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Selection for freezing tolerance in St. Augustinegrass through somaclonal variation and germplasm evaluation

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Abstract

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is the least cold-hardy turfgrass species. Development of freezing-tolerant St. Augustinegrass cultivars would greatly benefit home owners in many southern states of the US. Towards this breeding goal, 7800 plants regenerated through tissue culture and 36 germplasm accessions were screened for improved freezing tolerance. Among the conditions tested, 1 week at 13°C followed by another week at 3°C, then freezing at −3 to −5°C for 3 h, was found to be suitable to distinguish genotypes in freezing tests. The experiments revealed that germplasm accession Elm4 was significantly more freezing-tolerant under a controlled environment than 'Raleigh', the current commercially available, most freezing-tolerant cultivar. In addition, out of 7800 regenerated plants from tissue culture, somaclonal variant SVC3 showed significantly more freezing-tolerant than its parent 'Raleigh'.

Key words: *Stenotaphrum secundatum* — freezing tolerance — germplasm — tissue culture

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] has good shade and salt tolerance, and is an important turfgrass in the tropics and subtropics. It has been reported to comprise 70% of the lawns in Florida (Busey 2003). However, the lack of freezing tolerance is one of its drawbacks, which hinders its production and distribution (Beard et al. 1980). St. Augustinegrass is adapted to the U.S. Department of Agriculture hardiness zones 8, 9 and 10 (Maier et al. 1994a). Currently, North Carolina is the north edge of its distribution range.

Germplasm collection and somaclonal variation (SV) induced from tissue culture are two effective approaches for cultivar development. In case of germplasm collections, for example, tall fescue (*Festuca arundineacea* Schreb.) cv. 'Kentucky31', one of the most popular tall fescue cultivars, and 'Raleigh', the most cold-tolerant St. Augustinegrass cultivar, were both selected from plants collected from the field (Maier et al. 1994a). SV is the induction of variants through plant tissue culture. Jain (2001) reported in an review article that 22 cultivars had been released from SV with improved traits including yield, plant architecture, colour, pest resistance, salt and heat tolerance. Examples are: 'He Zu No. 8' wheat (*Triticum aestivum* L.) with high yield, 'Yidan No. 6' maize (*Zea mays* L.) with improved grain quality, 'CIMAP/bio-13' aromatic grass (*Cymbopogon winterianus* Jowitt) with increased oil yield, and 'DAMA' rice (*Oryza sativa* L.) with *Picularia* spp. resistance. However, there has been no report regarding freezing tolerance through SV from tissue culture.

Freezing temperatures that result in ice formation within plant cells can cause multiple types of tissue damage and death of the entire plant under severe conditions (Livingston et al. 2006). During a period of low but non-freezing temperatures in a process called cold-acclimation (Thomashow 1999, Xin and Browse 2000, Livingston et al. 2006), plants can increase their ability to withstand freezing temperatures. In nature, cold-acclimation is initiated by decreasing temperatures in late autumn or early winter.

Selecting plants with increased tolerance to winter freezing is an important aspect of plant improvement. However, fluctuating winter temperatures make it necessary for experiments to be conducted in multiple locations and years (Tcacenco et al. 1989). Such tests are costly and time-consuming. Consequently, procedures have been developed to evaluate freezing tolerance in which plants are acclimated and frozen under controlled conditions. Fuller and Eagles (1978) accurately predicted freezing tolerance of mature plants with controlled freezing treatments of perennial ryegrass (*Lolium perenne* L.) seedlings at the two-leaf stage (14 days after planting). Based on those results, they were able to rank the field survival of cultivars. Anderson et al. (1993) evaluated bermudagrass [*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burt-Davy] genotypes by exposing them to freezing temperature following 4 weeks of acclimation at 8/2ºC in a controlled environment. In St. Augustinegrass, Maier et al. (1994a) acclimated plants in the field and then froze them in a chamber at various temperatures. They found the freezing survival of 'Raleigh' (at $>60\%$), was much better than 'Floratam' and 'FX-332' ($<20\%$). In addition, the inheritance of cold tolerance in St. Augustinegrass was studied by a complete diallel mating with reciprocals (Philley et al. 1998).

The purpose of this study was to develop a system to evaluate freezing tolerance among St. Augustinegrass lines and accessions using controlled acclimation and freezing, and to identify more freezing-tolerant genotypes from germplasm collections and SV induced through tissue culture.

