ABSTRACT

LI, RUYU. St. Augustinegrass Improvement for Freezing Tolerance and Semi-Dwarf growth Habit. (Under the direction of Dr. Rongda Qu).

The sod industry is an important sector of the North Carolina turfgrass industry. A significant crop produced by the industry and sold in many states is 'Raleigh' St. Augustinegrass, which was developed by North Carolina State University in the 1980's. It is a popular cultivar and is known to be one of the most freezing tolerant among St. Augustinegrass cultivars. However, like most St. Augustinegrass cultivars, Raleigh has broad leaf blades, long internodes, and coarse plant architecture. The NC sod industry demands new St. Augustinegrass cultivars with further improved freezing tolerance and finer plant architecture (semi-dwarf growth habit). The objective of this project was to use various approaches, including germplasm collection, induction of somaclonal variations, and irradiation mutagenesis, to create variations and to screen for plant clones with improved freezing tolerance and/or semi-dwarf growth habit.

Techniques were developed to achieve these goals, which include establishment of a freezing tolerance test system under controlled conditions and improved tissue culture conditions. It was found that treating plants at 13ºC for a week, followed by 3ºC for another week was an effective condition for cold acclimation of St. Augustinegrass in a controlled environment for freezing tolerance test at or around -4°C for 3 hrs. An efficient tissue culture system was established for the somaclonal variation approach. In that effort, 11 explant tissues and four callus culture media were examined for tissue culture response. The best response came from

immature embryo 7-14 days after pollination (DAP) on Murashige and Skoog (MS) medium containing 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} 6-benzyladenine. The callus induction and regeneration rates were 97.7% and 47.6%, respectively.

Thirty six germplasm accessions were collected. Nearly 8000 plants were regenerated through tissue culture, and 3300 plants recovered from mutagenized plant materials. They were screened for improved freezing tolerance and/or semi-dwarf growth habit. Using the freezing tolerance test system, it was revealed that Elm4, collected from downtown Raleigh, and SVC3, a somaclonal variant, had significantly improved freezing tolerance over cv. Raleigh. Ray, a collection from Polk County, had finer plant architecture and comparable freezing tolerance to Raleigh. In addition, nineteen somaclonal variants from tissue culture and 12 mutants from gamma ray mutagenesis showed semi-dwarf growth habit and grew vigorously. Most of them had shortened internodes and stolons, with some having finer leave texture. Eleven of these lines were subjected to field trials for development of potential new cultivars.

ST. AUGUSTINEGRASS IMPROVEMENT FOR FREEZING TOLERANCE AND SEMI-DWARF GROWTH HABIT

By
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BIOGRAPHY

Ruyu Li was born in Inner Mongolia in western China. She obtained her Bachelor of Science degree with a major in plant pathology at Shenyang Agriculture University, Shenyang, China, in 1994. After graduation, she worked in the Institute of Forestry, the Chinese Academy of Forestry for four years. With working on cell culture for taxol production, she became interested in plant cell and tissue culture. In 1998, she entered Chungbuk National University, Chungbuk, Korea, and received a Master of Science degree majoring in plant cell and tissue culture in 2000. She joined Dr. Rongda Qu's laboratory at Department of Crop Science, North Carolina State University, in 2003 to work on the current project. She is expected to complete her study for Degree of Doctor of Philosophy and graduate in August, 2007.

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INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a widely distributed coastal pioneer species in the tropics and subtropics, and an important turfgrass in those areas. It is a coarse-textured, stoloniferious species that produces dense and attractive dark blue-green turf and is well adapted to most soils. The rapidly spreading stolons form a tight canopy, which prevents weed encroachment.

The world's first known recorded planting St. Augustinegrass was on November 11, 1880, as a turf alongside an avenue at A.M. Reed's Mulberry Grove Plantation at Yukno, Florida (Busey, 2003). The origin of St. Augustinegrass is unknown. However, it was hypothesized that St. Augustinegrass was originated at the coastlines and islands of the Indian Ocean, and that European later brought it to the New World during the post-Columbian era (Busey, 2003).

A lack of freezing tolerance is one of the major drawbacks of St. Augustinegrass, compared to other warm-season turfgrasses (Beard et al., 1980). The most known cold-tolerant cultivar of the species is Raleigh (Busey et al., 1982), however, its use is limited to areas that rarely experience temperatures lower than -5ºC. North Carolina is the northern edge of its distribution range, but severe freezing injury may occur during some winters.

Besides a lack of freezing tolerance, the coarse plant architecture of St. Augustinegrass can be a drawback for lawns. The width of leaf blade can be 3 or 4 times wider than bermudagrass [*Cynodon dactylon* (L.) Pers.]. And internode can be 4 or 5 times thicker.

Cultivars with finer leaf textures and greater density can be an appealing feature to turf managers and home owners.

There have been limited breeding activities on St. Augustinegrass because it is a vegetatively propagated crop (Busey, 2003). Cultivar Raleigh was collected from the lawn of Dr. D. Timothy's residence in Raleigh, NC, and developed by Dr. W. B. Gilbert in the early 1980's. However, winter killing is still a major concern for this cultivar in North Carolina. Quite a few cultivars were developed for plant architecture improvement of St. Augustinegrass, such as 'Bitterblue' (Busey, 1986), 'Floratine' (Nutter, 1960) and 'Seville' (Riordan et al., 1980). However, these cultivars lack freezing tolerance. In addition, 'Floratam' was bred to improve resistance against southern chinch bug (*Blissus insularis* Barber) and St. Augustine decline virus (Horn et al., 1973), and now occupies 70% of the lawn area in Florida (Busey, 2003). However, this variety was very sensitive to freezing.

Collection, somaclonal variation from tissue culture, and mutagenesis are means to develop a large pool of genetic variations for selection and new cultivar development, and have been widely used for crop improvement (Chahal and Gosal, 2002). In this research, we have employed these approaches to screen for improved freezing tolerance and semi-dwarf growth habit among St. Augustinegrass plant lines.

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OBJECTIVE

The sod industry is an active and profitable sector of the North Carolina's turfgrass industry. 'Raleigh' is an important cultivar for sod production in NC because of its freezing-tolerance. However, winter killing is still a concern for Raleigh, especially in cold winters. In addition, like most other St. Augustinegrass cultivars, Raleigh has broad leaf blades, long internodes, and is overall considered coarse-textured. The NC sod industry demands new St. Augustinegrass cultivars with improved freezing tolerance and semi-dwarf growth habit. The objective of this project was to use various approaches, including germplasm collection and evaluation, induction of somaclonal variations and irradiation mutagenesis, to create a larger germplasm pool for selection of plant lines with improved freezing tolerance and/or semi-dwarf growth habit.

CHAPTER 1. ESTABLISHMENT OF A FREEZING TEST SYSTEM IN A CONTROLLED ENVIRONMENT

1.1 Introduction

Freezing causes ice formation in intercellular spaces and cell walls in plant tissues. Ice formation can result in multiple type of tissue damage including disruption of membrane structure (Guy, 1990; Webb et al., 1994). Plants can increase their ability to withstand freezing temperatures in response during a period of low but non-freezing temperatures. This process is called cold acclimation (Xin and Browse, 2000). In nature, cold acclimation is initiated by decreasing temperatures in late autumn or early winter. In winter annuals such as cereals and legumes, the primary site for freezing injury and recovery is the crown tissue, and its ability to tolerate freezing and subsequently to recover from whatever injury occurred during freezing determines winter survival of plants (Tanino and Olien, 1981; Livingston et al., 2006).

Freezing tolerance is a quantitative, complex trait, which has polygenic nature with mainly additive gene action (Marshall, 1982; Fowler et al., 1993). Often, tests in multiple locations and years are required to overcome unpredictable freezing test winters (Tcacenco et al., 1989). However, such tests are costly and time-consuming. Consequently, alternative ways to evaluate freezing tolerance have been developed, in which plants are acclimated in a growth chamber, followed by exposure to a range of temperatures in a freeze chamber. Such approaches have been used to evaluate freezing tolerance in turfgrasses. For instance, Fuller and Eagles (1978) reported that controlled freezing of perennial ryegrass seedlings at the two-leaf stage (14 d after sowing) accurately predicted freezing tolerance of mature plants

and ranked the field survival of cultivars. Anderson et al. (1993) evaluated bermudagrass genotypes LT_{50} of bermudagrass genotypes by exposing to freezing temperature in cone-tainers in a freezer following 4 weeks acclimation at 8/2ºC in a controlled-environment.

However, there's no report on acclimating St. Augustinegrass in a controlled environment. A combined approach with plants acclimated in the field and freezing in a controlled freeze chamber have been used in St. Augustinegrass (Maier and Lang, 1994b). They compared the freezing tolerance of Raleigh and Floratam from field-grown stolons, sampled between October and March, and frozen at -4ºC for 1 h. Raleigh exhibited over 60% survival in December and January, whereas Floratam had a survival rate less than 20%. However, field-grown plant is not reproducible and is subject to seasonal constraints.

In addition, electrolyte leakage technique and differential thermal analysis have also been used to predict freezing tolerance (Maier and Lang, 1994a; Fry et al., 1991). But, electrolyte leakage only assesses freeze injury of cells, which could not represent freezing tolerance of whole plant. Therefore, it is necessary to develop a freezing test system using controlled conditions for St. Augustingrass to mimic field acclimation conditions and to select freezing tolerant germplasm.

1.2 Materials and methods

Raleigh was used as a control and to determine treatment conditions. Plants of Raleigh were grown in flat trays (52.07 cm x 25.91cm x 6.03 cm) for 6 months in a greenhouse. Plants were then subjected to different acclimation treatments by placing the trays in a growth

chamber set at 13ºC for 12 h photoperiod or 3ºC for 10 h photoperiod with a light intensity of 285-290 μmol $m^{-2}s^{-1}$. Single node cuttings were collected after two week's acclimation, and soil was washed off with tap water. Leaves and roots were trimmed to 3-4 cm. Fifteen single node cuttings were put in a plastic container (12.7 cm x 6.99 cm x 5.08 cm) for each freezing treatment. The boxes with plants were randomly placed on the same shelf inside a modified commercial freezer with thermocouples inserted in boxes to monitor temperature. Ice shavings were added to each box to promote nucleation and prevent supercooling (Livingston et al., 2006). Freezers were calibrated before each freezing test and programmed to keep the plants at 1°C for an hour, and then lower the temperature at the rate of 1°C/h until it reached the target temperature. Freezer was kept at the target temperature for 3 hours before the temperature was brought up to 3°C at the rate of 2°C/h. Thawed plants were dipped into Daconil® fungicide (Syngenta, Crop Protection, Greensboro, NC) solution (2.63 ml L^{-1}) to prevent fungal diseases, and transplanted into small pots (17.78 cm x 13.34 cm x 6.03 cm). The plants were allowed to grow at 25°C for a month in a greenhouse, and freezing survival was visually assessed on the basis of regrowth of shoot and root. A plant with obvious regrowth was scored as 1 (fully survived). If the shoots stayed green but had no obvious regrowth, the plant was scored as 0.5 (partially survived), and dead plants had a score of 0. Survival rate was calculated as a percentage of surviving plants among the total treated plants.

A preliminary test was conducted to identify the target freezing temperature by freezing the field grown plants at -2ºC, -4ºC, -6ºC and -8ºC for 3 hrs (Livingston et al., 2006). To identify effective acclimation parameters, four treatment combinations were tested, which included

13ºC for 2 weeks, 3ºC for 2 weeks, 13ºC for one week followed by 3ºC for another week, or no acclimation. The test was replicated twice. ANOVA was carried out by using SAS software (ver.9.1, SAS Institute. 2003). When significant differences ($p<0.05$) were observed, the least significant difference (LSD, Steel et al., 1996) test was applied to detect differences between treatments.

1.3 Results

In the preliminary test, -4ºC was selected as the test temperature since all the plants were killed at -6ºC and -8ºC while 90% survived at -2ºC and 33.3% survived at -4ºC. Among the four acclimation treatments, significant higher survival rates (26.7-65%) were obtained with the cold acclimation over the non-acclimated plants (3.3%) (Fig. 1 and Table 1). Plants treated at 13ºC for a week followed by 3ºC for another week had the highest survival rate (65%). Plants acclimated at 3ºC had significant higher survival rates than the non-acclimated plants, but, lower than the treatment of combination of 13ºC and 3ºC. Treating plants only at 13ºC for two weeks did not improve the freezing survival. The combination of 13ºC followed by 3ºC was selected as the acclimation protocol for all subsequent screening of St. Augustinegrass.

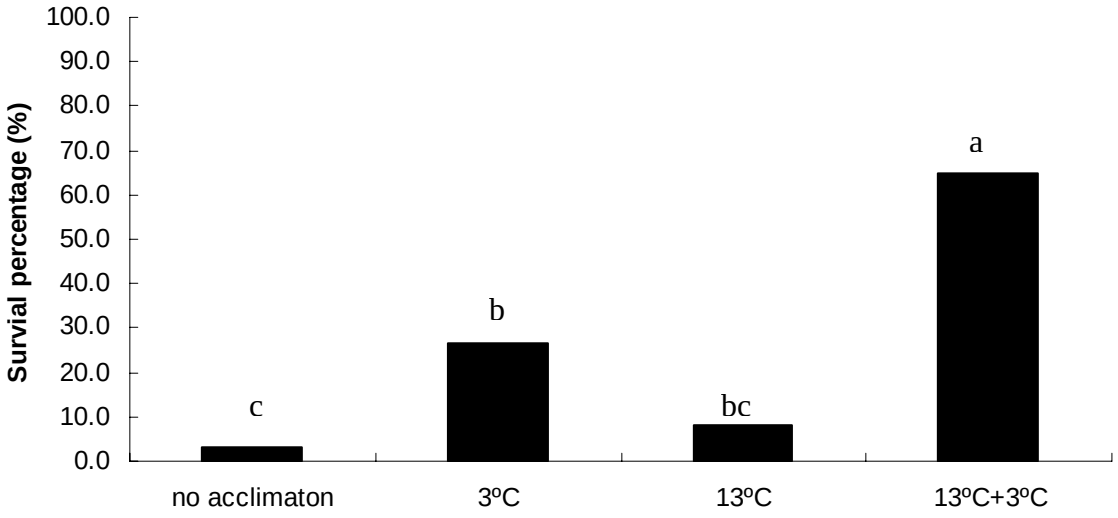

Fig. 1.3. The effect of cold acclimation on the survival of Raleigh St. Augustinegrass six weeks after freezing at -4ºC for 3 hrs. Columns represent means of the two replicates of 15 single cutting nodes per treatment. Values followed by the same letter were not significantly different from each other at the 5% level by LSD.

Table 1.3. ANOVA analysis for cold acclimation effect on the survival of Raleigh St. Augustinegrass six weeks after freezing at -4ºC for 3 hrs.

Source	DF	SS	MS	F Value	Pr > F
Acclimation condition	3	99.33	33.11	25.63	0.0378
Replication	1	0.01	0.01	0.01	0.9323
Error	2	2.58	1.29		
Total	6	101.93			

1.4 Discussion

In previous studies on St. Augustinegrass freezing tolerance, field grown plants with natural acclimation were evaluated for freezing tolerance (Maier and Lang, 1994b). Physiological

assays such as electrolyte leakage and differential thermal analysis were used in other studies to predict freeing tolerance (Fry et al., 1991; Maier and Lang, 1994a; Philley et al., 1995). A two-step acclimation process in a controlled environment was found to have the highest survival rate, and should serve well when screening large number of genotypes in this study. The process consists of acclimating plants at 13ºC for one week followed by additional week at 3ºC.

During cold acclimation, an increase in membrane stability (Cyril et al., 1998; Samala et al., 1998), cold-regulated protein synthesis (Gatschet et al., 1996) among other biochemical changes have been reported to be important in freezing tolerance of turfgrass. It was observed that a slight increase in stolon sucrose levels occurred when Floratam St. Augustinegrass entered dormancy (Fry et al., 1991). Crown tissue is the primary site of freezing injury in cereals (Livingston et al., 2006). Studying biochemical changes of St. Augustinegrass crown during cold acclimation in a controlled environment might provide insight into relationship of biochemical changes to freezing tolerance. In addition, *CBF/DREB1*-like genes which control the expression of a regulon of cold-induced genes that increase plant freezing tolerance were identified in monocot plants such as rice (Dubouzet et al. 2003), wheat (Jaglo et al. 2001), and ryegrass (Tamura and Yamada, 2007). Isolating homologs of these genes from St. Augustinegrass may help to understand freezing tolerance in St. Augustinegrass at molecular level**.**

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CHAPTER 2. GERMPLASM COLLECTION AND EVALUATION FOR FREEZING TOLERANCE AND SEMI-DWARF GROWTH HABIT

2.1 Introduction

Germplasm collections are excellent resources for genetic variation. They can be incorporated into breeding programs by crossing with elite lines or by directly introduced into cultivation (Simpson, 1994; Burow et al., 2001). Some cultivars have a profound effect on global food production and economy. One remarkable case is the wheat 1B/1R translocation, which was identified as a simple transfer between rye and wheat in the former Soviet Union cultivar 'Kaukza'. The translocation carries a number of genes from rye and confers resistance to various diseases and adaptation to marginal environments (Villareal et al., 1991). Over 60 wheat varieties had been incorporated with this translocation. These varieties were grown in over 50% of the wheat production field in the developing countries, almost 40 million hectares (Skovmand et al., 1997).

