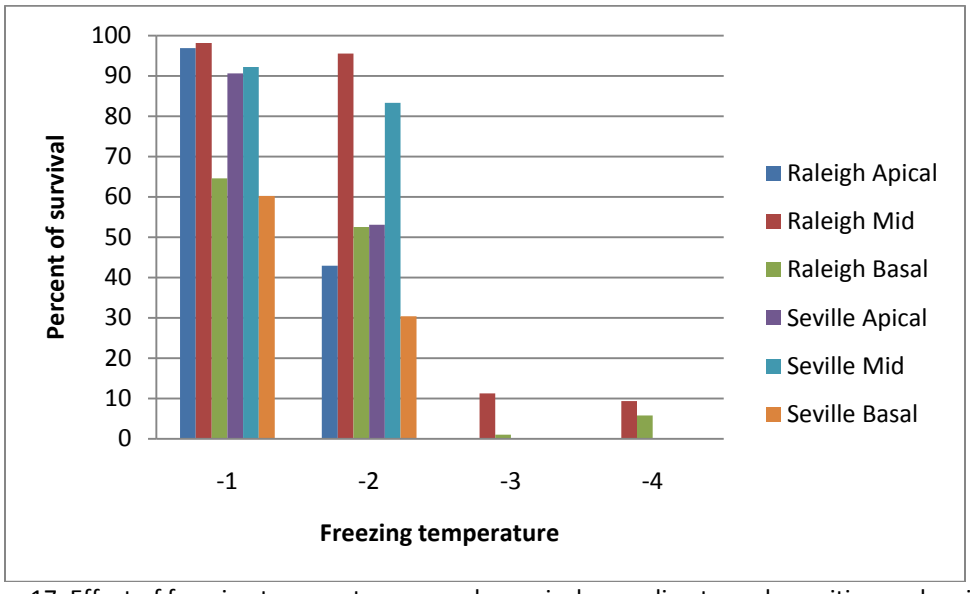

Figure 17. Effect of freezing temperature on node survival according to node position and variety.

Results from this experiment indicate that even when looking at a wider range of temperatures, -2^{o}C is the most suitable temperature for evaluating freezing survival in St. Augustinegrass when using the rolls method. Secondly, a stabilization step of 4.5 h at -1^{o}C before proceeding to the freezing process seems to be an important factor to include in the freezing protocol in order to allow the nodes to reach sub-zero temperatures. Lastly, our analysis confirms once more that the mid region of the stolon presents the best survival rates among all three regions.

Experiment 4: *Comparison of Freezing Methods.*

SNS in conetainers gave a 100% survival for all treatments (data not shown). Given that in this method the soil mix is included in the freezing procedure, most likely the target temperature was affected. Moreover, the moisture content of the soil mix may have acted as a buffer protecting the node from desiccation and reducing freeze damage. Therefore, it would be important to consider serious modifications in the protocol for future studies.

For the rolls method, the ANOVA shows differences between varieties ($p<0.001$) and differences between node-position ($p<0.0001$). In both cases the trend is the same as discussed in previous experiments (data not shown).

For the SNS in sponges method, results were highly variable, even tough there were differences between varieties ($p=0.0325$) (Table 9), but covariance parameter estimates (Table 10) gave the highest value (in a ratio of 3 times more) for sponges. Therefore most of the

variation observed was due to differences between sponges. Moreover, it was observed that stolons placed in sponges containing higher amounts of water appeared to have better survival rates.

Table 9. Effect of variety in plant survival after freezing at -2°C as SNS in sponges.

Effect	Num DF	Den DF	F Value	Pr > F
Var	1	15	5.55	0.0325

Table 10. Covariance parameter estimates for SNS in sponges' freezing survival after freezing at -2°C.

Cov Parm	Ratio	Estimate
sponge	3.8363	0.001715
var*sponge	0.04567	0.000020
Residual	1.0000	0.000447

In order to test this hypothesis, a sub-experiment was designed in order to test three different moisture contents for sponges (15g, 30 g and 45g of water applied before freezing) using non-acclimated plants of Raleigh and Seville in five replicates. The ANOVA (Table 11) showed that there were significant differences among the three moisture contents (p=0.0009) , but not between varieties (p=0.8513). Regarding moisture content, 45 g and 15 g were the treatments with the highest (96.7%) and lowest (20.0%) survival rates, respectively (Fig. 18, 19).

Table 11. Effect of moisture content using the sponge method for cvs. Raleigh and Seville frozen at -2°C.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	12	13.17	0.0009
Variety	1	72	0.04	0.8513
Variety*treat	2	72	0.88	0.4172

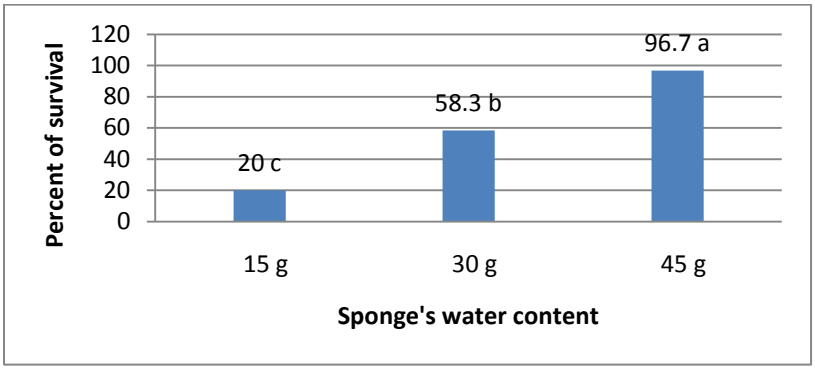

Figure 18. Effect of sponges' water content across varieties after freezing at -2°C. Values followed by the same letter were not significantly different from each other at the 5% level.