Materials and Methods

Development of a screening protocol for freezing tolerance in St. Augustinegrass: Stolons of 'Raleigh' were collected from the field in August, 2004, and transplanted in four plastic trays (52 × 26 × 6 cm, with 5–10 stolons per tray) filled with potting media (Metro-Mix-200; Scotts, Marysville, OH, USA). They were grown for 6 months in a greenhouse at 25 ± 5°C. Plants were then subjected to

acclimation treatments by placing trays in a growth chamber (Model M36, EGC; Chagrin Falls, OH, USA) at 13ºC with a 12 h photoperiod or 3ºC with a 10 h photoperiod at a light intensity of 285–290 μmol m^{-2} s^{-1} photosynthetically active radiation. The acclimation conditions were designed to simulate the natural acclimation in late fall or winter. Single-node cuttings with leaves and roots trimmed to 3–4 cm were collected after acclimation and washed with tap water and blotted dry. Fifteen such cuttings from each line or accession were placed in a plastic container (13 × 7 × 5 cm) for the freezing treatment. The containers with plants were randomly placed on the second shelf inside a commercial freezer (model FF20, GE; Fairfield, CT, USA) that was modified to allow microprocessor-controlled ramp and soak cooling. Thermocouples were placed inside the container to monitor the air temperature. Ice shavings were added to each box to promote nucleation and prevent supercooling. The temperature of the freezers was lowered at 1°C/h to the target temperature where they were maintained for 3 h. The temperature was then raised to 3°C at 2°C/h and kept there for about four hours before moving the plants to the greenhouse.

Thawed plants were dipped in Daconil® fungicide (active ingredient: chlorothalonil; Syngenta Crop Protection, Greensboro, NC, USA) solution (2.63 ml/l) to prevent fungal diseases, and transplanted into pots (18 × 13 × 6 cm) filled with Metro-Mix-200 media. Recovering plants were grown at 25°C for a month in a greenhouse, and freezing survival was visually assessed on the basis of shoot and root regrowth. A plant with obvious regrowth received a score of 1 (full survival). If shoots were green but had no obvious regrowth, the plant received a score of 0.5 (partial survival), and dead plants received a score of 0. Survival rate was calculated as a percentage of surviving plants among the total treated plants.

This test was conducted twice over time. ANOVA was performed using SAS software (ver.9.1, 2003; SAS Institute, Cary, NC, USA). When significant differences (P = 0.05) were observed, the least significant difference (LSD) test (Steel et al. 1996) was applied to detect differences between treatments.

Freeze test of collected germplasm: 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. Thirty-six germplasm accessions were collected from North Carolina, Virginia, South Carolina and Texas. Each line was planted in Metro-Mix-200 media and maintained in a greenhouse. They were subjected to a preliminary test at −4ºC to screen for lines with freezing tolerance better than, or comparable with 'Raleigh'. Six lines (Table 1) were chosen for further testing and propagated in the field at the NCSU Lake Wheeler Turf Field Lab located in Raleigh, NC. Two standard cultivars, freeze-tolerant 'Raleigh' and freeze-sensitive 'Floratam' were planted as controls. The freeze test was conducted in mid-December, 2005, after plants had experienced natural acclimation. Single-node cuttings of the plants were collected from established field plots, washed and trimmed. A replicate consisted of fifteen cuttings from each accession. The samples were frozen at −4ºC for 3 h as described above. The plants were allowed to recover at 25°C for 4 weeks in a greenhouse. Survival rate was calculated as the percentage of plants that survived among total plants frozen. Three replicates were conducted, and the data were statistically analysed by ANOVA. Differences between accessions were evaluated by an LSD test at the 5% level.

The three accessions with the highest freezing tolerance were further tested. Stolons were grown in the greenhouse at 25 ± 5°C. After 2 months, single-node cuttings of similar size were collected and grown in the greenhouse for a week. This test was replicated four times over time with 20 single-node cuttings in each replicate, and the results were statistically analysed by ANOVA. Differences between accessions were evaluated by an LSD test at the 5% level.

Plant tissue culture: Plant tissue culture was carried out in the summer of 2004 using a previously described protocol (Li et al. 2006), in which calli were induced from immature embryos of 'Raleigh' on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 1 mg/l of 2,4-dichlorophenoxyacetic acid and 0.5 mg/l of 6-benzyladenine or 6-benzylaminopurine (BA), and regenerated on MS medium supplemented with 1 mg/l of BA, 0.2 mg/l of α-naphthaleneacetic acid and 0.5 mg/l of gibberellic acids. Regenerated plantlets were grown in Metro-Mix-200 soil in a lighted culture room at 25 ± 3°C. After 2 weeks, the plantlets were transferred to a greenhouse and maintained with regular watering and trimming. Two grams of Osmocote® slow release fertilizer (Scotts) per pot were applied monthly.