A small germplasm collection of St. Augustinegrass is preserved in the National Plant Germplasm System (USDA/ARS, 2001). This collection consists of 21 accessions including 14 genotypes that are resistant to southern chinch bug (*Blissus insularis* Barber) and sting nematode (*Belonolaimus longicaudatus*). However, little genetic diversity exists among these accessions (Busey, 1995). Most available sources for breeders are released cultivars and active breeding populations outside national collections (Busey et al., 1982).

St. Augustinegrass is adapted to the U.S. department of Agriculture hardiness zones 8, 9, and 10 (Maier and Lang, 1994). Currently, North Carolina is the north edge of its distribution range. It was expected that plants growing in colder areas might have improved freezing tolerance. Therefore, collections were mainly made in NC and evaluated for freezing tolerance and semi-dwarf growth habit.

2.2 Materials and methods

Germplasm collection: Germplasm collection was solicited to TCNC members and county extension agents for support. When collected, they were transplanted into flat trays (10.16 cm x 35.56 cm x 50.8 cm) with Metro-Mix-200 soil (Scotts, Marysville, OH) and maintained in a greenhouse.

Establishment of a field trial of eight accessions: The freezing survival rates of germplasm accessions Co2, Ray, WS, Elm2, Elm4, and Craig were found to be higher than, or comparable to, Raleigh in the preliminary freezing test at -4ºC. To evaluate their field performance, a formal field trial was established at Lake Wheeler Turf Field Lab for these six accessions along with Raleigh (as a freezing tolerant standard) and Floratam (as a freezing sensitive control) on July 1, 2005. A randomized complete block design with three replicates was used in the field trial (Fig 2.2a). The soil was fine sandy loam with pH 6.0. Twenty-four stolons with 3 or 4 nodes for each accession were planted in a plot (1.22 m x 1.83 m). Plots were irrigated three times daily for the first month to accelerate establishment and then irrigated daily to prevent dryness. Forty-eight kg ha^{-1} of nitrogen (34-0-0; N-P_2O_5-K_2O%) was applied on August 2 for faster establishment. Fungicide Heritage (Azoxy Strobin:

Syngenta) was sprayed at a rate of 15.8 g/100 m^2 when gray leaf spot (*Pyricularia grisea*) appeared. Plot borders were trimmed monthly to prevent plants from growing into neighboring plots.

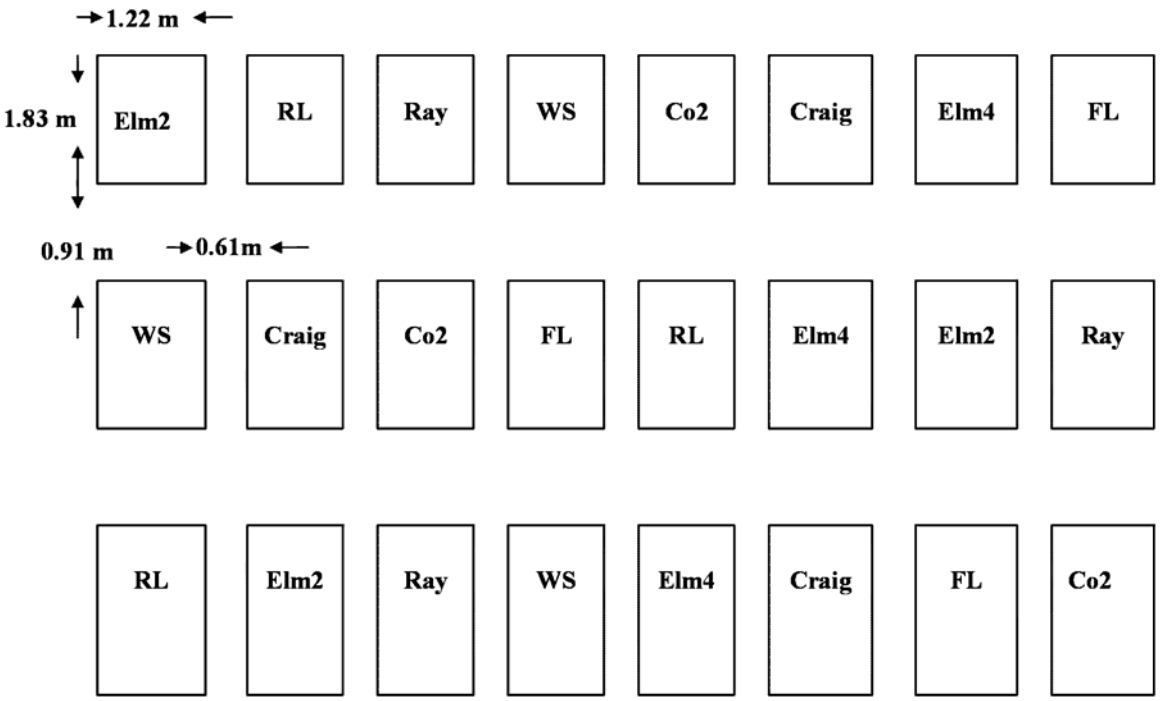

Fig 2.2a. Field trial of eight germplasm accessions with a randomized complete block design.

The ground coverage was monitored during the establishment from August 1 to September 26, 2005, using digital image analysis techniques (Fig. 2.2b), in which the color threshold feature in the SigmaScan Pro (v. 5.0, Systat, Inc., Richmond, CA 94804) software allows the user to search a digital image for a specific color or a range of color tones which could distinguish the green color of plant tissues from the brown color of soil surface by adjusting hue and saturation. Pictures were taken at 3:00-4:00 pm weekly with a Nikon E4300 digital camera (Nikon, Japan) mounted on a monopod to insure shooting from a consistent height

(Fig. 2.2b A). To selectively identify green leaves in the images, hue range was set from 42 to 100 and a saturation range from 3 to 100 (Fig. 2.2b B). After developing a fingerprint of the green areas of the image (Fig. 2.2b C & D), the total selected green pixels were counted by the software package. The number of the green pixels in each image was then divided by the total pixel count of the image to determine ground coverage percentage in the image.

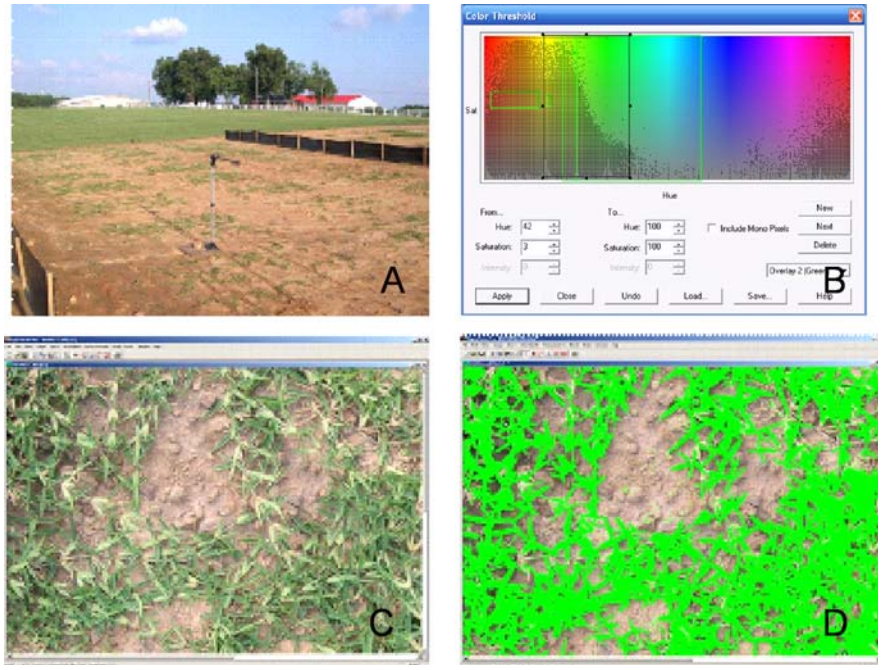

Fig. 2.2b. Ground coverage percentage analysis by SigmaScan Pro. A. Photo-taking with a digital camera mounted on a monopod; B. Adjusting hue and saturation to identify green leaves in the image; C & D. A green coverage before and after pixilation.

Freezing test of the eight germplasm accessions: The freezing test was conducted in mid-December, 2005, after plants had experienced natural acclimation. Single node cuttings of the plants were collected from the above established field plots, washed, and trimmed. Fifteen cuttings from each accession were used as replicates and were frozen at -4ºC for 3 hrs. The survival rates were calculated as described in Chapter 1. The test was repeated 3

times, and the data were statistically analyzed by ANOVA. Differences between accessions were evaluated by an LSD test at the 5% level.

Based on the initial freezing test, accessions Ray, Elm4, and WS exhibited freezing tolerance higher than, or comparable to, Raleigh, and were further tested after acclimation under controlled conditions. Stolons were grown in the greenhouse for two months. Single node cuttings of similar size were collected and grown for a week, and then subjected to acclimation and freezing. However, considering the younger age of plant materials, they were frozen at -3ºC instead of -4ºC. This test was replicated 4 times with 20 single node cuttings for each replicate, and the results were statistically analyzed by ANOVA. Differences between accessions were evaluated by an LSD test at the 5% level.

Morphological characterization of the accessions: Besides visual selection, measurements were conducted for the third internode length in the winter of 2004 for 24 germpalsm accessions collected by that time. Morphological characteristics of accessions Elm4, Ray, WS, together with Raleigh, were further investigated on September of 2006. Four to five stolons with 3-4 nodes were collected and transplanted into a flat (62 cm x 26 cm x 6 cm) containing Metro-Mix-200 soil (Scotts) and fertilized with ~12 g Osmocote (16-4-8; N-P_2O_5-K_2O %) per flat. The flats with stolons were maintained in the greenhouse for a month until plants were well established. The flats were then transferred to the field at the Lake Wheeler Turf Field Lab. The ground was covered with weed mat to prevent stolons from growing into the soil. Plants were irrigated twice a day. Heritage was applied at 15.8 g/100 m^2 when gray leaf spot disease occurred. After two months, the longest stolon in each flat was measured for

morphological characters which included: the third node leaf sheath length, the third internode length and thickness, stolon length (the length from the shoot tip to the fourth node), inflorescence length, culm length, florets per inflorescence, inflorescence number per flat and plant height at maturation. Each flat was considered a replicate. Nine replicates were measured for each accession, and statistical analysis was performed by ANOVA and LSD at the 5% level.

2.3 Results

Germplasm collection: Substantial efforts were spent to collect St. Augustinegrass germplasm within North Carolina and in the Southeast U.S. with courtesies of our colleagues, extension agents, and turf industry supporters. Since Jan 2003, a total of 36 germplasm accessions were collected from Florida, California, North Carolina, Texas, Tennessee**,** South Carolina, and Virginia, with a majority from NC. The complete collections are listed in Table 2.3a.

Table 2.3a. The list of germplasm collections.

	Name	Location	Note
1	313 W	313 Waldo St. Cary, NC	Provided by Jennifer Platt Established over 6 years
2	2825 E	2825 Eastburn, Charlotte, NC	Established 30 years in Charlotte
3	Clemson	Clemson University, SC	Established many years, provided by Dr. Bert McCarty
4	Riverside	1012 Riverside Ave, Elizabeth City, NC	Established several years ago
5	Craig	1100 Riverside Ave (named as Craig & Foreman), Elizabeth City, NC	It is probably Raleigh
6	Roanoke	Roanoke Bible College, Elizabeth city, NC	Unknown clone, long time established
7	718 G	718 Avondale St. Gastonia, NC,	Established for over 30 years
8	3129 64 A	3129 64 Alternate, Nash County, NC	Unknown
9	Ro	Original Raleigh: Raleigh, NC	In the front yard of Dr. David Timothy's house
10-16	Cl-C7	NCSU turf field lab: Raleigh, NC	Seven clones still remained green in late Dec.2002
17	Elm2	200 Elm St., Raleigh downtown, NC	In Raleigh downtown, found by Dr. Charles Peacock
18	Elm4	408 Elm St., Raleigh Downtown, NC	Found by Dr. Art Bruneau and Dr. Charles Peacock
19	Jones St.	503 Jones St., Raleigh Downtown, NC	Found by Dr. Charles Peacock
20	1972 W	1972 Western Blvd, Raleigh, NC	Found by Dr. Charles Peacock
21	T644	Tennessee (north of Nashville),	Provided by Dr. Wayne Hanna
22	T638	Halletteville, TX	Provided by Dr. Wayne Hanna
23	T672	Halletteville, TX	Provided by Dr. Wayne Hanna
24	1800S	1800 S. Woodside Lane,VB, VA	In Virginia beach 20 years old , info from Tom Tracy

Table 2.3a. Continued.

	Name	Location	History
25	418 D	418 Discovery Rd., VB, VA	Installed 8 years, info from Tom Tracy
26	421 D	421 Discovery Rd., VB, VA	Established for 5 years, info from Tom Tracy
27	WS	1525 Bunkerhill_Sandy Ridge Rd, Kernersville, NC	Near Winston-Salem, established over 40 years.
28	LA	Los Angles, CA	I collected from a home lawn in Los Angles
29	FL	Belle Glade, Florida	Floratam cultivar provided by Dr. Russell Nagata
30	Ray	Columbus, NC	Established over 42 years provided by John Vining (agent) and Ray Skipper (lawn owner)
31	Co2	Columbus, NC	Established 25-30 years in Harry Goodheart's lawn, info from John Vining
32	Pineview	1508 Pineview Dr., Raleigh, NC	In Dr. Don Morland's home lawn for over 30 years
33	Country club	1504 Country Club, High Point, NC	Established near 30 years
34	Laurel Hills	4200 Laurel Hills, Raleigh, NC	Established for 40-50 years, info from Mr. Matthew Marttin
35	Barbara	1040 Barbara Dr., Garner, NC	Not known
36	Stockton	5104 Stockton, Raleigh, NC	I collected them and was told it was originally from Wilmington

Establishment of a field trial for eight accessions: A formal field trial was conducted for germplasm accessions Co2, Ray, WS, Elm2, Elm4, and Craig, which showed survival rates higher than, or comparable to, Raleigh among all the accessions in the preliminary screening. During the period of establishment, the ground coverage percentage was digitally imaged and analyzed by SigmaScan Pro software. All the germplasm accessions had comparable ground coverage to Raleigh during the time course (Fig. 2.3a & b). The ground coverage of Raleigh reached 22% after a month. The coverage increased linearly afterwards, with an increase of 8% per week until September 26, 2005, when it reached 82%. The ground coverage did not increase further since the temperature started to drop in late September and plants stopped growing.

Fig. 2.3a. Field establishment of eight germplasm accessions in 2005.

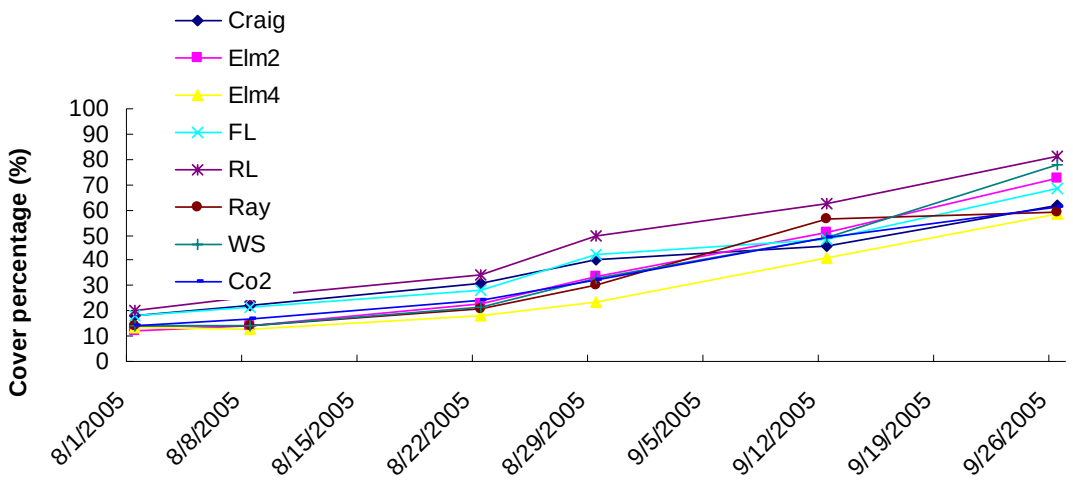

Fig. 2.3b. The ground coverage of eight accessions during establishment in 2005. Symbols represent means of the three replicates of each accession. Statistical analysis was performed and no significant difference among accessions was detected at the 5% level by ANOVA.