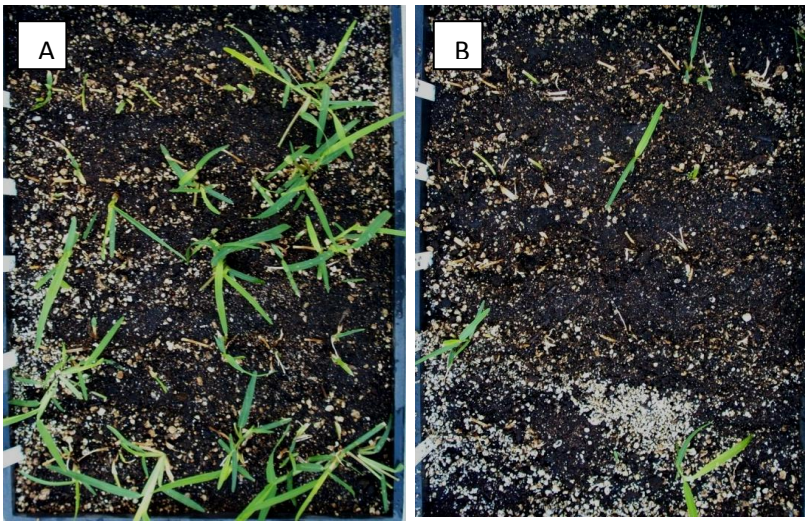

Figure 19. Comparison of sponge's moisture content treatments A) 45 g, and B) 15 g .

Results obtained in experiment 4 indicated that: (i) combined analysis of three methods (rolls, SNS in sponges, and SNS in conetainers) was not possible due to intrinsic differences among the three methods, (ii) the rolls method gave the same results, in terms of differences in freezing survival for varieties and node-positions, as in previous experiments, (iii) the SNS in conetainers method needed an adjustment in protocol since it gave 100% survival for all

treatments, and (iv) survival response of SNS in sponges was highly affected by moisture content of the sponge, being the sponges with higher moisture content the ones that gave the highest survival rates.

Experiment 5: *Using SNS in conetainers to compare acclimation treatments*

Although the protocol for SNS in conetainers method was adjusted and the freezing temperature was lowered to -4^{o}C, 100% node survival was observed. This result indicated that further re-adjustments of the protocol are necessary. There is the need to determine not only the most suitable target freezing temperature, but also to elucidate what is the freezing point temperature. Moreover, it may be better to use -3^{o}C instead of -1^{o}C for the first step, and to increase the period of time for stabilization from 9 to 21h.

Experiment 6: *Comparison of varieties and acclimation treatments*

In contrast with our previous attempts to use the SNS in conetainers method, this new protocol showed some differential responses to freezing, even though the survival rates obtained were still high (between 60 to 90%).

Considering the freezer effect as part of the model, results showed no significant differences between both freezers (p=0.3623). Furthermore, no significant interaction was found between freezers and any of the other factors (data not shown). Therefore, the analysis was performed considering all four replicates without differentiating freezer effects. The ANOVA (Table 12) showed no differences between acclimation treatments (p=0.7467),

significant differences between genotypes (0.0136), and highly significant differences among node-positions (p=0.0004). Moreover, borderline differences were found in the interaction of genotype by position (p=0.0597).

Table 12. Effect of cold-acclimation and node position on freezing survival of five genotypes of St. Augustinegrass after freezing at -6^{o}C using the conetainers method.

Effect	NumDF	DenDF	F Value	Pr > F
acclimation	1	70	0.11	0.7467
genotype	4	70	3.39	0.0136
genotype*acclimation	4	70	1.71	0.1572
position	2	140	8.42	0.0004
acclimation*position	2	140	1.86	0.1594
genotype*position	8	140	1.93	0.0597
genotype*acclimat*pos	8	140	1.44	0.1840

In the case of genotypes, there was no difference between Raleigh, Seville, Floratam and GF2 (survival values above 85%). However, there was a significant difference between these cultivars and Texas Common, which showed a survival of only 70% (Fig. 20). This result is contrary to previous reports (Wilson et al, 1977; Maier et al, 1994b; Duble, 1996; Philley, 1998; Li et al, 2010; and Moseley, 2010) and even to results obtained by preceding experiments in this study. Showing Raleigh and Seville at the same level of survival questions the accuracy of this method to assess for differences among treatments. Moreover, it shows the role of the soil media in retaining moisture around the nodes and reducing freeze damage. Further studies are needed to elucidate these effects in order to achieve a good freezing protocol to be used with conetainers.

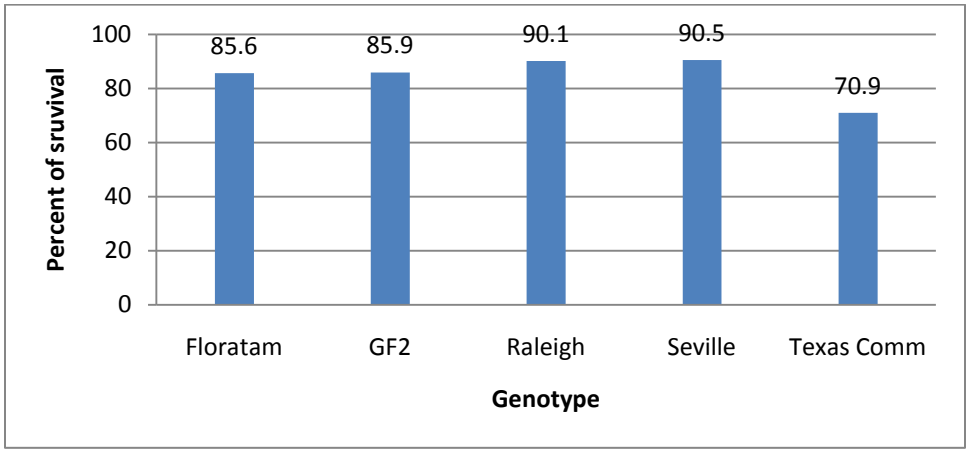

Figure 20. Freezing survival of five St. Augustinegrass' genotypes across node positions and acclimation treatments after freezing at -6°C in conetainers.