Selection of plants regenerated from tissue culture for freezing tolerance: Screening of freezing-tolerant lines among regenerated plants through tissue culture was conducted in four rounds under controlled conditions. All the plants were subjected to the two-step acclimation protocol (13ºC for a week followed by 3ºC for another week) before the 3 h freezing treatment. The freezing temperature used for the treatment varied from −3 to −5ºC based on the age of the plants. In the first round, approximately 7800 plants, which were about 6 months old, were frozen at −5ºC. The surviving plants were grown in the greenhouse for 2–3 months and then frozen at −4ºC in the second round of screening. For the second and third rounds, 4–8 plants per line were used depending on availability of plant materials. In the 3rd and 4th round, the cuttings were grown for 1 week and subjected to freezing at −3ºC. The fourth round screening test was a replicated one, in which three replicates with 15 cuttings per replicate per line were included. ANOVA and LSD analysis was performed for the fourth round test.

Results

Establishment of a freezing test system in a controlled environment

A preliminary test was conducted by freezing field-acclimated plants of 'Raleigh' at −2, −4, −6 and −8ºC for 3 h. In the

Table 1: Six germplasm collections tested for freezing tolerance

Germplasm	Location	Coordinate	Distinct morphological characteristics	Site information
Co2	Columbus, NC	35°15′2″N, 82°12′8″W	Yellow stigma	Established home lawn near 30 years
Craig	Elizabeth City, NC	36°17′44″N, 76°13′30″W	Purple stigma	Established home lawn (years unknown)
Elm2	Raleigh, NC	35°49′8″N, 78°38′41″W	Yellow stigma	Established home lawn over 20 years
Elm4	Raleigh, NC	35°49′8″N, 78°38′41″W	Yellow stigma	Established home lawn over 35 years
Floratam	Belle Glade, FL	26°41′7″N, 80°40′17″W	Purple stigma	Commercial cultivar
Ray	Columbus, NC	35°15′2″N, 82°12′8″W	Purple stigma, shorter leaf blade and inflorescence, shorter and thinner internodes, lower height	Established home lawn over 42 years
Raleigh	Raleigh, NC	35°49′8″N, 78°38′41″W	Yellow stigma	Commercial cultivar
WS	Kernersville, NC	36°6′58″N, 80°4′55″W	Yellow stigma	Established home lawn over 40 years

preliminary test, −4ºC was selected as the optimum temperature for testing as all the plants were killed at −6 and −8ºC while 90% survived at −2ºC and 33.3% survived at −4ºC. To identify effective cold-acclimation parameters under controlled conditions, four treatments were tested: (i) no acclimation, (ii) 13ºC for 2 weeks, (iii) 3ºC for 2 weeks and (iv) 13ºC for a week followed by 3ºC for another week.

Among the four acclimation treatments, significantly higher survival rates were obtained in cold-acclimated plants of 'Raleigh' over non-acclimated plants followed by freezing at −4ºC for 3 h (Fig. 1). The fourth treatment (13ºC, 1 week plus 3ºC, 1 week) had the highest survival rate (65%). Plants acclimated at 3ºC for 2 weeks had significantly higher survival rates than the non-acclimated plants but not as high as the combination of 13 and 3ºC. Result of acclimation at 13°C was not different from no acclimation. The fourth treatment (referred hereafter as the 'two-step acclimation' protocol) was then selected as the acclimation protocol for further screening experiments.

Evaluation of germplasm accessions

Among 36 germplasm collections, six were selected in a preliminary freezing test for further evaluation. They were Co2, Elm2, Elm4, Craig, Ray and WS (Table 1). These accessions, together with 'Raleigh' and 'Floratam', were grown in the field and were naturally acclimated before being collected in mid-December for freeze testing. Among them, Elm4 had the highest survival rate (73%) after freezing at −4ºC for 3 h, which was comparable with WS, but significantly higher than 'Raleigh' (51%) and other accessions (Fig. 2). WS, Ray and Co2 had survival percentages comparable with 'Raleigh'. The survival rates of Craig, Elm2 and 'Floratam' were significantly lower than 'Raleigh'.