Evaluation of freezing tolerance of eight germplasm accessions: To investigate freezing tolerance of the field grown accessions, naturally cold-acclimated single node cuttings were frozen and differences in survival were evaluated. It was found that Elm4 had the highest survival rate (73%) and was significantly higher than Raleigh (51%) among the eight germplasm accessions (Fig. 2.3c and Table 2.3b). Co2, Ray and WS showed comparable survival rates to Raleigh. The survival percentage of Craig (35.7%), and Elm2 (29.0%) and Floratam (2.3%)were significantly lower than Raleigh.

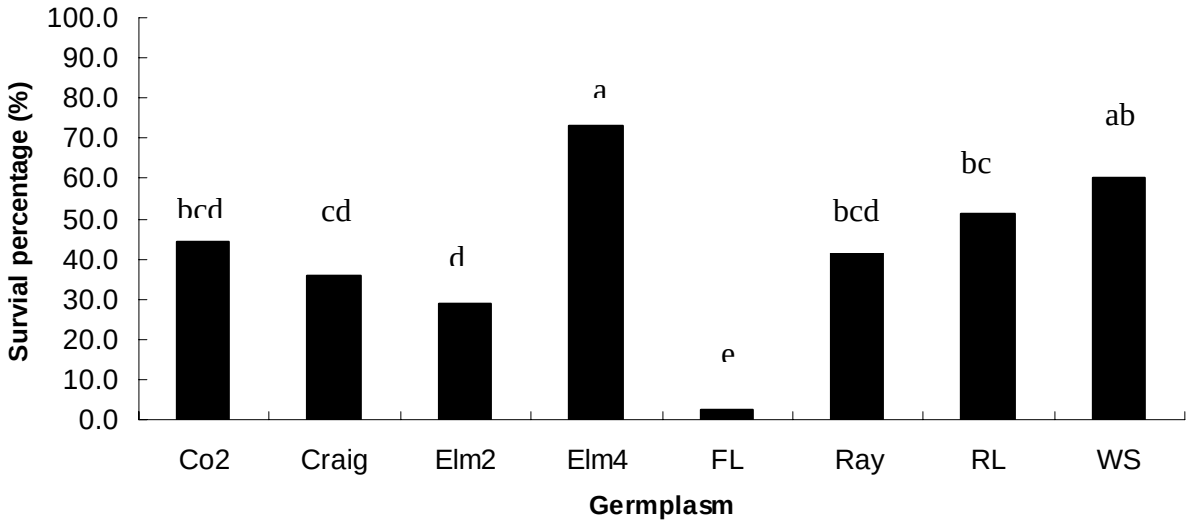

Fig. 2.3c. Survival percentage of eight accessions frozen at -4°C for 3 hrs after field acclimation. Columns represent means of three replicates of 15 single cutting nodes of each germplasm. Values followed by the same letter were not significantly different from each other at the 5% level by LSD.

Table 2.3b ANOVA analysis for survival percentage of eight accessions frozen at -4°C for 3 hrs after field acclimation.

Sources	DF	SS	MS	F Value	Pr > F
Germplasm	**7**	**219.96**	**31.42**	**10.56**	**0.0001**
Replication	**2**	**8.33**	**4.17**	**1.40**	**0.2791**
Error	**14**	**41.67**	**2.98**		
Total	**23**	**269.96**			

Based on the above freeze test results, collections Elm4, WS, and Ray were further tested after acclimation under controlled conditions. Because cuttings from these plants had a shorter period of growth and may not be hardy enough, they were frozen at -3ºC instead of -4ºC. Significant differences were observed among the accessions (Fig. 2.3d) and the ranking

of genotypes was consistent with those of the field-acclimated plants (Fig. 2.3c and Table 2.3b). The survival percentage of Elm4 was still the highest (41.3%), significantly higher than Raleigh (27.5%). The survival rates of Ray (33.8%) and WS (22.8%) were similar to Raleigh, whereas only 1.3% Floratam plants survived.

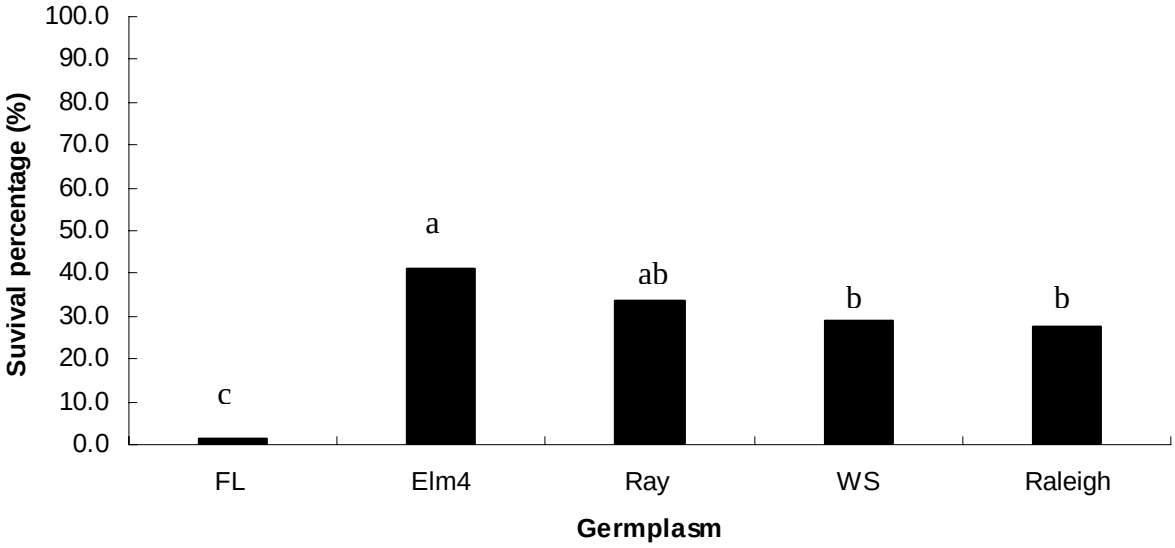

Fig. 2.3d. Survival percentage of five accessions frozen at -3ºC for 3 hrs after acclimation in the controlled environment. Columns represent means of the four replicates of 20 single node cuttings for each accession. Statistical analysis was performed among accessions. Values followed by the same letter were not significantly different from each other at the 5% level by LSD.

Table 2.3c. ANOVA analysis of survival percentage of five accessions frozen at -3ºC for 3 hrs after acclimation in the controlled environment.

Source	DF	SS	MS	F Value	Pr > F
Germplasm	4	146.2	36.55	15.34	0.0001
Replication	3	15.4	5.13	2.15	0.1466
Error	12	28.60	2.38		
Total	19	190.20			

Morphological characteristics of the germplasm collections: Internode length was measured in winter of 2004. Raleigh had the internode length of 3.72 ± 0.44 cm. No significant difference was observed on internode length among the 24 accessions collected by that time (Fig. 2.3e).

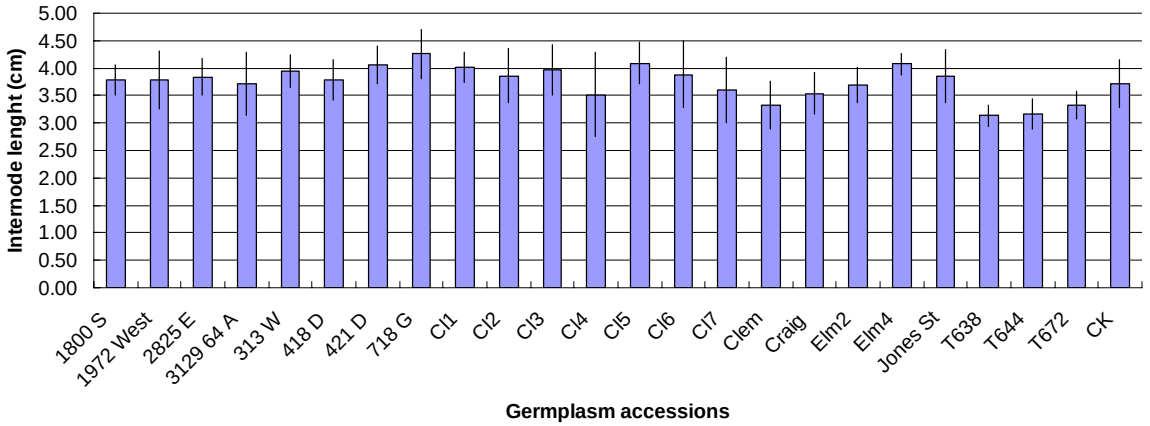

Fig. 2.3e. The internode length of 24 germplasm accessions in Nov. 2004 at Lake Wheeler Turf Field Lab. Each column represents means of the 20 stolons from each accession, and the bar represents the standard deviation.

Stigma color difference was noted among the accessions. Ten accessions (LA, FL, Ray, Jones St, 2825 E, 718 G, 1972 West, Craig, Country Club, and Laurel Hills) had purple stigmas whereas the other 26 accessions had white.

Further morphological measurements were performed in 2006 for germplasm accessions Elm4, Ray, and WS since they had better or comparable freezing tolerance to Raleigh in the tests, which may have potential for new cultivar development. Elm4 and WS did not show significant difference from Raleigh in nine morphological traits measured (internode diameter, internode length, stolon length, sheath length, culm length, inflorescence length,

plant height at maturity, floret number per inflorescence, and inflorescence number) (Table 2.3d). However, Ray had significantly shorter and thinner internodes. In addition, its inflorescence length and height at maturity were also significantly shorter than Raleigh. Leaf blade was not measured this time in the field. In a measurement taken in a greenhouse, lengths of leaf blade were about 1/3 shorter than the Raleigh's. However, during that measurement, no significant difference was observed for internode thickness, and internode and stolon lengths (Table 2.3b). In addition, stigma color of Ray (purple) was also different from Raleigh (white).

Table 2.3d. Morphological characteristics of Elm4, Ray and WS. Each value represents means of nine replicates. Statistical analysis was performed among the genotypes. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also shown.

Genotypes	ID† (mm)	IL (cm)	SL (cm)	SSL (cm)	CL (cm)	InL (cm)	MH (cm)	FI	IN
Elm4	3.62 [a]	4.56 [a]	15.21 [a]	2.49 [a]	7.01 [a]	12.34 [a]	15.83 [a]	22.11 [a]	31.33 [a]
Ray	3.28 [b]	3.84 [b]	13.03 [b]	2.16 [b]	6.79 [a]	10.87 [b]	13.61 [b]	22.33 [a]	29.33 [a]
WS	3.67 [a]	4.20 [ab]	13.54 [ab]	2.43 [a]	7.10 [a]	12.81 [a]	16.76 [a]	23.25 [a]	54.22 [a]
Raleigh	3.49 [a]	4.42 [a]	16.39 [a]	2.46 [a]	7.45 [a]	13.55 [a]	16.63 [a]	20.75 [a]	48.67 [a]
CV%	6.53	14.22	10.77	12.07	11.52	14.62	11.53	17.08	48.27

†ID: Internode diameter; IL: Internode length; SL: Stolon length; SSL: Sheath length; CL: Culm length; InL: Infloresence length; MH: Height at maturity; FI: Florets no. per inflorescence; IN: Inflorescence number.

Table 2.3e. Morphologyical characteristics of Ray grown in greenhouse. Each value represents means of eight stolons. Statistical analysis was performed among genotypes. Values followed by the same letter were not significantly different from each other at the 5% level by t-test. Coefficient of variance is also shown.

Genotype	LL(cm)	LW (cm)	ID (mm)	IL (cm)	SL (cm)	SSL (cm)
Ray	1.35 [b]	0.51 [a]	2.89 [a]	5.48 [a]	17.74 [a]	2.39 [a]
Raleigh	1.99 [a]	0.64 [a]	3.12 [a]	6.74 [a]	23.06 [a]	3.38 [a]
CV%	11.54	8.70	4.18	14.49	5.24	12.80

†LL: Leaf blade length; LW: Leaf blade width; ID: Internode diameter; IL: Internode length; SL: Stolon length; SSL: Sheath length.

2.4 Discussion

Germplasm collection and evaluation can be an effective way for developing new cultivars. For example, two major cultivars, Floratam, the cultivar with the largest acreage in Florida (Busey, 2003), and Raleigh, the most cold tolerant cultivar, were both selected out of the collections (Maier and Lang, 1994). In this project, germplasm collections were an important genetic source for selection. A total of 36 accessions were collected, and two accessions, Elm4 and Ray, with improved freezing tolerance or plant architecture were identified. In freezing test of the accessions, two types of acclimation (natural acclimation in the field and two-step acclimation in controlled environment) were applied, and the ranking of the accessions were consistent: Elm4 demonstrated higher survival than Raleigh, and Ray was similar to Raleigh. Elm4 had 73% survival from the field grown plants while Raleigh was 51%. However, Elm4 had only 41.3% survival from plants acclimated in the controlled environment while Raleigh had 27.5%. The discrepancy between the two treatments was most likely because higher light level, better light quality and optimum sequence of temperatures in field may have prepared plants better for freezing stress. Although Ray had similar freezing tolerance to Raleigh, it had somewhat finer plant architecture. Both Elm4 and Ray have potential for new cultivar development.

The eight germplasm accessions, including Elm4 and Ray, all survived well in the field trial during the winters of 2005 and 2006 at Lake Wheeler Turf Field Lab, probably due to mild temperatures in the two winters. In the field trial, digital image technique, which has been used successfully to quantify turfgrass coverage (Richardson et al., 2001) and color (Karcher

and Richardson, 2003), was used to monitor ground coverage. It was effective for quantifying the ground coverage of St. Augustinegrass. By using this technique, it was found that the ground coverage of Elm4 and Ray was similar to Raleigh (Fig. 2.3c), which suggested the growth and establishment of the two accessions were not affected while having improved cold tolerance or finer plant architecture.

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CHAPTER 3. IMPROVEMENT OF TISSUE CULTURE RESPONSE OF ST. AUGUSTINEGRASS

An article published in Plant Breeding 125: 52-56, 2006.

Improved plant regeneration and *in vitro* somatic embryogenesis of St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze]

St. Augustinegrass is widely used as a lawn and pasture grass in warm, subtropical, and tropical climate regions (Busey 2003). The propagation is usually vegetative, by stolon cuttings, plugs, and sod (Busey and White 1993). Most studies of St. Augustinegrass have been focused on genetic diversity, conventional breeding, and the responses of cultivars to the biotic and abiotic stresses (Busey 2003). Despite the importance of St. Augustinegrass as a turf and pasture grass species, its tissue culture response has not been intensively studied. Only one report dealing with the topic has been published thus far (Kuo and Smith 1993). In that article, immature embryos of cultivar 'Texas Common' were cultured on MS medium (Murashige and Skoog 1962) containing 1 mg/l 2,4-D, followed by 4 weeks culture with 0.5 mg/l 2, 4 D plus 0.25 mg/l kinetin. Approximately one third of the induced calli were able to regenerate into plantlets. Successful tissue culture is often a prerequisite for plant improvement via genetic transformation (Birch 1997). Plant tissue culture is also an effective means to induce somaclonal variations for crop improvement (Brown and Thorpe 1995). In order to broaden the opportunities for genetic manipulation of St. Augustinegrass through tissue culture, we have tested various explant tissues and culture media and have studied the

developmental pathway of its *in vitro* regeneration. The results are reported in this correspondence.

Material and Methods

Plant materials: Cultivar Raleigh (Busey et al. 1982) was used in the study. Eleven types of explants including young shoot, young leaf, young leaf sheath, nodal segment, root, young inflorescence, early immature embryo, immature embryo, young shoot base, mesocotyl, and anther were tested for their tissue culture response. Most of the explants were randomly collected from an experimental plot on campus except for the young shoot base and mesocotyl, which were collected from the germinating embryos in culture medium.

Tissue culture: Stolons were collected from the field and washed with tap water. The materials were rinsed with 70% ethanol for 30 seconds, followed by sterilization in 50% Clorox (6% sodium hypochlorite, Clorox, Oakland, CA, USA) for 30 min (20 min under vacuum, and 10 min with stirring), and then five time rinses with sterile distilled water. Young shoots, young leaves, young leaf sheaths, nodal segments and roots were separated and sliced into 0.5 to 1 cm segments. Young inflorescences, 1.0 to 4.5 cm in length, were also used in the study after being sliced into 0.2-0.3 cm segments. "Early immature embryos" were collected from seeds approximately 3 days after pollination when the endosperm was in a watery stage. "Immature embryos" were collected 7 to 14 days after pollination when the endosperm was in the milky stage. Young shoot bases and mesocotyls (0.7 to 1 cm in length) were sliced from 7-day-old seedlings germinated from immature embryos in dark on MS medium without hormone supplements. For anther culture,

inflorescences with microspores in uni-nucleate stage were collected and were cold treated at 4°C for a week (Nitsch and Norreel 1973) before the anthers were excised and cultured on callus induction medium.