For node-position, results showed mid region had the best survival with 94.1% compared with 81.4 and 78.3% for basal and apical, respectively (data not shown). As seen in previous experiments, these differences are clear. This trend is also constant for the genotype by position interaction, where mid region is the hardiest for all genotypes (Fig. 21).

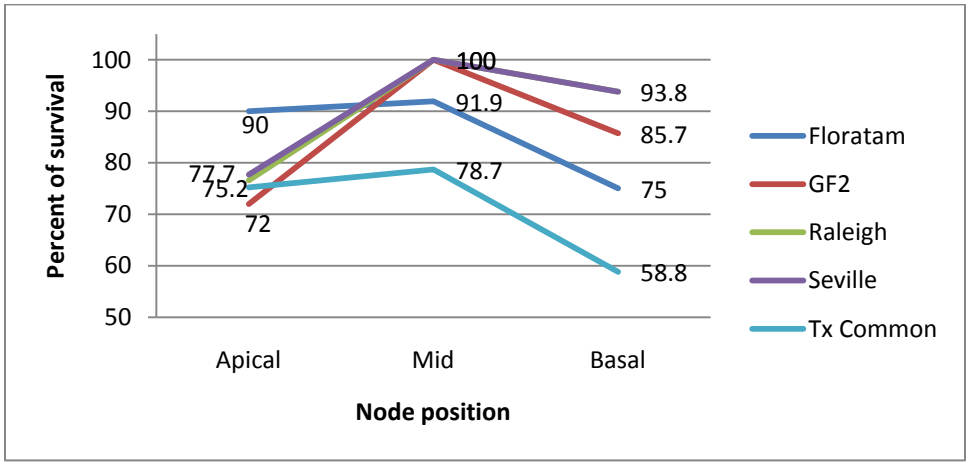

Figure 21. Effect of node position on freezing survival of five St. Augustinegrass' genotypes across acclimation treatments after freezing at -6°C in conetainers.

Results obtained in experiment 6 indicate that: (i) even at lower freezing temperatures, the SNS in conetainers method still yielded high survival rates, (ii) there seems to be a buffer effect by the soil surrounding the nodes, which masks freezing response, and (iv) the mid-region continues to be the most hardy, with higher survival rates than the other two regions.

Histology analysis

1. Nodal Structure.

As stated previously, St. Augustinegrass is a stoloniferous grass which has stem modifications with nodes and long, thin internodes (Beard, 1973; Duble, 1996). Nodes contain meristematic regions for root and shoot development, and are analogous to crowns of cereals such as wheat, barley and oats. To better understand the structure of St. Augustinegrass nodes and their relationship to freezing survival, frozen and unfrozen nodes from Seville and Raleigh were analyzed histologically.

This analysis revealed that the node of St. Augustinegrass corresponds to the structure of a typical C_4 monocot (Turgeon, 1999; Busey, 2003) (Fig. 22) with well defined bundle vessels of xylem and phloem surrounded by a bundle sheath. Each bundle of vessels was surrounded by conjunctive tissue or cortex, similar to maize and other cereals (Mauseth, 1988; Shane et al, 2000). Bundle vessels were scattered in an arrangement called an “atactostele” (Mauseth, 1988) with the phloem oriented towards the outside and the xylem oriented towards the center of the node (Fig 22C).

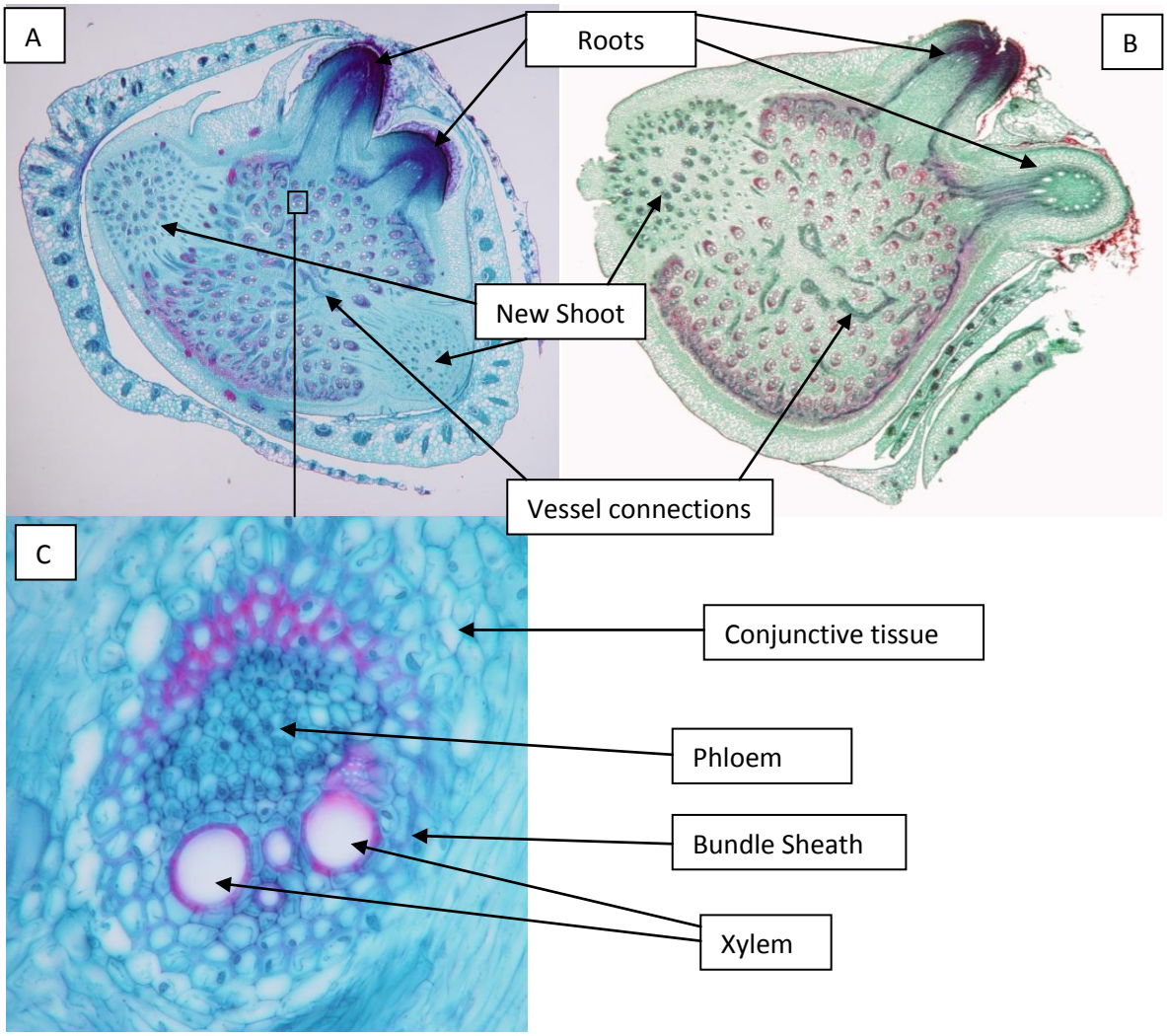

Figure 22. Cross section of (A) Seville and (B) Raleigh showing roots and shoots connections to the main stem, and (C) bundle vessels showing basic C4 structure.

The bundles generally run parallel to the node and internode, but in the center of the node there are some bundles which are perpendicular. This is similar to other monocots where bundle vessels form a network of joints and branches ultimately leading into leaves (Hoppe et al., 1986, Mauseth, 1988; and Shane et al, 2000). The continuity of the vessels becomes clear

when the internal structure of nodes are viewed in three dimensions. In a 3D video of Seville, a series of bundles clearly proceed from basipetal regions and are distributed into the leaves. In addition to vascular bundles within the nodes, a layer of cells forming a ring surround the vascular bundles just inside the epidermis (Fig. 23C). This ring stained a darker red at the basipetal end of the node (Fig. 24). Saffranin reportedly stains lignin compounds (Johansen, 1940; Livingston et al, 2005). For that reason we predict that basipetal regions of the node contain more lignin compounds. However, more research is necessary to confirm this.

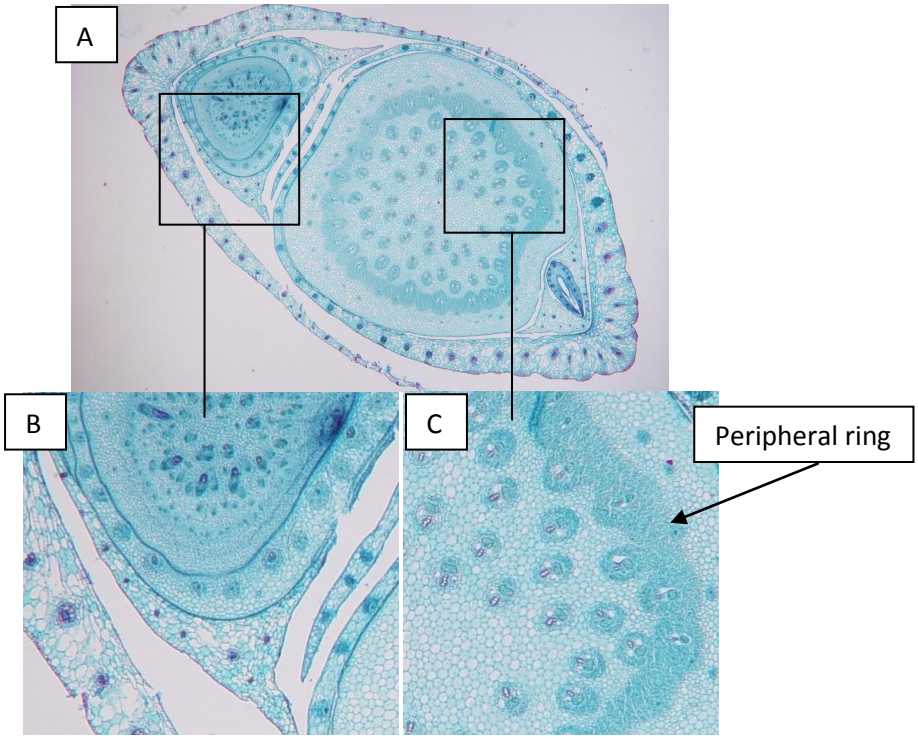

Figure 23. Cross section of Seville's node, showing (A) entire section, (B) new shoot amplification, and (C) main-stem amplification.