The callus induction/subculture was on the MS basal medium (Caisson Laboratories, Rexburg, ID, USA), with 30 g/l sucrose, 3.2 g/l Phytagel, and 1 mg/l 2, 4-D, either alone or in combination with 0.01 or 0.1 mg/l BA. The regeneration medium was MS medium supplemented with 1 mg/l BA, 0.2 mg/l NAA, and 0.5 mg/l GA. The best medium treatment reported for St. Augustinegrass tissue culture by Kuo and Smith (1993) was used in the experiments as a reference. In that treatment, MS medium supplemented with 1 mg/l 2, 4-D was used for callus induction, MS medium supplemented with 0.5 mg/l 2, 4-D and 0.25 mg/l kinetin for subculture, and MS medium with 0.25 mg/l 2, 4-D and 0.5 mg/l kinetin for regeneration. The rooting medium used for all the treatments was the same, i.e., half strength MS medium with no addition of phytohormones. The pH of all media was adjusted to 5.8 with 0.2 N KOH prior to autoclaving. All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except otherwise specified. The culture medium was autoclaved at 121°C for 25 min. The sterile growth regulator solutions (BA, GA, kinetin, and NAA) were added to the autoclaved media after they cooled down to 50°C.

For callus induction, the explants were cultured for 4 weeks and subcultured for an additional 4 weeks on callus induction medium. The cultures were maintained in the dark at 25°C in a culture chamber (I-36NL, Percival Scientific, Boone, IA, USA). For regeneration, all the calli of eight-week-old were transferred onto the regeneration medium and maintained in a lighted

culture chamber (CU-32L, Percival) with a 16-h photoperiod (140 μmol/m^2/s cool white fluorescent irradiance) for three weeks at 25°C. At the end of this period, callus with clearly differentiated shoots was scored as 'regenerating' callus. Each piece of regenerating callus was counted as one regardless the number of shoots. The regeneration rate of a treatment is presented as the percentage of regenerating calli out of the total number of the induced calli. Regenerated plantlets were transferred to the rooting medium and placed in a lighted chamber. Once a substantial root system was developed, the plant was transplanted to a pot containing Metro-Mix-200 soil (Scotts, Marysville, OH, USA) and kept in a lighted culture room for acclimation. When established, the plantlets were transferred to a greenhouse.

Experimental design and statistical analysis: Completely randomized design was employed in the tissue culture experiments. A total of 42 to 55 explants were used per replicate in each treatment. Each experiment was carried out with three replicates except for the BA concentration comparison experiment (between 0.1 and 0.5 mg/l), which has two replicates for each treatment. Two factor ANOVA analysis (Steel et al. 1996) was carried out to test for main and interaction effects for explant type and medium by SAS program (SAS Institute Inc. 1999). When a significant difference ($p<0.05$) was observed, the least significant difference test (Fisher LSD, Steel et al. 1996) was applied to detect differences among treatments or means within each type. Most of the work reported here was performed in 2003. The BA concentration comparison experiment between 0.1 and 0.5 mg/l was performed in 2004.

SEM procedure: Callus samples for scanning electron microscopy (SEM) were collected from culture media containing BA (0.01 mg/l) two months after the culture and from the regeneration medium a week after the transfer. Samples were fixed in 6% buffered gluteraldehyde for a week at 4°C, washed in 0.05 M potassium phosphate buffer (pH 6.6) for an hr, dehydrated in a graded cold ethanol series (30%, 50%, 70%, 95% and 100%) for 24 hr each at 4°C, critical point dried in liquid carbon dioxide for 15 min, affixed to aluminum stubs with silver paint and coated with 35 nm gold palladium (Bradley et al. 2001B). The mounted specimens were examined with a Philips 505T scanning electron microscope (JEOL, Peabody, MA, USA) at 15 KV and images were captured digitally.

Results

It has been demonstrated in many cases that 2, 4-D is usually the choice of auxin for callus induction and subculture of grasses (Bhaskaran and Smith 1990, Chaudhury and Qu 2000). Lately, more and more experimental results indicated that the addition of a low concentration of cytokinin, particularly BA, in callus culture medium often enhances callus regeneration (Chaudhury and Qu 2000, Altpeter and Posselt 2000, Cho et al. 2000, Bai and Qu 2001, Bradley et al. 2001A). For some grasses such as St. Augustinegrass and bermudagrass, lower concentrations of 2, 4-D also facilitates later regeneration (Kuo and Smith 1993; Chaudhury and Qu 2000; Li and Qu 2004). Since the optimum 2, 4-D concentration on St. Augustinegrass immature embryo culture has been determined (Kuo and Smith 1993), this project was focused on the effect of addition and concentration of BA in the callus induction and subculture medium and on the explant type used in the experiment. Moreover, we

employed scanning electron microscopy to study the developmental pathway of the regenerated plants.

Three callus induction/subculture media containing 1 mg/l 2, 4-D and 0, 0.01, or 0.1 mg/l BA were designed as medium treatments and were compared to the reported procedure. Eleven types of explants were tested for callus induction. Usually, calli started to appear 3 or 4 days after the explants were cultured. Young shoots, immature embryos (Fig. 3.3a A), young shoot bases, mesocotyls and young inflorescences had much higher callus induction frequencies, ranged from 71% to 100%, whereas no callus was induced from the culture of young leaves, roots, and anthers. In between were nodal segments (50%), early (3 DAP) immature embryos (12-18%), and young leaf sheath (7-13%) on various medium treatments. The calli were often watery and soft and had loose structures (Fig.3.3a B). In some cases, embryogenic calli were observed, which were pale white or yellowish, compact, granular, and friable (Fig. 3.3a C). They were mostly generated from the culture of immature embryos, and shoot bases of the young seedlings. Most of them were formed within 4 weeks. Plantlet regeneration was only observed from calli induced from these three explants.

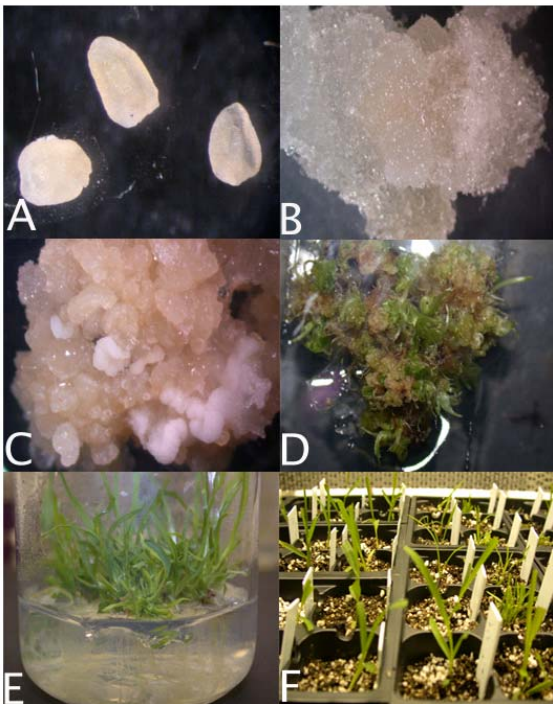

Figure 3.3a. Immature embryo culture of St. Augustinegrass. A. Cultured immature embryos. The bar in the figure represents 0.5 mm. B. Non-embryogenic callus. C. Embryogenic callus. D. Shoot formation from embryogenic callus 3 weeks in regeneration medium. E. Regenerated shoots developed roots in rooting medium. F. Regenerated plantlets growing in soil.

Subsequently, a randomized experiment was conducted to evaluate the BA treatment in callus induction/subculture media using these three explants. Statistical analysis suggested callus induction was highly significantly affected by the explant type, but not affected by the medium treatment or the interaction of the two factors (Table 3.3). In contrast, callus regeneration ability was highly significantly different among the medium, explant type and the interaction of the two (Table 3.3). The best result in the experiment came from the 7-14 DAP immature embryos cultured on medium containing 1 mg/l 2, 4-D and 0.1 mg/l BA, in which callus induction rate was 98.5%, and 40% of the calli were regenerated. The callus regeneration rate of this treatment was significantly higher than other treatments for the two age groups of immature embryos (Table 3. 3B). However, the young shoot bases reacted very

differently. Higher concentration of BA (0.1 mg/l) reduced callus induction significantly, and BA failed to enhance regeneration of callus from this explant type (Table 3.3B).

Although callus from early immature embryos had relatively high regeneration rates, the explant type suffered from low callus induction rate (below 20%). A majority of the explants turned brown within 3 or 4 days in all the treatments before callus formation.

Shoots were developed from calli with compact structures in the regeneration medium. They grew slowly and the young leaves looked curled (Fig. 3.3a D). However, when placed at the rooting medium, the growth of shoots and roots was much accelerated (Fig. 3.3a E). The occurrence of albino plantlets was extremely low, around 0.2%. One month later, the green plantlets were transplanted into soil. They all survived the transplantation and grew well later in the greenhouse and in the field (Fig. 3.3a F). Nearly all of them flowered and had seed setting. However, the seed development deteriorated before maturation, similar to the case of St. Augustinegrass plants in the field.

In the following year, BA concentration effect on embryogenic callus formation was further evaluated in immature embryo (7-14 DAP) culture by comparing 0.1 mg/l and 0.5 mg/l of BA, respectively, in the callus induction/subculture medium. The callus induction rates were 96.5% and 97.7%, respectively, suggesting no harmful effect on callus induction with a higher concentration of BA. Moreover, 0.5 mg/l BA further enhanced callus regeneration (47.6% vs. 27.1% for 0.1 mg/l BA). The difference was significant at $P<0.1$ level.

Table 3.3. Statistical analysis of effects of media and explants in St. Augustinegrass tissue culture.

A. ANOVA analysis

Source	DF	MS	F value	P value
Callus induction				
Medium (M)	3	40.9	1.5	0.2346
Explant type (E)	2	23178.1	861.7	<0.0001
M x E	6	45.7	1.7	0.1647
Callus regeneration				
Medium (M)	3	983.2	20.2	<0.0001
Explant type (E)	2	623.3	12.8	0.0002
M x E	6	188.8	3.9	0.0076

B. LSD test

Explant type	Medium Treatment*	Callus induction rate (%) **	Callus regeneration rate (%)
Early immature embryo (3 DAP)	1	13.9 ^{d}	14.4 ^{bc}
	2	12.1 ^{d}	3.7 ^{cd}
	3	18.7 ^{d}	9.8 ^{bcd}
	4	13.9 ^{d}	38.3 ^{a}
Immature embryo (7-14 DAP)	1	100.0 ^{a}	6.8 ^{cd}
	2	99.2 ^{a}	11.4 ^{bcd}
	3	97.0 ^{a}	18.6 ^{b}
	4	98.5 ^{a}	40.0 ^{a}
Young shoot base	1	84.8 ^{b}	2.6 ^{d}
	2	81.8 ^{b}	5.5 ^{cd}
	3	78.8 ^{bc}	5.0 ^{cd}
	4	71.2 ^{c}	9.4 ^{bcd}

***1: MS medium + 1.0 mg/l 2, 4-D, culture for 4 weeks, subculture for an additional four weeks on the same medium**

2: MS medium + 1.0 mg/l 2, 4-D, culture for 4 weeks, subculture for an additional four weeks on MS medium with 0.5 mg/l 2, 4-D and 0.25 mg/l kinetin (Kao and Smith 1993)

3: MS medium + 1.0 mg/l 2,4-D + 0.01 mg/l BA, culture for 4 weeks, subculture for an additional four weeks on the same medium

4: MS medium + 1.0 mg/l 2,4-D + 0.1 mg/l BA, culture for 4 weeks, subculture on the same medium for an additional four weeks

**** Values represent means of the three replicates of 55 early immature embryos, 44 immature embryos and 44 young shoot bases per replicate. Statistical analysis was performed among treatments within each explant type group. Values followed by the same letter within each column were not significantly different from each other at the 5% level.**

In vitro somatic embryogenesis of St. Augustinegrass, with some similarities to those reported in wheat (Ozias-Akins and Vasil 1982), bermudagrass (Chaudhury and Qu 2000), perennial ryegrass and tall fescue (Bradley et al. 2001B), was observed by scanning electron microscopy (SEM) examination (Fig. 3.3b). On the surface of watery, non-embryogenic calli, long, tubular, and loosely-held cells were observed (Fig. 2A). On the compact, embryogenic calli, it seems St. Augustinegrass did not develop large, easily recognizable scutellum tissue as observed in bermudagrass, perennial ryegrass, and tall fescue. The embryogenesis proceeded faster than those grasses and the development varied a great deal among the calli. Two months in culture, various developmental stages of embryogenesis were observed which included granular, somatic embryo clusters (SEC) similar to the ones observed in bermudagrass (Fig. 3.3b B); dense coleoptiles (CO) formed by circularization of the granular protuberances on compact structures (Fig. 3.3b C); and early shoots (SH) often seen at the center of the developing, circular coleoptiles (Figure 3.3b C, D). They developed into one or more shoots (Figure 3.3b E, F). Trichomes were observed at this stage on the coleoptile surface as well as on the true leaves. The differentiation was accelerated when the calli were transferred to the regeneration medium and more leaves were formed (Figure 3.3b F, G, H).

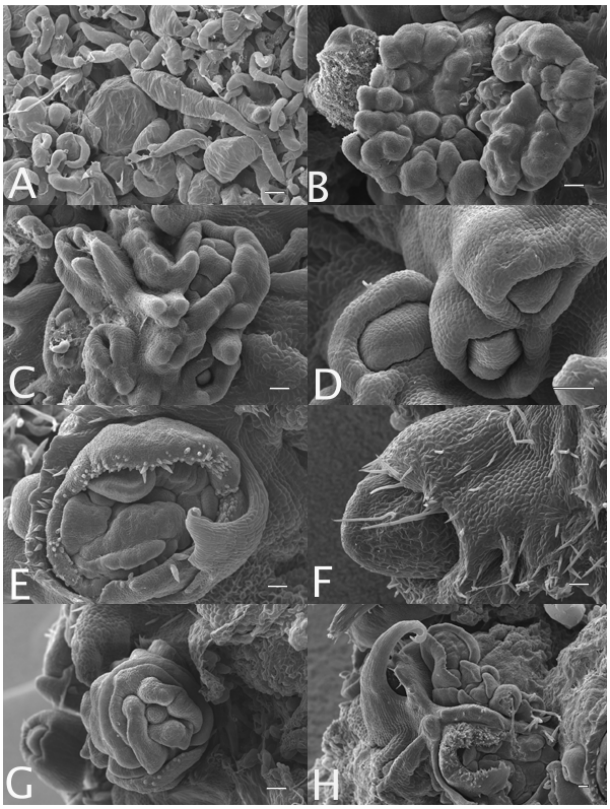

Figure 3.3b. Scanning electron micrographs of somatic embryogenesis of St. Augustinegrass. Bar in each figure represents 100 µm. A. Non-embryogenic callus showing long, tubular and loosely held cells on its surface. B. C. D. and E. Embryogenic calli 2 month on callus induction/subculture medium showing various stages of development of somatic embryogenesis: granular somatic embryo clusters (B), coleoptiles (CO) and shoot (SH) formation (C, D), and early leaf (LF) formation (E). Trichomes start to appear in later developmental stages. F. G. H. Embryogenic calli, 1 week in regeneration medium, showing shoot formation.

Discussion

In this study, 11 explant types of St. Augustinegrass were tested for their tissue culture responses. Three of them (young leaf, root, and anther) did not induce any callus although callus can often be induced from their counterparts of other grasses (Bhaskaran and Smith 1990). Moreover, calli from only two tissues (two age groups of immature embryo and young leaf base of seedlings) were able to regenerate. The BA concentration clearly made a difference in callus regeneration ability. In immature embryo culture, addition of 0.1 mg/l BA

in callus induction/subculture medium had significantly higher regeneration rates (2-6 fold) over 0 and 0.01 mg/l. When compared to the previously reported medium treatment of St. Augustinegrass tissue culture (Kuo & Smith 1993), supplement of 0.1 mg/l BA in the medium significantly improved callus regeneration over supplement of 0.25 mg/l kinetin. In immature embryo culture, the regeneration rate from 0.1 mg/l BA supplement (40%) was 3.5 fold of the one from the kinetin supplement (11.4%). Moreover, the addition of BA up to 0.5 mg/l further enhanced callus regeneration without adversely effecting callus induction from immature embryos. Interestingly, BA negatively affected callus induction from the young leaf bases and failed to enhance regeneration of the calli induced from this explant type, suggesting a strong interaction between BA and the explant source.

The SEM work revealed that somatic embryogenesis is the major developmental pathway for St. Augustinegrass *in vitro* regeneration although some detailed differences were observed between St. Augustinegrass and other grasses reported. These differences most likely reflect the different tissue responses to the phytohormones added in the culture media among the grasses.

In summary, we investigated tissue culture responses of various tissues of St. Augustinegrass, identified immature embryos being the most suitable explant for its tissue culture, and optimized the culture medium. The callus regeneration rate could be as high as 47.6%. The work helps to pave the road for isolation of somaclonal variants through tissue culture for St. Augustinegrass breeding, as well as for the genetic transformation of the species.

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CHAPTER 4. SOMACLONAL VARIATION FOR IMPROVEMENT OF FREEZING TOLERANCE AND SEMI-DWARF GROWTH HABIT

4.1 Introduction: application of somaclonal variation in plant breeding

Somaclonal variation (SV) has been defined as the variation induced by tissue culture in regenerated plants (Larken and Scowcroft, 1981). It is a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones (soma = vegetative, clone = identical copy). It was first reported in sugarcane (*Saccharum officinarum* L.) plants derived from cell culture by researchers at the Hawaiian Sugar Planters Association Experiment Station (Heinze et al., 1969). They observed considerable variation in chromosome number and enzyme activities among regenerated plants. Since then, SV has been widely observed in a large number of plant species, such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), potato (*Solanum tuberosum* L.), and tobacco (*Nicotiana tabacum* L.), and used for selection of various agronomic traits, including disease resistance, yield, plant height, and tolerance to adverse soil and climatic conditions (Jain et al., 1998).

The exact mechanism of SV formation is not clear. However, the plant tissue culture is considered a mutagenic system because cells experience traumatic effects when being isolated and cultured *in vitro*. McClintock (1984) suggested that when cells face trauma, they reset the genome expression, and may not follow the same orderly sequence that occurs under natural conditions. Instead, the genome is abnormally reprogrammed or restructured. This restructuring can give rise to a wide range of altered phenotype in newly regenerated

plants. Phillips et al. (1994) also speculated that a stress–response mechanism in which the culture media and confined environment could impose stress on plants.

Somaclonal variation can be associated with a vast array of genetic changes. At the chromosomal level, chromosomal number could change during tissue culture, which can be observed by light microscopy with simple staining techniques. Lee and Phillips (1988) reviewed chromosomal basis of somaclonal variation. For instance, a large number of regenerated plants in maize (*Zea mays* L.) were observed to have chromosomal aberrations, such as ploidy level changes and translocation. At the gene level, Dennis et al. (1987) showed that a somaclonal variant derived from tissue culture of maize embryos had a single base change in the coding region of the gene which converts a lysine codon (AAG) to a stop codon (TAG). In addition, organelle DNA changes were observed during tissue culture. Rode et al. (1987) found that a fraction of the mitochondrial genome was lost in callus culture induced from wheat embryos. Epigenetic changes could occur too. By using methylation sensitive restrictive enzyme *Hpa*II and probing with genomic clones and cDNA clones, Kaeppler and Phillips (1993) demonstrated that DNA methylation status changed in 39% of families of the regenerated plants from maize embryo culture. In addition, they found that demethylation also took place at a high frequency, which may explain the cause of the instability of some somaclonal variations.

Using SV in breeding could shorten the generation time normally needed by conventional breeding, and be able to handle a large number of cells at the same time (Jain, 2001). In addition, there is a distinct advantage of somaclonal variants in vegetatively propagated plants, where conventional breeding is difficult or impossible through sexual crossing.

Despite of the unpredictable nature and some genetic instability of SV, heritable SVs have been used for improvement of important agronomic traits. By 2001, 22 cultivars had been released from selected somaclones (Jain, 2001). Improved traits include yield, plant architecture, color, pest resistance, and salt and heat tolerance. No cultivar with improved freezing tolerance through somaclonal variation has been released to date. Among 22 released cultivars, seven belong to the *Gramineae* family, which include 'He Zu No.8' wheat with high yield, 'Yidan No. 6' maize with better grain quality, 'CIMAP/bio-13' aromatic grass (*Cymbopogon winterianus* Jowitt) with increased oil yield, 'DAMA' rice with *Picularia* resistance, 'FR13A' rice with sub-emergence tolerance, and 'LSBR-33' rice and 'LSBR-5' rice with *Rhizoctonia* resistance. These somaclonal variants are all derived from somatic embryogenesis after callus induction from different explants, including embryos (wheat and maize), leaf sheath (aromatic grass), and mature seed (rice). However, there has been no report, to my knowledge regarding cultivar development in St. Augustinegrass through tissue culture.

Kuo and Smith (1993) cultured immature embryos of 'Texas Common' St. Augustinegrass on MS medium containing 1 mg L^{-1} 2,4-D, followed by 4 weeks culture with 0.5 mg L^{-1} 2, 4 D plus 0.25 mg L^{-1} kinetin and found that approximately one third of the induced calli were able to regenerate into plantlets. We (Li et al. 2006) improved the tissue culture response by culturing immature embryos on MS medium containing 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} 6-benzyladenine, in which the callus induction and regeneration rates were 97.7% and 47.6%, respectively. In our research, mass tissue culture was carried out using the improved tissue

culture system to create somaclonal variants for selection of improved traits of St. Augustingrass.

4.2 Materials and methods

Mass tissue culture: Mass tissue culture was carried out in the summer of 2004 by using a protocol developed by us (Li et al.,2006) by culturing immature embryos on MS medium containing 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} 6-benzyladenine. Regenerated plantlets were transplanted to Metro-Mix-200 soil and kept in a lighted culture room. The plantlets were transferred to a greenhouse and maintained with regular watering and trimming. Two grams of fertilizer Osmocote (16-4-8; N-P_2O_5-K_2O%) per pot were applied monthly.

Mass screening for freezing tolerance: Screening of regenerated plants for freezing tolerance was conducted four rounds in a controlled environment. The freezing temperature used for the screening varied from -5ºC to -3ºC based on the age and growth condition. In a preliminary test, freezing treatment at -5ºC was optimum for screening of 6 months old plants. Therefore, in the first round when the regenerated plants were about 6 months old, about 7800 plants were subjected to -5ºC for 3 hrs after the two-step acclimation as described in Chapter 1. Surviving plants were subjected to a second round of screening at -4 ºC after 2-3 months of growth. In the third and fourth rounds, the surviving plants were allowed to recover for one week and were subjected to freezing treatment at -3ºC. For the second and third rounds, 4 to 8 plants per clone were used depending on availability of the plant materials. The fourth round screening was focused on four clones, SVC1, SVC1, SVC3, and

SVC4, having 3 replicates with 15 plants per replicate. The data were statistically analyzed using ANOVA. LSD tests were used to determine differences between clones.

Morphological characteristics for somaclonal variants: Before the first-round screening for freezing tolerance, about 100 plants with lower height and shorter leaves than Raleigh were selected and planted at the Lake Wheeler Turf Field Lab in the summer of 2005. Selection for similar phenotypes was also conducted on the 380 plant lines, which survived the first round of the freezing test. The selected plants were grown in pots for 1-3 months in the greenhouse, and the ones clearly showing morphological differences from Raleigh St. Augustinegrass were transferred to flats for further growth to confirm the altered phenotype. A complete set of measurements as described in Chapter 2 was conducted on somaclonal variants SV20 and SV15. For all others, only the lengths of the stolon and the third internode were measured.

4.3 Results

Mass screening for freezing tolerance: Approximately 7900 plants were regenerated from tissue culture efforts. Among them, about 100 plants with finer plant architecture were directly grown in the field for further selection, the rest of 7800 plants were subjected to the first round of freezing screening test -5ºC. Three hundred and eighty plants survived the first round of screening, and15 plants were alive after the second screening at -4ºC. After the third round screening at -3ºC, four clones, SVC1, SVC2, SVC3, and SVC4, showed higher survival rate and entered the fourth screening, where they were evaluated in replicated experiments. The survival percentage of SVC3 (60%) was significantly higher than Raleigh

(28.9%) (Fig 4.3a and Table 4.3a), and is considered a somaclonal variant with improved freezing tolerance.

SVC3 was subjected to morphological characterization, and no significant difference was observed on internode length, interonode thickness, and stolon length between SVC3 and Raleigh (Table 4.3c). Actually, SVC3 had a significantly longer (4.68 cm) and wider (0.95 cm) leaf blade and longer leaf sheath (4.52 cm), when compared with Raleigh (3.07 cm, 0.83 cm, and 3.02 cm, respectively) in a greenhouse measurement.

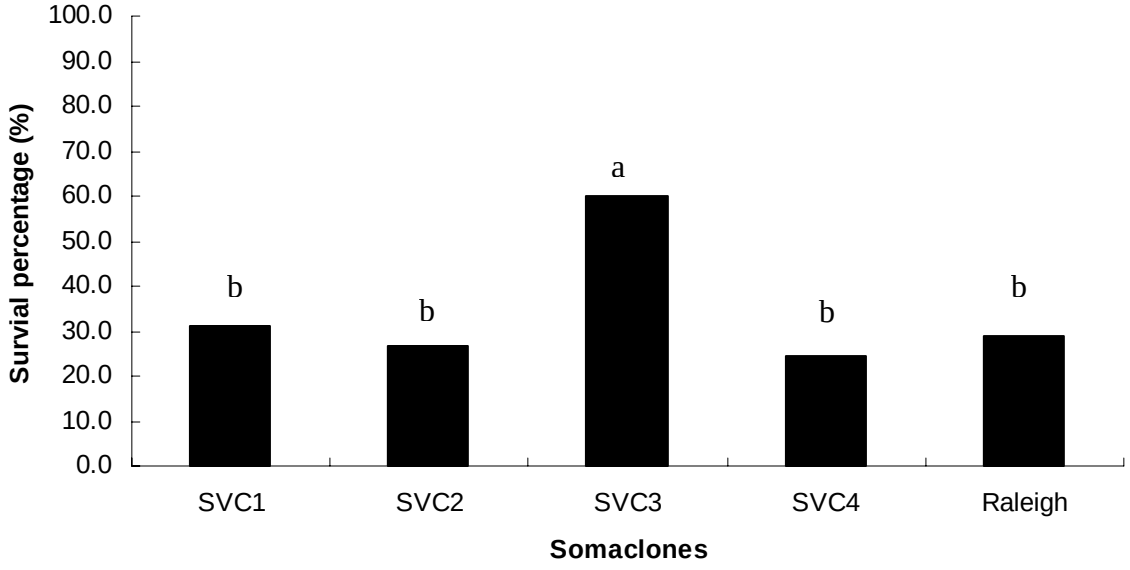

Fig 4.3a: Survival percentage of four somaclones one month after freezing treatment at -3ºC. Columns represent means of three replicates with 15 single node cuttings from each somaclones. F test and LSD tests were performed among the somaclones. Values followed by the same letter were not significantly different from each other at the 5% level by LSD.

Table 4.3a: ANOVA analysis of survival percentage of four somaclones one month after freezing treatment.

Source	DF	SS	MS	F Value	Pr > F
Somaclones	4	0.36	0.09	7.24	0.0091
Replication	2	0.01	0.01	0.48	0.6337
Error	8	25.07	3.13		
Total	14	95.73			

Table 4.3b. Morphological characteristics of SVC3. Each value represents the mean of six stolons of each genotype. Statistical analysis was performed among genotypes. Values followed by the same letter were not significantly different from each other at the 5% level by t-test. Coefficient of variance is also shown.

Genotype	LL† (cm)	LW (cm)	SSL (cm)	ID (mm)	IL (cm)	SL (cm)
Raleigh	3.07[b]	0.83[b]	3.02[b]	3.18[a]	7.65[a]	24.23[a]
SVC3	4.68[a]	0.95[a]	4.52[a]	3.70[a]	8.17[a]	26.73[a]
CV%	13.70	4.10	6.28	10.17	7.16	3.99

†**LL: The third internode leaf blade length; LW: Leaf blade width; SSL: Sheath length; ID: Internode diameter; IL: Internode length; SL: Stolon length.**

Morphological characteristics of somaclonal variants with semi-dwarf growth habit: Before the plants were subjected to freezing tolerance screening, about 100 plants with lower height and shorter leaves were identified. Fifty plants survived the winter of 2005 in the field. Except SV27, most of these plants did not exhibit acceptable vigor in growth and were not pursued any further.

Eighteen somaclonal variants with altered morphology were identified from the 380 plants that survived the first round of freezing test. They are: SV20, SV15, SV27, T4, T5, T7, 106T1-T3, 904AT1 - AT5, TD1, and TCo. Clones SV20, SV15, SV27, T4, T5, and T7 have been grown in the field and their data will be presented in Chapter 6. The morphological characteristics of the other 12 clones, as observed in the greenhouse conditions, are described below.

Clones 106T1, 106T2, 106T3, 904AT1 - 904AT5, 904T1, and 904T2 all had significantly reduced internode length and stolon length (Table 4.3c, d, e). They looked more compact, and some reductions were remarkable. For example, 904AT3 had nearly 40% reduction in stolon length and 43% reduction in internode length. 904T1 had nearly 40% shorter stolon and about 50% reduced internode length. Clone 106T2 had approximately 40% decrease in both stolon and internode lengths.

Table 4.3c: Internode and stolon length of five St. Augustinegrass somaclonal variants. Each value represents the mean from 10 stolons of a clone. Statistical analysis was performed among the clones and Raleigh. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also shown.

Somaclonal variants	Internode length (cm)	Stolon length (cm)
904AT1	4.85 [cd]	17.29 [b]
904AT2	5.27 [bc]	18.15 [b]
904AT3	4.50 [d]	13.59 [c]
904AT4	5.72 [b]	18.01 [b]
904AT5	5.31 [bc]	19.22 [b]
Raleigh	7.96 [a]	21.89 [a]
CV%	13.31	12.15

Table 4.3d. Internode length and stolon length of SVs 904T1 and 904T2. Each value represents the mean of 10 stolons from a clone. Statistical analysis was performed among the clones and Raleigh. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also shown.

Somaclonal variants	Internode length (cm)	Stolon length (cm)
904T1	3.84 [b]	14.71 [b]
904T2	4.61 [b]	15.83 [b]
Raleigh	7.48 [a]	23.52 [a]
CV%	20.12	14.81

Table 4.3e. Internode and stolon lengths of 106T1 – 106T3 somaclonal variants. Each value represents means of 10 stolons from each clone. Statistical analysis was performed among the clones and cv. Raleigh. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also shown.

Somaclonal variants	Internode length (cm)	Stolon length (cm)
106T1	4.05 [b]	13.56 [b]
106T2	3.69 [b]	13.42 [b]
106T3	3.90 [b]	14.02 [b]
Raleigh	6.32 [a]	22.18 [a]
CV%	16.11	15.49

In addition, a somaclonal variant, TCo, with yellow stripes on the leaves, was observed (Fig 4.3b). Somaclonal variant TD1 was found to have significantly thicker internodes than Raleigh (Fig. 4.3c). These two variants might have value for ornamental uses.

Fig. 4.3b. Somaclonal variant TCo with yellow stripes in its leaves.

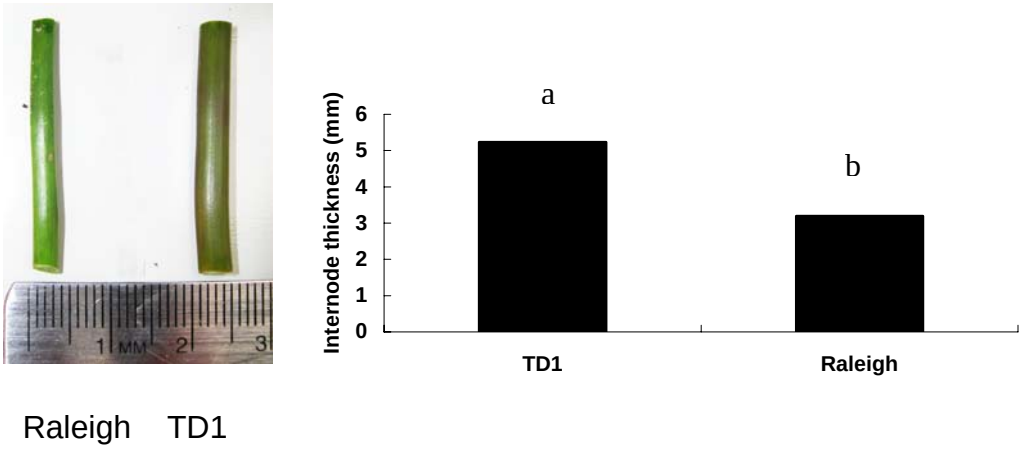

Fig. 4.3c. Somaclonal variant TD1 with thicker stem. Columns represent means from 10 stolons of each clone. Statistical analysis was performed among genotypes. Values followed by the same letter were not significantly different from each other at the 5% level by t-test.

4.4 Discussion

Breeding through somaclonal variation has yielded many new cultivars. However, to my knowledge, this is the first time somaclonal variation has been used for St. Augustinegrass improvement. The improvement of tissue culture conditions (Li et al., 2006) made it possible to regenerate nearly 8000 tissue culture plants, which created a significant variation pool. After four rounds of screening, SVC3 was identified as a clone with improved freezing tolerance. The survival percentage of SVC3 in our experiments (60%) was twice as high as cv. Raleigh (29%, Fig. 4.3a), and would be a promising candidate for a cultivar with improved freezing tolerance. A replicated test is underway to evaluate its field performance.