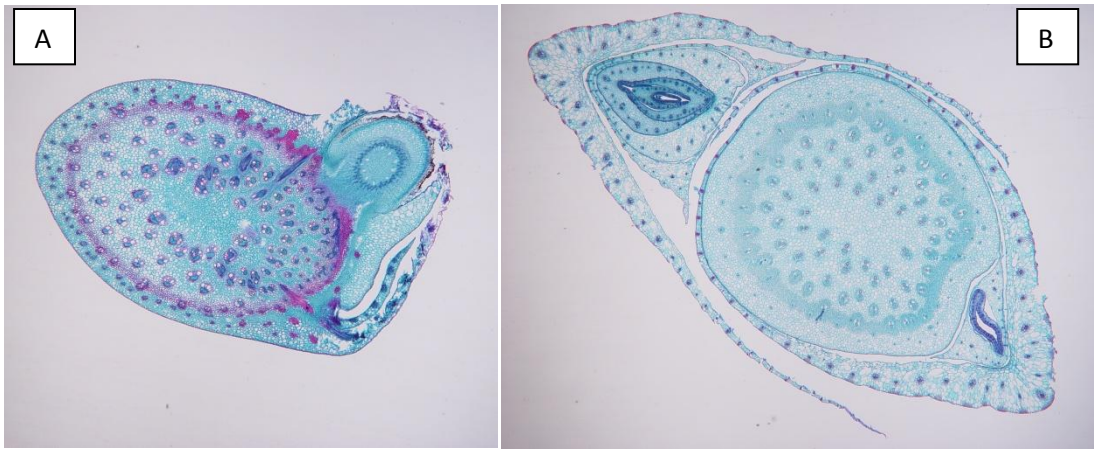

Figure 24. Seville's node cross section, showing (A) a basipetal section compared to (B) an acropetal section where differences in lignin content are appreciated.

2. Responses to Freezing

2.1 Lignin. It is possible that differences in lignin content could affect freezing rates which could in turn affect damage to the tissue. In maize leaves, more rigid cell walls (presumably with more lignin) in the epidermis and bundle sheath seemed to provide an opposing force to water loss and thus prevent desiccation due to freezing (Ashworth and Pearce, 2002). We found less structural damage in basipetal regions of the node (Fig. 25) which were stained a darker red, before (Fig. 24A) and after (Fig. 25A) freezing. It is possible that differences in freezing rates described by Stier et al (2003) as ice moved from roots into crowns and up into stems and leaves of grasses was caused by differences in lignin content within the crown.

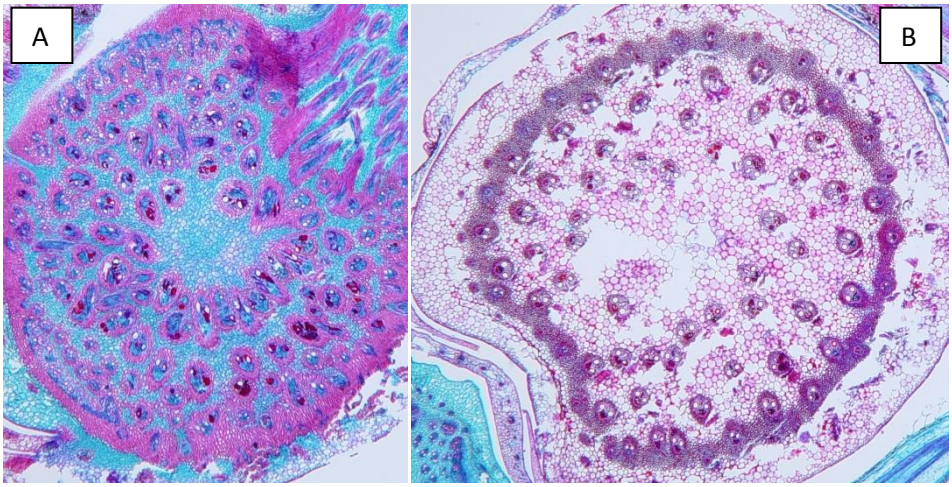

Figure 25. Cross section of frozen Seville's node showing (A) a basipetal section with dense lignified tissue surrounding the plugged vessels with almost intact intracellular space in the cortex, compared to (B) an acropetal section were this lignified tissues are almost absent and the damage is devastating.

2.2 Meristems. A more detailed analysis of Seville showed that new shoots have smaller and more compact cells than those at the center of the node (Fig. 23B). If freezing initially occurs extracellularly this would suggest that new shoots might freeze after the main stem and if temperatures did not reach a critical point, new shoots may survive a lower temperature than the main stem. In fact, in tissues recovering from freezing, more tissue disruption and discoloration of cells was observed in the stem than in the new shoot (Fig 26B). Almost no tissue disruption is visible in new shoots and the cells are dark green, suggesting that cells are alive and had survived freezing. This suggests that the most freezing tolerant area of the node is the meristematic region where new shoots and roots originate (Fig. 27). This is analogous to the

transition zone in winter cereals which is also the most freezing tolerant part of the crown (Olien, 1981; Tanino and McKersie, 1985; Livingston et al, 2005b)