In addition, among 7900 regenerated plants, 19 somaclonal variants were identified as morphological variants with most of them having semi-dwarf growth habit showing shorter

leaf blades, internodes, stolons while still keeping vigorous growth. The plants grow in a more compact pattern and may contribute to an improved turf quality.

A major drawback of utilizing somaclonal variation is that some variations are caused by epigenetic effects, and the phenotypes may not be stable (Kaeppler and Phillips, 1993). To avoid such interference, plants from each clone were transplanted into at least two different containers and let grow for 3-6 months to confirm that the altered phenotype was stable. Some SV clones, such as SV15, SV20, T4, T5, and T7, were evaluated in formal field trials with three replicates. Moreover, clone SV27 has been measured in different years at different locations. All of these clones showed consistent performance, suggesting the phenotypes of these SV clones are stable and can be used for future breeding efforts.

Although somaclonal variants with changes such as improved freezing tolerance and shortened internode were discovered, the exact mechanism of SV formation is not clear. Tissue culture is considered to be a mutagenic system since *in vitro* culture can place the cells under severe stress when being moved from their *in vivo* tissue sites. The stress induces the genome to undergo reprogramming and restructuring, which would give rise to a wide range of altered phenotypes (McClintock, 1984), which could be associated with a vast array of genetic changes, such as chromosomal aberrations (Lee and Phillips, 1988), single base changes (Dennis et al., 1987), or changes of organelle DNA (Rode et al., 1987).

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CHAPTER 5. MUTAGENESIS FOR IMPROVEMENT OF ST. AUGUSTINEGRASS

5.1 Application of irradiation mutagenesis in turfgrass breeding

Irradiation mutagenesis has been extensively used in turfgrass breeding. For example, 'Coastcross II', which gives denser, better turf quality was derived from mass irradiation of freshly cut green stems of 'Coastcross-I' bermudagrass at the dosage of 70 Gy (Burton et al, 1980; Burton, 1981; Burton, 1985). Two widely used turfgrass cultivars, 'TifBlair' centipedegrass with improved vigor and cold tolerance, and 'TifEagle' bermudagrass with dwarfness were also obtained from gamma ray irradiated seeds (120 Gy) or stolons (70 Gy) respectively (Hanna, et al., 1997; Hanna and Elsner, 1999).

Irradiation mutagenesis has also been reported in St. Augustingrass. Busey (1980) investigated dosage effects of gamma rays on different cultivars. Stolons with four nodes from five genotypes including '1806', Floratam, Floratine, Bitterblue, and 'FA243' were treated with six dosages (0, 30, 40, 45, 50, and 60 Gy), and 45 Gy were found to be optimum (LD_{50}) for most genotypes. A total of 22 mutants were recovered, which included altered traits like dwarfness, chlorophyll deficiency, narrow-leaf, early-flowering, wrinkled-leaf, tall inflorescence, or altered stigma color. However, their field performance is not reported. At the same time, Powell and Toler (1980) treated 2,693 nodes at a dosage of 58.3 Gy. Fourteen mutants were obtained with shortened internodes. However, most of them were less vigorous, and incapable of surviving under field conditions. Two of these mutants, 'TXSA 8202' and 'TXSA 8212' (Toler et al., 1985) were later released with multiple disease

resistance against *Pyricularia*, *Ceratosphaeria*, *Scherophthora* and *Tanatephorus*. They are the only two released mutant cultivars in St. Augustinegrass.

Up to now, based on FAO/IAEA database, ten mutant cultivars have been released in turfgrasses (Table 5.1), which came from creeping bentgrass (*Agrostis palustris* Huds.), centipedegrass [*Eremochloa ophuiroides* (Munro) Hack], bermudagrass [*Cynodon dactylon* (L.) Pers.] and St. Augustinegrass (*Stenotaphrum secundatum* Kuntze). The improved traits include heat tolerance, disease resistance, vigor, dwarfness, leaf quality and nematode resistance. The most common used mutagen in turfgrass mutagenesis was gamma ray, and the dosage used depended on the species and the tissues used for the treatment.

Table 5.1. Turfgrass cultivars developed through induced mutagenesis (based on FAO/IAEA database, 2007).

Species		Cultivar released	Year	Country	Starting material, mutagen & dosage	Trait improved
Creeping bentgrass	*Agrostis sp.*	Springs	1983	Japan	Gamma rays	Heat tolerance
Creeping bentgrass	*Agrostis sp.*	Chiba Green B-2	1997	Japan	X-rays	Resistance to brown patch
Centipedegrass	*Eremochloa ophuiroides* Hack.	Tifblair	1995	U.S.A	Gamma rays, 120 Gy	Vigor
Centipedegrass	*Eremochloa ophuiroides* Hack.	AU Centennial	1983	U.S.A	Caryopses, gamma rays, 300-400 Gy	Dwarfness
Bermudagrass	*Cynodon sp.*	Tifton 94	1995	U.S.A	Gamma rays, 80 Gy	Leaf qualities
Bermudagrass	*Cynodon sp.*	Tifeagle (TW-72)	1995	U.S.A	Stolons, Gamma rays , 70 Gy	Dwarfness
Bermudagrass	*Cynodon sp.*	Tifway II	1981	U.S.A	Dormant rhizomes Gamma rays, 90 Gy	Nematode resistance
Bermudagrass	*Cynodon sp.*	Tifgreen	1983	U.S.A	Gamma rays	Vigor
St. Augustinegrass	*Stenotaphrum secundatum Kuntze*	TXSA 8202	1985	U.S.A	Stolon sections Gamma rays, 58.3 Gy	Disease resistance
St. Augustinegrass	*Stenotaphrum secundatum Kuntze*	TXSA 8212	1985	U.S.A	Stolon sections Gamma rays, 58.3 Gy	Disease resistance

5.2 Materials and methods

Dosage effect on single node cutting plants and calli: Cultivar Raleigh was used for mutagenesis. Dosage effect was investigated on both single node cuttings and calli from tissue culture.

To investigate dosage effect on single node cuttings, stolons were randomly collected from a field plot at Lake Wheeler Turf Field Lab, and the soil was washed off with tap water. The stolons were cut into single node cuttings. Shoots and roots were trimmed to 3-4 cm in length to make it easy to handle in the limited irradiation treatment space. The plant materials were sealed in plastic bags and irradiated with various dosages of gamma ray from a ^{60}Co source that provided dosage rate at approximately 80 Gy/hr, at the Nuclear Engineering Department, NCSU. The single node cuttings were planted to Metro-Mix-200 soil promptly after irradiation treatment for recovery in the greenhouse.

In the first experiment, the cuttings were treated at a range of dosages of 0, 15, 45, 100, 200, 400, 800, and 1600 Gy. Twenty cuttings were used for each treatment. To measure the effect of gamma ray dosage on plant growth, the fresh weights of the plants before the treatment and 6 weeks afterwards were recorded for each treatment. Growth index was calculated as final fresh weight minus the initial fresh weight divided by the initial weight.

Following the above experiment, a more formal experiment was performed to determine the LD_{50} and LD_{20} dosages, which included treatments of 0, 50, 60, 70, 80, 90, and 100Gy.

Twenty single node cuttings were used as a replicate and three replicates were conducted. Survival of the treated plants was scored 6 weeks later, and Probit analysis (Finney, 1971) was conducted to determine LD_{50} and LD_{20} by SAS program, respectively.

Gamma ray dosage effect on calli was determined by treating the calli at the dosages of 0, 25, 50, 100, and 200 Gy. Ten pieces of calli were used per treatment. Calli were induced from immature embryos of Raleigh on the MS basal medium supplemented with 30 g L^{-1} sucrose, 3.2 g L^{-1} phytagel, 1 mg L^{-1} 2, 4-D, and 0.1 mg L^{-1} BA. The fresh weight of calli was determined and the calli were then placed in a sterile petri dish containing a wet filter paper for maintaining moisture. The petri dishes were sealed and subjected to irradiation treatment at different dosages. The treated calli were then transferred to the same culture medium and maintained in the dark at 25°C in a culture chamber (I-36NL, Percival). After three weeks, the calli were weighed again and transferred to a regeneration medium, which was similar to the culture medium but with different phytohormones (1 mg L^{-1} BA, 0.2 mg L^{-1} NAA, and 0.5 mg L^{-1} GA). The cultures were maintained in a lighted culture chamber (CU-32L, Percival) with a 16-h photoperiod (140 μmol/m^2/s cool white fluorescent irradiance). After 4 weeks, the regenerated shoots were transferred to the rooting medium (hormone-free MS medium). Eight weeks later, regenerated plantlets with shoot length over 0.5 cm were counted. Based on size and vigor, some of them were transplanted to pots containing Metro-Mix-200 soil and kept in a lighted culture room. The plantlets were transferred to a greenhouse when established.

Mass irradiation mutagenesis of dormant single node cuttings: Two hundred and fifty Raleigh dormant single node cuttings, collected from the Lake Wheeler Turf Field Lab in Jan, 2005, were treated with either 50 or 70 Gy rays. In addition, 2800 dormant single node cuttings of Raleigh were collected in Turf Field Lab (in three batches) and treated at 70 Gy in Feb, 2006. Irradiated plants were planted into soil and maintained for three months in the greenhouse to determine survival rate and to identify mutations.

Selection for morphological mutants: Plants obtained from irradiated dormant single node cuttings in Jan, 2005 or regenerated from the treated calli were subjected to the first round screening for freezing tolerance. One hundred and five plants survived this freezing treatment. These plants were planted in pots and grown in the greenhouse for 1-3 months. Each putative mutant, as determined by morphological changes, was then transferred to a flat to grow further to evaluate whether the observed changes were stable. Plants obtained from the other batch (2800 dormant single node cuttings, Feb, 2006) were directly screened for morphological changes. Mutants identified with stable changes were measured as described in chapter 2.

5.3 Results

Dosage effect on single node cuttings and calli: The single node cuttings were tested with a wide range of irradiation dosages (15, 45, 100, 200, 400, 800, and 1600 Gy). It was found that dosages greater than 100 Gy killed plants while lower dosages such as 15 and 45 Gy did not affect plant growth significantly (Fig. 5.3a). Later on, a more formal experiment was conducted to test dosages of 0, 50, 60, 70, 80, 90, or 100 Gy. Plant survival rate dropped to

near 0 when the dosage was over 80 Gy (Fig 5.3b). By Probit analysis, the dosages of 48.54 Gy and 72.59 Gy were found as LD_{50} and LD_{20}, respectively, for irradiation of Raleigh single node cuttings.

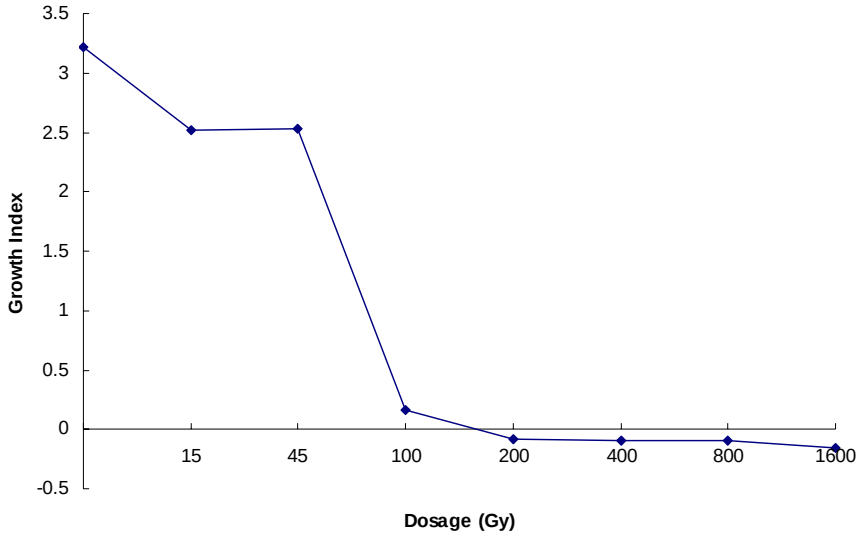

Fig. 5.3a. Gamma ray dosage effect on the growth of single node cutting plants of Raleigh. Each dot represents means of growth rate of 20 single node cutting plants.

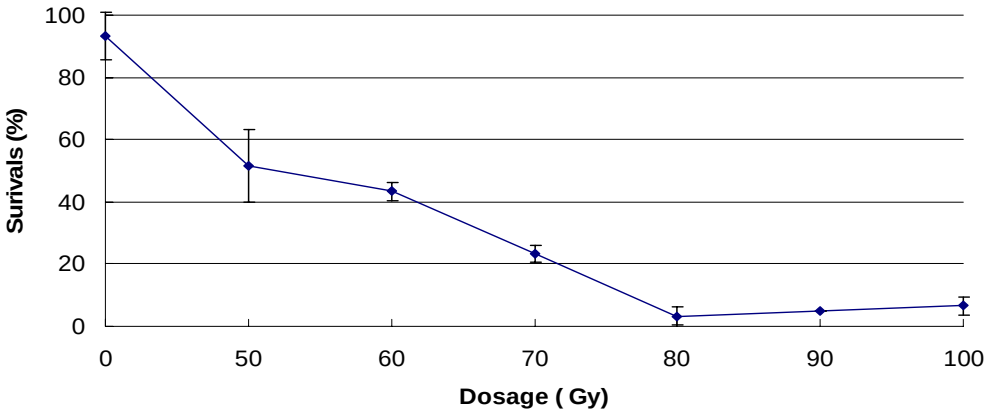

Fig. 5.3b. Gamma ray dosage effects on survival of Raleigh. Each value represents means of three replicates with 20 single node cuttings per replicate. Bars represent standard errors.

Embryogenic calli were irradiated with the dosages of 0, 25, 50, 100, or 200 Gy. It was found that higher dosages inhibited callus growth (Table 5.3a), with the growth index changing from 1.92 (0 Gy) to 1.33 (200 Gy). In addition, callus regenerations was affected by radiation dosage. Over 300 regenerated plants were recovered treatments of 25 Gy or 50 Gy, which were comparable to the control. However, only 39 plants were regenerated when calli were exposed to 100 Gy and no regeneration was observed after treatment of 200 Gy. A total of 1070 plants were regenerated from the experiment. Four hundred and ninety plants were selected basing on size and growth vigor and then transplanted in the greenhouse. Plants from these lines were subjected to freezing tests, but none survived the first two rounds of screening. However, three lines (904G4, G1 and GB1), regenerated from calli treated at 100 Gy, showed morphological changes.

Table 5.3a. Gamma ray dosage effect on callus growth and regeneration of cv. Raleigh.

Dosage (Gy)	Growth Index of calli $(W_1-W_0)/W_0$	Regenerated plants number
0	**1.92**	**336**
25	**2.11**	**351**
50	**1.51**	**344**
100	**1.43**	**39**
200	**1.33**	**0**

Mass irradiation mutagenesis with dormant single node cuttings: Dormant single node cuttings might be more radiation-sensitive than single node cuttings with vigorous growth. The irradiation was conducted on a preliminary basis (500 dormant single node cuttings) and later on a relatively large scale (2800 dormant single node cuttings). A total of 9 mutants were identified.

Six mutants with semi-dwarf growth habit, 106G1- 106G4, 904G1 and GF, were found among 386 recovered plants from 500 dormant single node cuttings irradiated at dosages 50 Gy or 70 Gy. Plants from these 386 lines were subjected to the first two rounds of freezing tolerance screening, but none of them survived. In another test, a total of 939 plants were recovered from 2800 irradiated dormant single node cuttings. Three mutants (904AG1, 904G2, 904G3) with altered growth habit were found. Therefore, a total of 9 mutants with semi-dwarf growth habit were identified among irradiated dormant single node cutting plants.

Morphological characteristics of mutants: A total of 12 stable morphological mutants were identified, which were G1, GF, 904AG1, 904G1-G4, 106G1-G4 and GB1 after screening plants recovered from the irradiation mutagenesis. It was observed that mutants often had significantly shortened internode length and stolon length. All the lines grown in the greenhouse are described below except lines G1, GF and 904AG1 which have been grown in the field and will be reported in chapter 6.

Mutant lines 904G1-G4 derived from irradiated dormant single node cuttings irradiated at 50Gy had shortened internodes and stolons (Table 5.3b). Most notably, 904G2 had about 50% shorter internodes and stolons than cv. Raleigh. However, this line grew less vigorously than Raleigh and the other three mutants.

Table 5.3b: Internode and stolon lengths of mutants 904G1-4. Each value represents means of 10 stolons per line. Statistical analysis was performed among the lines. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also presented for each category.

Mutants	Internode length (cm)	Stolon length (cm)
904G1	**5.82 [b]**	**19.34 [b]**
904G2	**3.53 [d]**	**11.47 [d]**
904G3	**4.30 [cd]**	**14.43 [cd]**
904G4	**5.59 [b]**	**18.90 [b]**
Raleigh	**7.48 [a]**	**23.52 [a]**
CV%	**16.73**	**14.55**

Mutant lines 106G1, 106G2, 106G3, and 106G4, from dormant single node cuttings irradiated at the dosage of 50 Gy, showed significantly shorter internodes and stolons than Raleigh (Table 5.3c).

Table 5.3c. Internode and stolon lengths of mutants 106G1-G4. Each value represents means of 10 stolons per line. Statistical analysis was performed among the lines. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also shown.

Mutants	Internode length (cm)	Stolon length (cm)
106G1	5.13 [b*]	20.07 [b]
106G2	4.38 [c]	16.96 [c]
106G3	4.27 [c]	15.03 [c]
106G4	4.38 [c]	16.96 [c]
Raleigh	6.32 [a]	22.18 [a]
CV%	13.63	11.96

Mutant GB1 was regenerated from callus irradiated with the dosage of 100 Gy. The plant grew vigorously but the growth pattern was greatly altered from horizontal spreading to a bunch-type growth. The plants were clustered together after six months. The plant had approximately 80% fewer stolons which were 90% shorter (Table 5.3d). Shoots and seed heads were much denser in GB1.

Table 5.3d: Morphological characteristics of GB1 measured on March 1, 2007. Each value represents means of 5 stolons of each genotype. Statistical analysis was performed among genotype. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also presented.

Genotypes	No. of stolons spreading out of the flat	Length of stolon spreading out of the flat (cm)	Stolon length within the flat (cm)	Internode length (cm)
GB1	5	13.4[b]	11.88[b]	3.3[b]
Raleigh	25	123.8[a]	27.04[a]	8.0[a]
CV%	-	22.72	7.60	5.76

5.4 Discussion

We explored the dosage range of gamma ray treatment and determined LD_{50} and LD_{20} for single node cuttings of Raleigh St. Augustinegrass. The LD_{50} (48.54 Gy) found in this study was similar to the results from Busey (1980), who found that 45 Gy was LD_{50} for most genotypes by irradiation of stolons. In addition, nine mutants with significantly reduced internode and stolon lengths were identified from 3300 irradiated dormant single node cuttings at dosage of 50 Gy and 70 Gy. It appears that dosages of 50 Gy and 70 Gy was effective for inducing mutations in dormant single node cuttings of St. Augustinegrass. They were similar to breeding of TifEagle bermudagrass (70 Gy), and TXSA 8202 and TXSA 8212 St. Augustinegrass (58.3 Gy, Toler et al., 1985).

Calli showed more tolerant to irradiation than single node cuttings. For example, thirty nine plants were regenerated from irradiated calli at the dosage of 100 Gy while single node cutting plants were all dead at this dosage. It appears that irradiation of calli might be an

effective approach for inducing mutations in St. Augustinegrass. Mutant lines G1 and GB1, regenerated from irradiated calli at 100 Gy, showed either a semi-dwarf growth habit (G1), or a more bunch growth habit (GB1).

Semi-dwarf mutants of St. Augustinegrass were observed by Powell and Toler (1980) from irradiated stolons of Floratam. However, most of them were less vigorous, and incapable to survive under field conditions (Reinert, 1981). Fortunately, the mutants we found showed stability and vigorous growth which had no problem in surviving under field condition, indicating that they could be used as for potential new cultivars.

Besides the value in breeding, it might also be interesting to use dwarf mutants to elucidate regulatory mechanisms for St. Augustinegrass growth and development. Dwarf mutants have been isolated in many species and have been extensively analyzed for their mode of inheritance and their responses to plant hormones (Murfet and Reid, 1997; Kwon and Choe, 2005). Various dwarf phenotypes have been shown to be associated with, for example, gibberellins (Ross et al., 1997), brassinosteroids (Noguchi et al., 1999), abnormal cell walls (Reiter, 1993), and abnormal cell elongation (Takahashi et al., 1995).

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CHAPTER 6. PRELIMINARY FIELD PERFORMANCE OF SOMACLONAL VARIANTS AND MUTANTS

6.1 Introduction

It is difficult to prove inheritance of traits caused by somaclonal variants and mutants in St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] without field trials, which can be used to examine trait stability and to determine if other cryptic changes may have taken place that could affect clonal performance. In addition, it is important to evaluate field performance such as cold tolerance, growth vigor, turf quality, and pest resistance.

The objective of this study was to determine if the somaclonal variation and mutations identified in greenhouse studies were stable in field trials, and to determine overall plant performance in the field.

6.2 Materials and methods

Two field trials were conducted for nine plant lines, either somaclonal variants from tissue culture or mutants from irradiation. Somaclonal variants SV15, SV20, SV27, and mutants G1 and GF were space planted on June 22, 2006 using a randomized block design (RBD) (Fig.6.2a). The trial of somaclonal variants T4, T5, T7, and mutant 904AG1 was planted using a RBD on Aug 8, 2006 (Fig. 6.2b). Both trials were conducted at Lake Wheeler Turf Field Lab in Raleigh, NC. For the trial on June 22, 2006, plugs were prepared by growing single node cuttings in small pots for about one month in the greenhouse. Twenty five to thirty plugs for each SV or mutant were then planted within each plot (0.91 m x 1.22 m). Since the August 8, 2006 trial was planted late in the growing season, 0.61 x 0.61 m pieces of

sod (produced in soil beds at greenhouse) were transplanted at the center of each plot in the field. Both field trials used RBD with three replicates. The soil was fine sandy loam soil with pH 6.0. After transplantation, plots were irrigated three times daily for the first month to accelerate establishment and then watered as needed to prevent wilt. The fungicide, Heritage (Syngenta), was sprayed (15.8 g/100 m^2) as needed to prevent infection of gray leaf spot (*Pyricularia grisea*). Plot borders were mechanically trimmed monthly.

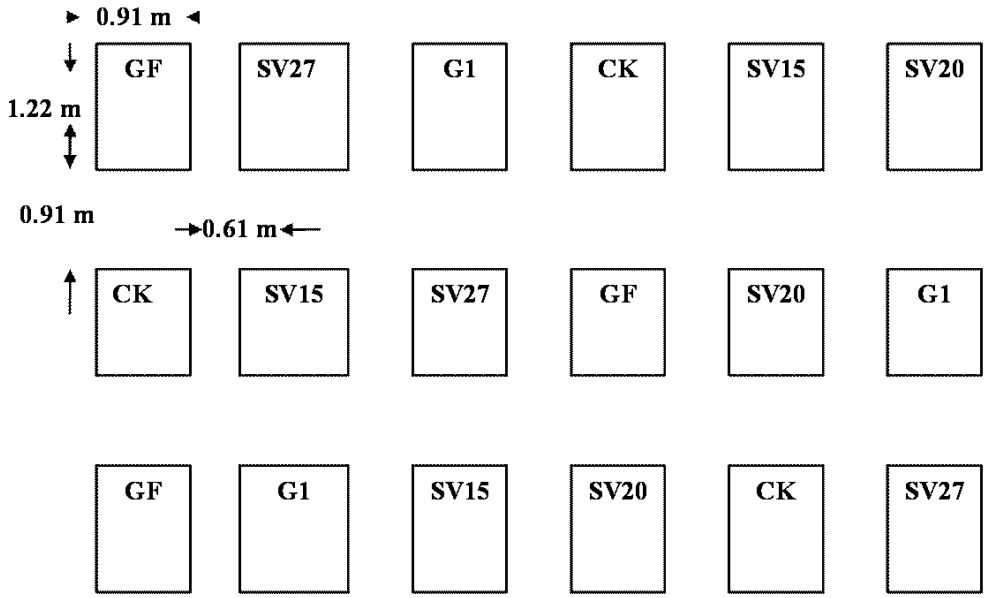

Fig. 6.2a. Field trial for 5 SVs and mutants planted on June 22, 2006.

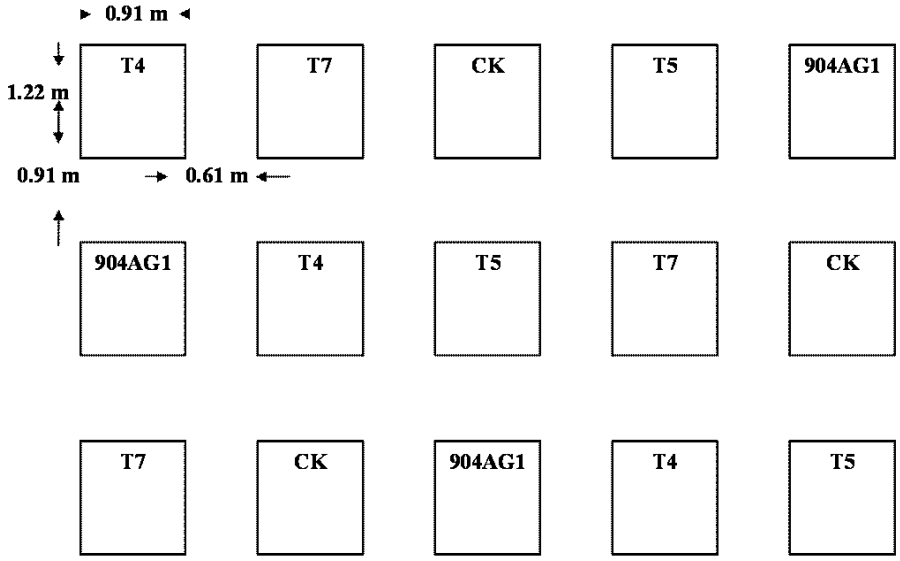

Fig 6.2b. Field trial for 4 SVs and mutants planted on August 8, 2006.

Morphological characteristics of somaclonal variants and mutants in the field: Measurements were taken in October, 2006, using ten stolons from each plot. The characters included the blade length and width taken from the shoot situated on the third node of each stolon, the length and thickness of the third internode, as well as the number of inflorescences per plot.

Winter color retention of somaclonal variants and mutants: Winter color retention is an important turf quality criterium. Therefore, the winter color retention was monitored weekly from Nov. 17 to Dec. 19, 2006 using digital image analysis for the trial started on June 22, 2006. The detailed procedure was the same as described in Chapter 2 except that the hue range was set from 52 to 100. The data were statistically analyzed by ANOVA.LSD tests were used to determine differences between lines at the level of 5%.

6.3 Results

Field trials for somaclonal variants and mutants: To investigate field performance of the somaclonal variants and mutants, nine lines were tested in two field trials. By visual observation, all of the SVs and mutants showed vigorous lateral growth during establishment (Fig. 6.3a). The morphological measurements were taken, and the winter color retention was also monitored.

Fig. 6.3a. Established field plots (October, 2006) of SV15, SV20, SV27, and mutants G1 and GF planted in June, 2006.

Morphological characteristics of somaclonal variants and mutants: Morphological measurements were conducted for nine lines. For somaclonal variants SV15, SV20, SV27, and mutants G1 and GF planted in June, 2006, the measurements are as follows:

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SV20 grown in field was 20% shorter in blade length than Raleigh, whereas the leaf width was similar to Raleigh. In addition, SV20 was 33 to 50% shorter in stolon and internode length when growing in both field (Table 6.3a) and flats compared to Raleigh (Table 6.3b). SV15 was similar to SV20 except that it produced significantly more inflorescences, which were two fold of SV20 and nearly five fold of Raleigh.

SV27 showed similar morphological characteristics as SV20 except that the blade width was narrower than Raleigh. The results were consistent with the measurements taken in September, 2005, in another field location (Table 6.3c), indicating that the performance of SV27 was stable at different locations and times.

Mutant line G1, regenerated from 100 Gy irradiated callus, was finer than Raleigh in all the measurements no matter grown in the field (Table 6.3a) or in flats (Table 6.3d). The blades were about 25% shorter and slightly narrower than Raleigh. G1 also had ~25% shorter internode, slightly shortened stolon, and thinner internodes than Raleigh. In addition, compared with Raleigh, G1 culm length was reduced by 40%, inflorescence shortened by 33%, and height at maturation reduced by 25%, and florets per inflorescence decreased by nearly 50%. G1 also produced nearly 2 fold of inflorescences over Raleigh.

The mutant GF, from dormant single node cutting irradiated at 50 Gy, also had significantly shorter leaf blade, internode, and stolon (Table 6.3a).

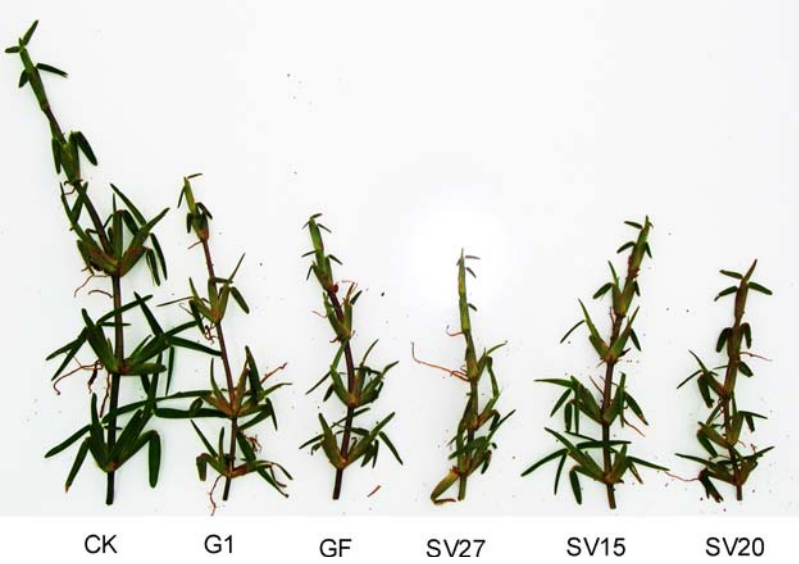

Fig. 6.3b. Stolons with four internodes from somaclonal variants and mutants grown in Lake Wheeler Turf Field Lab in 2006.

Table 6.3a: Morphological characteristics of somaclonal variants and mutants in field trial of 2006. Each value represents means of three replicates of 10 individual stolons. Statistical analysis was performed among treatments. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also presented.

SV & mutants	†LL (cm)	LW (cm)	ID (mm)	IL (cm)	SL (cm)	Inflorescence No./plot
G1	1.73^{b}	0.40^{bc}	2.43^{c}	3.67^{b}	14.66^{b}	123.33^{a}
GF	1.83^{b}	0.46^{a}	2.91^{a}	3.14^{c}	13.02^{bc}	0.67^{d}
SV15	1.65^{b}	0.34^{c}	2.84^{ab}	2.59^{d}	10.72^{c}	75.00^{b}
SV20	1.74^{b}	0.42^{ab}	2.69^{b}	2.60^{d}	11.39^{c}	33.33^{c}
SV27	1.63^{b}	0.34^{c}	2.93^{a}	2.59^{d}	11.21^{c}	32.33^{c}
Raleigh	2.16^{a}	0.46^{a}	3.05^{a}	4.88^{a}	18.53^{a}	16.33^{cd}
CV%	10.41	17.22	5.60	7.60	5.95	33.41

†LL: Leaf blade length; LW:Leaf blade width; ID: Internode diameter; IL: Internode length ; SL: Stolon length.

Table 6.3b. Morphological characteristics of somaclonal variants grown in a flat. Each value represents means of nine replicates. Statistical analysis was performed among lines. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also presented.

SVs	ID† (mm)	IL (cm)	SL (cm)	SSL (cm)	CL (cm)	InL (cm)	MH (cm)	FI	IN
SV15	3.50 [a]	2.27 [b]	8.52 [b]	1.79 [b]	6.84 [ab]	11.02 [a]	15.83 [a]	20.67 [a]	131.56 [a]
SV20	3.32 [a]	2.51 [b]	9.18 [b]	2.29 [a]	6.29 [b]	13.16 [a]	15.36 [a]	17.78 [a]	70.22 [b]
Raleigh	3.49 [a]	4.42 [a]	16.39 [a]	2.46 [a]	7.45 [a]	13.55 [a]	16.63 [a]	20.75 [a]	48.67 [b]
CV%	6.53	14.22	10.77	12.07	11.52	14.62	11.53	17.08	48.27

†ID: Internode diameter; IL: Internode length; SL: Stolon length; SSL: Sheath length; CL: Culm length; InL: Infloresence length; MH: plant height at maturation; FI: Florets no. per inflorescence; IN: Inflorescence number.

Table 6.3c: Morphological characteristics of SV27 measured in 2005 at other field location. Each value represents means of 10 stolons. Statistical analysis was performed between SV27 and Raleigh. Values followed by the same letter were not significantly different from each other at the 5% level by t-test. Coefficient of variance is also presented.

Time	Genotype	LL†(cm)	LW (cm)	IL (cm)	ID (mm)	SL (cm)
	SV27	1.69 [b]	0.33 [b]	2.4 [b]	2.41[a]	11.03 [b]
Sep-05	Raleigh	2.74 [a]	0.53 [a]	4.42[a]	2.60 [a]	19.4 [a]
	CV%	12.59	20.35	12.36	8.66	20.67

†LL: Leaf blade length; LW: Leaf blade width; IL: Internode length; ID: Internode diameter; SL: Stolon length.