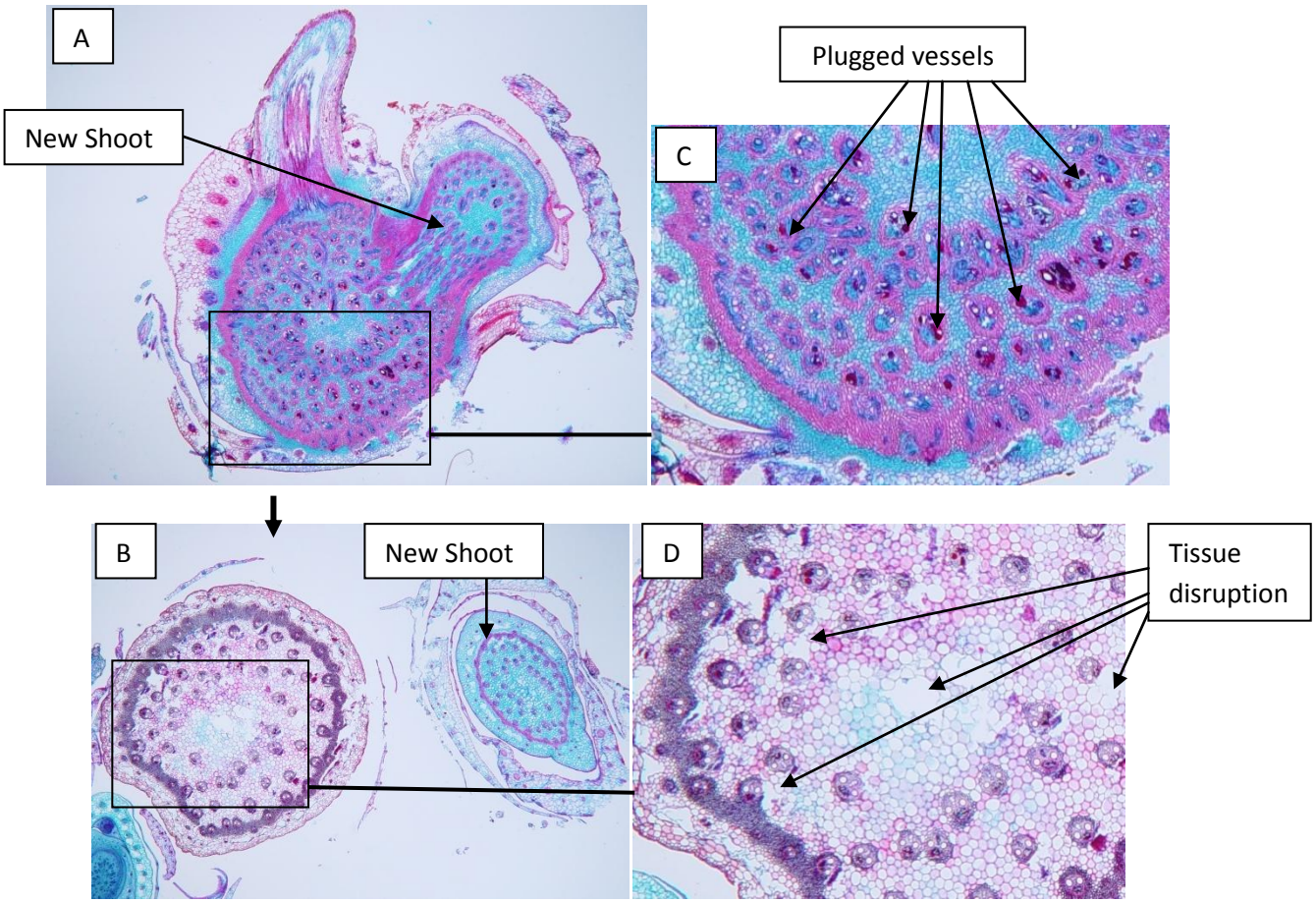

Figure 26. Cross section of frozen Seville node showing (A) a new shoot union to the main-stem, and (B) complete independence of the new shoot. Detailed magnification shows (C) plugged vessels, and (D) tissue disruption inside the main-stem.

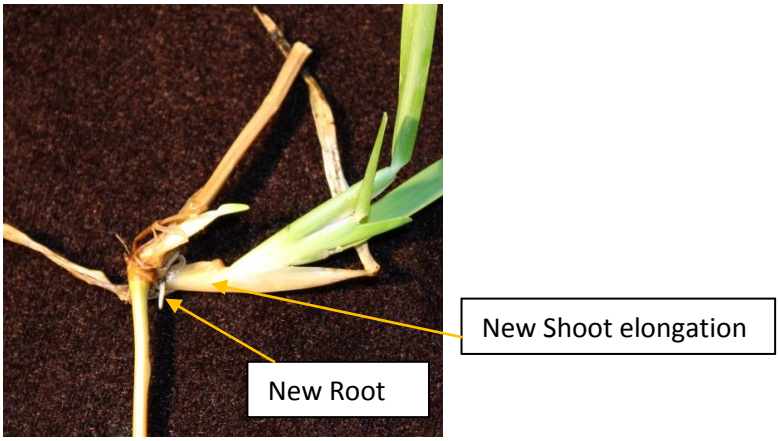

Figure 27. Raleigh's node after freezing, showing elongation of new shoot and new root formation beginning at new shoot.

2.3 Vessel plugging. Another freezing response reported by Olien (1961), Livingston et al (2005b), Livingston et al (2006) and Livingston et al (2009) is an apparent plugging of xylem vessels in the lower part of the oat crowns. We also observed vessel plugging in the basipetal region of nodes of St. Augustinegrass. A clear continuity of plugging is observed in the 3D reconstruction of frozen nodes with continuous plugging within the same vessel for at least 1/3 of the node. More research is needed to determine what the substance plugging vessels is composed of, as well as whether the plugging is a freezing protection mechanism or simply a freezing response. It may also be important to understand why only some vessels are plugged.

CONCLUSIONS

Based on all the presented experiments, five conclusions can be drawn from the results. First, contrary to previous reports, cold-acclimation showed a negative effect on plant survival under laboratory-based conditions. This might be explained by strong seasonal variations in cold acclimation and would indicate a possible role of photoperiod in St. Augustinegrass acclimation. The need for a third step in the laboratory-based cold-acclimation process should be considered in order to achieve an effective cold-acclimation in this turfgrass.

Desiccation seems to play an important role in freezing survival of St. Augustinegrass. Methods that provided a buffer between the nodes and the freezing air (*i.e.* the soil mix in the conetainers method) produced higher survival rates than those were nodes were directly exposed. Preservation of moisture around the node seems to be responsible for this.

Node position is of vital relevance for survival after freezing. As seen in all experiments, new, active nodes have higher survival rates than immature and old ones. This observation should serve as a basis for future discussions to refine protocols of stoloniferous grasses, given that it might indicate structural and metabolic differences that need to be taken into account.

Histological analysis of the node showed plugged vessels and disrupted tissue after freezing. Higher frequencies of these were observed in the main stem compared to new shoots. Re-growth begins at new shoots, which appear to act independently from the main stem.

Survival seems to be linked to reduction in water content and cell size as observed when comparing cross sections of main stems vs. new-shoot stems.

Finally, throughout all experiments we confirmed previous reports that Raleigh is more freezing tolerant than Seville.

REFERENCES

Ahring, R.M., and R.M. Irving. 1969. A laboratory method of determining cold hardiness in bermudagrass, *Cynodon dactylon* (L.) Pers. Crop Sci. 9:615-618.

Ahring, R.M.; W.W. Huffine, C.M. Taliaferro, and R.D. Morrison. 1975. Stand Establishment of Bermudagrass from Seed. Agronomy Journal 67:229-232

Alberdi, M.; L.J. Corcuera. 1991. Cold Acclimation in Plants. Review Article 62. Phytochemistry 30(10):3177-318

Anderson, J.A., C.M. Taliaferro, and D.L. Martin. 1993. Evaluating freeze tolerance of bermudagrass in a controlled environment. HortScience 28:955

Anderson, J.A.; C.M. Taliaferro, and D.L. Martin. 2003. Longer Exposure Durations Increase Freeze Damage to Turf Bermudagrasses. Crop Sci. 43:973–977

Ashworth, E.N., and R.S. Pearce. 2002. Extracellular freezing in leaves of freezing-sensitive species. Planta 214:798–805

Beard, J.B. 1966. Direct low temperature injury of nineteen turfgrasses, Q. Bull. Michigan Agr. Exp. Stn. 48, 377-383

Beard, J.B. 1973. Turfgrass: Science and Culture. Prentice Hall, Inc., Englewood Cliffs, N.J. p. 658

Busey, P. 1995. Genetic Diversity and Vulnerability of St. Augustinegrass. Crop Sci. 35:322-327

Busey, P. 2003. St. Augustinegrass. In: M.D. Casler and R.R. Duncan (eds). Turfgrass biology, genetics, and breeding. Wiley, Hoboken, N.J. 309-330

Duble, R.L. 1989. Southern Turfgrasses: Their Management and Use. Published by TexScape, Inc. College Station, Texas. Pp. 335

Duble, R.L. 1996. Turfgrasses, their Management and use in the southern Zone. Second Edition. Texas A&M University press.

Dudeck, A.E.; C.H. Peacock, and J.C. Wildmon. 1993. Physiological and Growth Responses of St. Augustinegrass Cultivars to Salinity. HortScience 28(1):46-48 pp.

Emmons, R.D. 2000. Turfgrass Science and Management. Third edition. Delmar Thomson Learning. NY. P. 528

Ensminger, I.; F. Busch, F.; and N.P.A. Huner. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Review. Physiologia Plantarum 126: 28–44

Fry, J.D., N.S. Lang, and R.G.P. Clifton. 1991. Freezing resistance and carbohydrate composition of 'Floratam' St. Augustinegrass. HortScience 26:1537-1539

Fry, J. and B. Huang. 2004. Applied turfgrass science and physiology. Hoboken, N.J. p. 310

Fujikawa, S; Y. Jitsuyama, and K. Kuroda. 1999. Determination of the Role of Cold Acclimation-Induced Diverse Changes in Plant Cells from the Viewpoint of Avoidance of Freezing Injury. J. Plant Res. 112: 237-244

Gray,G.R., Louis-Pierre Chauvin, F. Sarhan, and N. P.A. Hune. 1997. Cold Acclimation and Freezing Tolerance. A Complex Interaction of Light and Temperature. Plant Physiol. 114: 467-474

Green, R.L., A.E. Dudeck, L.C. Hannah, and R.L., Smith. 1981. Isoenzyme Polymorphism in St. Augustinegrass. Dep. of Omamental Horticulture, IFAS, Univ. of Florida, Gainesville, FL 32fi11. Published with the approval of the Director of the Florida Agric. Exp. Stn. as Journal Series Paper No. 2479

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annual Review of Plant Physiology and plant Molecular Biology 41:187-223

Harrison, J., C. Tonkinson, C. Eagles, and C. Foyer. 1997. Acclimation to freezing temperatures in perennial ryegrass (*Lolium perenne*). Acta Physiologiae Plantarum (APP) 19(4):505-515

Hoffman,L., M. DaCosta, J.S. Ebdon, and E. Watkins. 2010. Physiological Changes during Cold Acclimation of Perennial Ryegrass Accessions Differing in Freeze Tolerance. Crop Sci. 50:1037–1047

Hoppe, D.C., M.E. McCully, and C.L.Wenzel. 1986. The nodal roots of Zea: their development in relation to structural features of the stem. Canadian Journal of Botany 64: 2524-2537

Johansen DA. 1940. Plant microtechnique. New York/London: McGraw-Hill Book Co. p.