Table 6.3d. Morphological characteristics of mutant G1 grown in a flat, 2006. Each value represents means of 9 replicates. Statistical analysis was performed between G1 and Raleigh. Values followed by the same letter were not significantly different from each other at the 5% level by t-test. Coefficient of variance is also presented.

Genotypes	ID† (mm)	IL (cm)	SL (cm)	SSL (cm)	CL (cm)	InL (cm)	MH (cm)	FI	IN
G1	2.75 [b]	3.81 [a]	13.24 [a]	1.68 [b]	4.39 [b]	9.13 [b]	12.67 [b]	11.67 [d]	133.00 [a]
Raleigh	3.49 [a]	4.42 [a]	16.39 [a]	2.46 [a]	7.45 [a]	13.55 [a]	16.63 [a]	20.75 [a]	48.67 [b]
CV%	6.53	14.22	10.77	12.07	11.52	14.62	11.53	17.08	48.27

†ID: Internode diameter; IL: Internode length; SL: Stolon length; SSL: Sheath length; CL: Culm length; InL: Infloresence length; MH: plant height at maturation ; FI: Florets no. per inflorescence; IN:Inflorescence number.

For somaclonal variants T4, T5, T7 and mutant 904AG1 planted on Aug 8, 2006, the measurements of blade length, blade width, internode length, stolon length were taken in

December, 2006. It was found that all selections had significantly shorter leaf blades, internodes and stolons (Fig. 6.3c and Table 6.3e). The blades of the mutant and somaclonal variants were shorter than Raleigh by 20-25%. The selections, T7, had the shortest internodes and stolons (2.26 cm and 8.63 cm, respectively, compared to 5.31 and 14.39 cm of Raleigh). In addition, T5, T7 and 904AG1 had significantly thinner stolons compared to Raleigh.

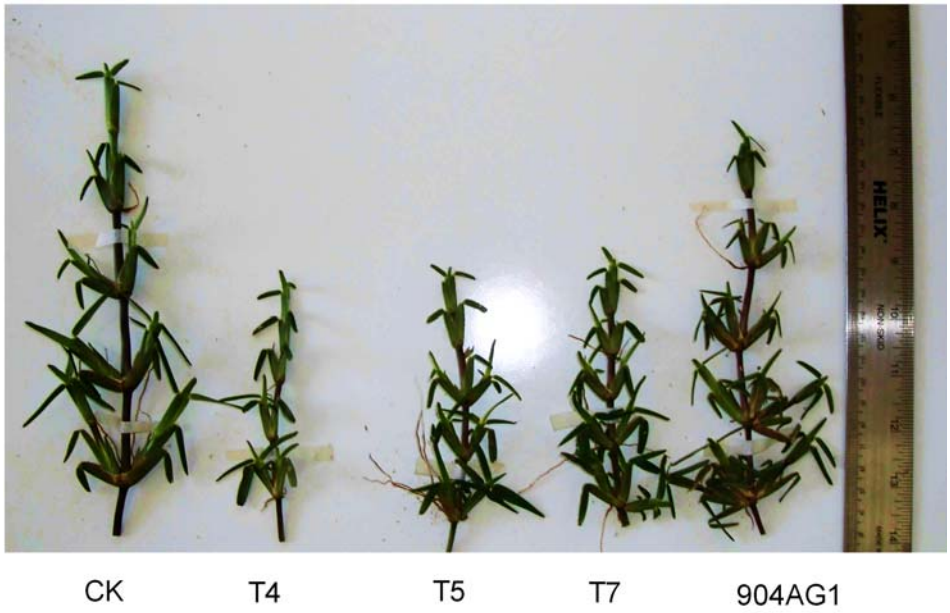

Fig. 6.3c: Stolons with four internodes from somaclonal variants and mutants grown in Lake Wheeler Turf Lab, Raleigh, 2006.

Table 6.3e: Morphological characteristics of SVs T4, T5, T7 and mutant 904AG1 in December, 2006. Each value represents means of three replicates, 10 stolons per replicate each genotype. Statistical analysis was performed among genotypes. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is also presented.

Genotype	LL (cm)†	LW (cm)	IL (cm)	ND (mm)	SL (cm)
T4	1.78 ^{b*}	0.45 ^{a}	2.70 ^{bc}	3.11 ^{a}	9.10 ^{c}
T5	1.92 ^{b}	0.45 ^{a}	2.79 ^{bc}	2.99^{a}	10.13 ^{bc}
T7	1.84 ^{b}	0.42 ^{a}	2.26 ^{c}	2.98 ^{a}	8.63 ^{c}
904AG1	1.73 ^{b}	0.44 ^{a}	3.50 ^{b}	2.75 ^{a}	11.94 ^{b}
Raleigh	2.32^{a}	0.47 ^{a}	5.31 ^{a}	3.35 ^{a}	14.39 ^{a}
CV%	6.37	5.59	16.68	6.23	10.66

†**LL: Leaf blade length; LW: Leaf blade width; ID: Internode diameter; IL: Internode length; SL: Stolon length.**

Winter color retention of somaclonal variants and mutants in the field: Winter color retention is an important character of turf quality. Digital image techniques were applied to monitor winter color retention for somaclonal variants SV15, SV20, SV27, and mutants G1 and GF from November 17 to December 19, 2006. On November 17 when the air temperature and soil temperature was 10°C and 13°C, respectively (Figure 6.3d), plants started to go dormant with the green retention for Raleigh at 57.3%. Thereafter, green retention of Raleigh decreased to 7.91% by mid-December (Fig 6.3e & Table 6.3f). Somaclonal variants, SV27 and SV20, and mutant line GF showed comparable green

retention ability to Raleigh. It seemed that SV15 and G1 had significantly lower green color, starting from 37.2% and 45.8%, respectively, on November 17. However, the low green retention could be partially attributed to the significantly more inflorescences produced by these two lines (Table 6.3a), which were yellow or brown, and would thus reduce the green color percentage substantially in the plots measured.

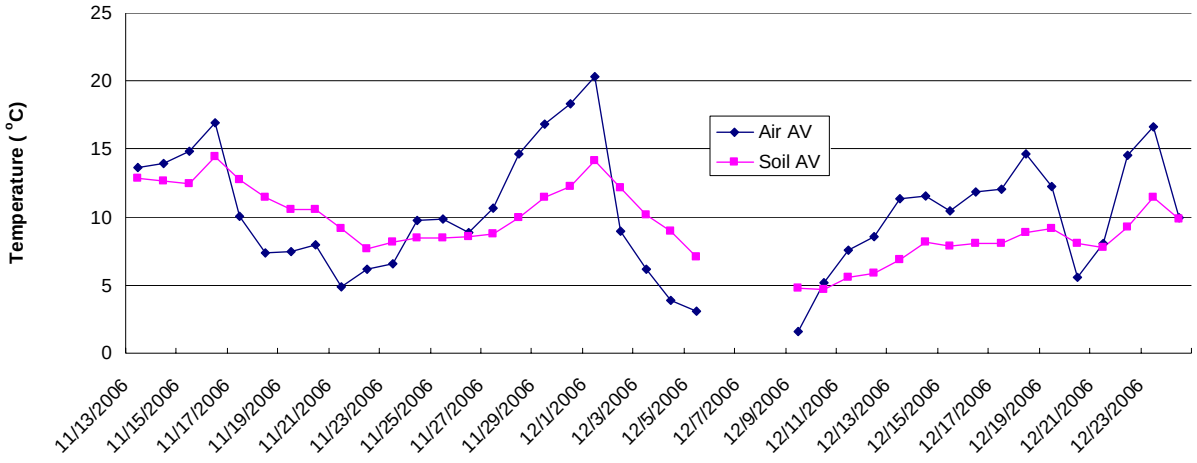

Fig. 6.3d. Daily average air and soil temperature changes from November 17 to December 19, 2006. Source: North Carolina Climate Retrieval and Observations Network of the Southeast Database (NC CRONOS): http://www.nc-climate.ncsu.edu/cronos.

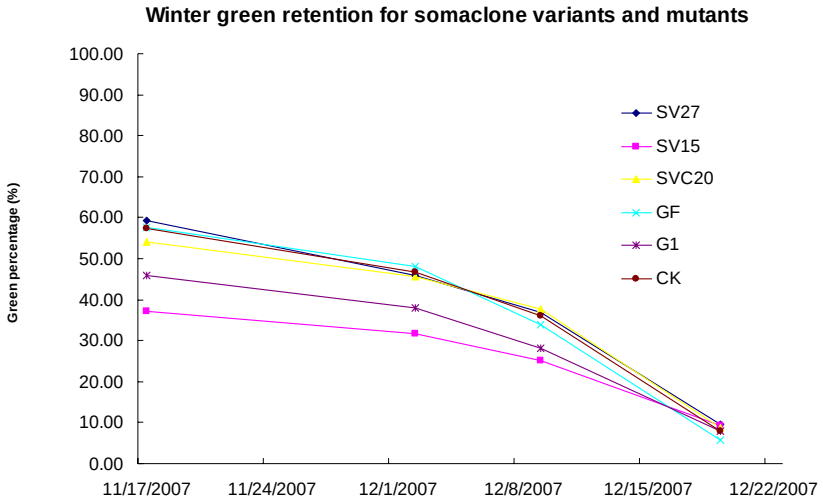

Fig. 6.3e. Green retention for somaclonal variants and mutants. Each symbol represents means of three replicates of each genotype. CK is Raleigh.

Table 6.3f. Green retention of somaclonal variants and mutants. Each value represents means of three replicates of each genotype. Statistical analysis was performed among genotypes by ANOVA. Values followed by the same letter were not significantly different from each other at the 5% level by LSD. Coefficient of variance is shown.

SVs & Mutants	Green retention percentage (%)			
	17-Nov	2-Dec	9-Dec	19-Dec
SV27	59.27[a]	45.95 [a]	36.77 [a]	9.56 [a]
SV15	37.23[c]	31.82 [c]	25.20 [c]	9.28 [a]
SVC20	53.98[a]	45.54 [a]	37.79 [a]	8.65 [a]
GF	57.73[a]	48.06 [a]	33.91 [ab]	5.78 [b]
G1	45.79[b]	37.97 [b]	28.01 [b]	7.83 [ab]
RALEIGH	57.31[a]	46.71 [a]	36.12 [a]	7.91 [ab]
CV%	6.83	6.77	11.28	14.98

6.4 Discussion

Field trials were performed for nine mutants and somaclonal variants. The altered phenotypes of these lines were shown to be stable in the field. There was no plant loss during establishment. It seemed that their ground coverage was comparable to Raleigh. Mutants and somaclonal variants had winter color retention similar to Raleigh. Overall, these SVs and mutants provided promising materials for future cultivar development.

Freezing tolerance was not evaluated for these lines. To investigate their field performance on winter survival, it is necessary to carry out field trials at multiple locations in multiple years since freezing tolerance is a complex, quantitative trait and the evaluation could be affected by unpredictable environmental factors (Tacenco et al., 1989). In addition, St. Augustine decline and southern chinch bug have been serious pest problems in St. Augustinegrass (Busey, 2003). Therefore, it would be necessary to investigate the pest resistance performance of these lines.

Most SVs and mutants had semi-dwarf growth type. It might be interesting to compare them with the current marketed dwarf cultivars such as ‘Amerishade’ and ‘Delmar’ (Trenholm et al., http://edis.ifas.ufl.edu/LH010) to investigate the advantages of these lines.

References

Busey, P. 2003. St. Augustinegrass, *Stenotaphrum secundatum* (Walt.) Kuntze. p. 309-330 In: Casler, M. D. and R.R. Duncan. (eds.) Biology, breeding, and genetics of turfgrasses. John Wiley & Sons, Inc, Hoboken, NJ.

Tcacenco, F.A., C.F. Eagles, and B.F. Tyler. 1989. Evaluation of winter hardiness in Romanian introductions of *Lolium perenn*. J. Agric. Sci. (Cambridge) 112:249-255.

Trenholm L.E., J.L. Cisar, and J.B. Unruh. St. Augustinegrass for Florida Lawns. http://edis.ifas.ufl.edu/LH010. Checked on April, 2007.

SUMMARY

To simulate natural cold acclimation and to perform selection in a more efficient and consistent manner, a freezing test system was developed under controlled conditions. It was found that plants treated at 13ºC for a week followed by 3ºC for another week was suitable for cold acclimation of St. Augustinegrass in a controlled condition, and -4°C for 3 hrs for evaluation of freezing tolerance.

To improve tissue culture response, 11 explant tissues and four callus induction/subculture media were examined. The best response came from 7-14 DAP immature embryo on MS medium containing 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} 6-benzyladenine. The callus induction and regeneration rates were 97.7% and 47.6%, respectively.

After screening 36 germplasm collections, nearly 8000 tissue culture regenerated plants, and 3300 mutagenized plants, it was determined that accession Elm4 and somaclonal variants SVC3 had significantly improved freezing tolerance compared to the standard cultivar Raleigh. Replicated field trials were initiated to investigate their performance under field conditions.

Regarding turf quality, accession Ray collected from Polk County, showed finer leaf texture and more dwarf growth habit compared Raleigh but was similar in freezing tolerance compared to Raleigh. In addition, nineteen somaclonal variants and 12 mutants, isolated through tissue culture and gamma ray irradiation approaches, showed semi-dwarf growth habit and vigorous lateral growth, and are promising plant lines for future cultivar

development. Field trials have been started for some of the variants and mutants, most of which had shortened internode and stolon while some also had finer leaf texture. The detailed characteristics of these lines are described in Table a, b and c.

It appeared that plants with semi-dwarf growth habit were more likely to occur from induced somaclonal variation (~0.25%) and irradiation mutagenesis (~0.36%). The frequency for improved freezing tolerance was much lower, but we did identify one clone (SVC3) that exhibited improved freeze tolerance out of ~8,000 tissue culture regenerated plants. The germplasm collections were also proven to be very valuable and yielded with an accession showing improved freezing tolerance and another with finer leaf texture.

Table a: List of plant lines with improved freezing tolerance.

Lines	**Source**	**Characteristics**
Elm4	**Germplasm collection**	**Improved freezing tolerance** **Survival rate was 2-fold of Raleigh** **Comparable establishment** **Similar plant architecture**
SVC3	**Tissue culture (TC)**	**Improved freezing tolerance** **>30% higher survival rate than Raleigh** **Vigorous growth with larger leaves**

Table b. The list of plant lines with semi-dwarf growth habit.

	Lines	Source	Characteristics	Note
1	Ray	Germplasm collection	Finer leaf blade	Freezing tolerance comparable to Raleigh
2	SV15	TC	Shortened LL, LW, IL, SL, CL	In field trial, 2006
3	SV20	TC	Shortened LL, IL, SL, CL	In field trial, 2006
4	SV27	TC	Shortened IL, SL	In field trial, 2006
5	T4	TC	Shortened LL, IL, SL	In field trial, 2006
6	T5	TC	Shortened LL, ID, IL, SL	In field trial, 2006
7	T7	TC	Shortened LL, ID, IL, SL	In field trial, 2006
8	904AT1	TC	Shortened IL, SL	
9	904T2	TC	Shortened IL, SL	

†LL: Leaf blade length; LW: Leaf blade width;ID: Internode diameter; IL: Internode length ; SL: Stolon length; SSL: Sheath length; CL: Culm length.

Table b. continued.

	Lines	Source	Characteristics	Note
10	904AT3	TC	Shortened IL, SL	
11	904AT4	TC	Shortened IL, SL	
12	904AT5	TC	Shortened IL, SL	
13	904T1	TC	Shortened IL, SL	
14	904T2	TC	Shortened IL, SL	
15	106T1	TC	Shortened IL, SL	
16	106T2	TC	Shortened IL, SL	
18	106T3	TC	Shortened IL, SL	
19	G1	γ-ray 100 Gy on calli	Everything smaller	In field trial, 2006
20	GF	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	

LL: Leaf blade length; LW: Leaf blade width;ID: Internode diameter; IL: Internode length ; SL: Stolon length; SSL: Sheath length; CL: Culm length.

Table b. continued.

	Lines	Source	Characteristics	Note
21	904G1	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	
22	904G2	γ-ray 70 Gy on dormant single node cutting	Shortened IL, SL	
23	904G3	γ-ray 70 Gy on dormant plants	Shortened IL, SL	
24	904G4	γ-ray 100 Gy on calli	Shortened IL, SL	
25	904AG1	γ-ray 70 Gy on dormant single node cutting	Shortened LL, ID, IL, SL	In field trial, 2006
26	106G1	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	
27	106G2	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	
28	106G3	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	
29	106G4	γ-ray 50 Gy on dormant single node cutting	Shortened IL, SL	

†LL: Leaf blade length; LW: Leaf blade width;ID: Internode diameter; IL: Internode length ; SL: Stolon length; SSL: Sheath length; CL: Culm length.

Table c. List of plant lines with other morphological changes.

	Lines	Source	Characteristics
1	GB1	γ-ray 100 Gy on calli	Bunch-type growth Less stolon formation
2	TCo	TC	Striped leaves
3	TD1	TC	Thicker stem