Li, R.; R.Qu, A.H. Bruneau; and D.P. Livingston. 2010. Selection for freezing tolerance in St. Augustinegrass through somaclonal variation and germplasm evaluation. Plant Breeding 129:417-421

Livingston, D.P. III; C.R. Olien, and R.D. Freed. 1989. Sugar Composition and Freezing Tolerance in Barley Crowns at Varying Carbohydrate Levels. Crop Sci. 29:1266-127

Livingston, D.P., III, S.P. Tallury, S. Owens, J.D. Livingston, and R. Premakumar. 2006. Freezing in nonacclimated oat: thermal response and histological observations of crowns during recovery. Can. J. Bot. 84: 199–210

Livingston, D.P., III, S.P. Tallury, R. Premakumar, S. Owens, and C.R. Olien. 2005a. Changes in the histology of cold-acclimated oat crowns during recovery from freezing. Crop Sci. 45: 1545– 1558

Livingston, D.P., S. P. Tallury, R. Premkumar, S. A. Owens, and C. R. Olien. 2005b. Changes in the Histology of Cold-Hardened Oat Crowns during Recovery from Freezing. Crop Sci. 45:1545–1558

Livingston, D.P., T. D. Tuong, C. H. Haigler, U. Avci, and S. P. Tallury. 2009. Rapid Microwave Processing of Winter Cereals for Histology Allows Identifi cation of Separate Zones of Freezing Injury in the Crown. Crop Sci. 49:1837–1842

Maier, F. P., N. S. Lang, and J.D. Fry. 1994a. Evaluation of an electrolyte leakage technique to predict St. Augustinegrass freezing tolerance. HortScience 29(4):316–318

Maier, F. P., N. S. Lang, and J.D. Fry. 1994b. Freezing tolerance of three St. Augustinegrass cultivars as affected by stolon carbohydrate and water content. J. Amer. Soc. Hort. Sci. 119:473-476

Mauseth, J.D. 1988. Plant Anatomy. Reprint of first edition. Menlo Park, Calif. Benjamin/Cummings Publ. Co. p. 560

Moseley, D., A. Patton, J. Trappe. 2010. Leaf and stolon characteristics of commercially available and experimental St. Augustinegrass cultivars. Arkansas Turfgrass Report 2009, Ark. Ag. Exp. Stn. Res. Ser. 579:64-68

Olien, C.R. 1961. A method of studying stresses occurring in plant tissue during freezing. Crop Sci. 1:26–28

Pearce, R.S., and M.P. Fuller. 2001. Freezing of barley studied by infrared video thermography. Plant Physiol. 125:227–240

Patton, A.J. and Z.J. Reicher. 2007. Zoysiagrass Species and Genotypes Differ in Their Winter Injury and Freeze Tolerance. Crop Sci. 47:1619-1627

Philley, H.W., C.E. Watson Jr., J.V. Krans, J.M. Goatley Jr., and F.B. Matta. 1995. Differential thermal analysis of St. Augustinegrass. HortScience 30:1388-1389

Philley H, Watson C, Krans J, Goatley J, Maddox V, and Tomaso-Peterson M. 1998. Inheritance of cold tolerance in St. Augustinegrass. Crop Sci. 38: 451—454

Qian, Y.L. , S. Ball, Z. Tan, A.J. Koski, and S.J. Wilhelm. 2001. Freezing Tolerance of Six Cultivars of Buffalograss. Crop Sci. 41:1174–1178

Ruzin, S.E. 1999. Plant microtechnique and microscopy. p. 2.Oxford Univ. Press, New York.

Santos, A.G., D. P. Livingston III, E. N. Jellen, D. R. Wooten, and J. P. Murphy. 2006. A Cytological Marker Associated with Winterhardiness in Oat. Crop Sci. 46:203–208

Shahba, M.A., Y. L. Qian, H.G. Hughes, A. J. Koski, and D. Christensen. 2003. Relationships of Soluble Carbohydrates and Freeze Tolerance in Saltgrass. Crop Sci. 43:2148-2153

Shane, M.W., M.E. MsCully and M.J. Canny. 2000. The Vascular System of Maize Stems Revisited: Implications for Water Transport and Xylem Safety. Annals of Botany 86: 245-258

Stavang, J., M. Hansen, and J.E. Olsen. 2008. Short term temperature drops do not enhance cold tolerance. Plant Growth Regul 55:199–206

Stier, J.C., D. L. Filiault, M. Wisniewski, and J. P. Palta. 2003. Visualization of freezing progression in Turfgrassses using infrared video thermography. Crop Sci. 43:415–420

Stier, J.C.; and S. Fei. 2008. Cold-Stress Physiology and Management of Turfgrasses. In: Handbook of Turfgrass Management and Physiology. Edited by Mohammad Pessarakli. CRC Press. Taylor & Francis Group. Az. p. 473-495

Turgeon, A.J. 1999. Turfgrass Management, Fifth Edition. Prentice Hall. Upper Saddle River, NJ. 392 p

Webb,M.S.; M. Uemura, and P. Steponkus. 1994. A Comparison of Freezing lnjury in Oat and Rye: Two Cereals at the Extremes of Freezing Tolerance. Plant Physiol. 104: 467-478

Wilson, C.A., J.A. Reinert, and A.E. Dudeck. 1977. Winter survival of St. Augustinegrasses in north Mississippi. Quarterly News Bul. Southern Turfgrass. Assn. V. 12:20

Wood, G.M. and R.P. Cohen. 1984. Predicting cold tolerance in perennial ryegrass from subcrown internode length. Agronomy Journal 76(4):516-517