Elm4, WS and Ray were then selected for further tests using the two-step acclimation protocol. Significant differences were observed among the accessions (Fig. 3) and the ranking of them was consistent with that observed previously (Fig. 2). Elm4 still had the best survival rate (41%) and it was still significantly higher than 'Raleigh' (27.5%), the most cold-hardy cultivar commercially available. Ray and WS were similar to 'Raleigh' while the survival rate of 'Floratam' (1.3%) was significantly lower than the other genotypes (Fig. 3). The results also demonstrated the reliability of the two-step acclimation protocol in identifying freezing-tolerant germplasm in St. Augustinegrass.

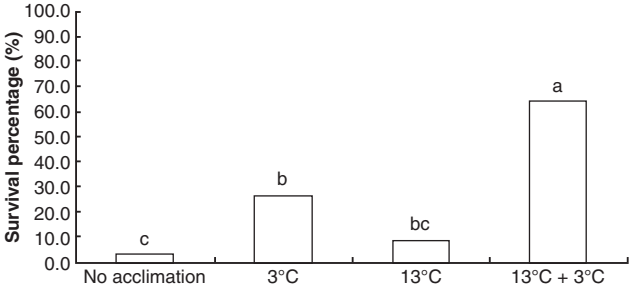

Fig. 1: The effect of cold-acclimation on the survival of 'Raleigh' St. Augustinegrass six weeks after freezing at −4ºC for 3 h. Columns represent means of two replicates with 15 single-node cuttings per replicate of the treatments. Values followed by the same letter were not significantly different from each other according to the least significant difference (P = 0.05)

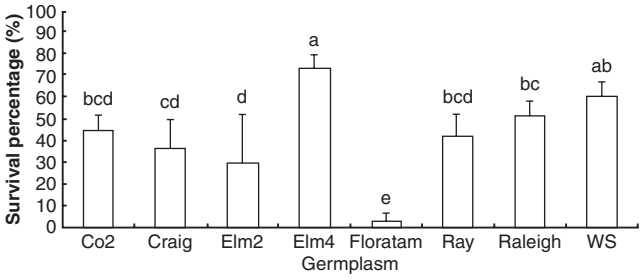

Fig. 2: Survival percentage of eight germplasm accessions frozen at −4°C for 3 h after field acclimation. Columns represent means of three replicates, 15 single-node cuttings per replicate, of the accessions. Values followed by the same letter were not significantly different from each other according to the least significant difference (P = 0.05)

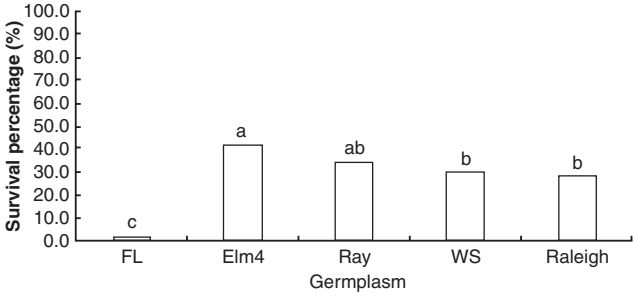

Fig. 3: Survival percentage of five germplasm accessions frozen at −3ºC for 3 h after acclimation under controlled conditions. Columns represent means of four replicates, with 20 single-node cuttings per replicate, of the accessions. Values followed by the same letter were not significantly different from each other according to the least significant difference (P = 0.05)

Screening tissue culture plants for somaclonal variation in freezing tolerance

Approximately 7800 individual plants regenerated from tissue culture of 'Raleigh' were subjected to the two-step acclimation protocol followed by freezing tests in four rounds of screening. In the first round, 380 plants survived. Among them, 15 plants were alive after the second round of screening. Four clones, named SVC1, SVC2, SVC3 and SVC4, showed higher survival rates in the third round screening, and entered the fourth round, where they were evaluated in replicated experiments. The survival rate of SVC3 (60%) was significantly higher than its parent 'Raleigh' (28.9%) (Fig. 4), and is thus a somaclonal variant with improved freezing tolerance.

Discussion

North Carolina is the northern edge of St. Augustinegrass distribution range. 'Raleigh', a release from North Carolina State University, is considered the most cold-tolerant cultivar of the species (Busey et al. 1982). However, the use of 'Raleigh' is limited to areas that rarely experience temperatures lower than −5ºC. Severe freezing injury still occurs in some winters.

In our efforts to develop more freezing-tolerant cultivars of St. Augustinegrass, two sources of plant material were used:

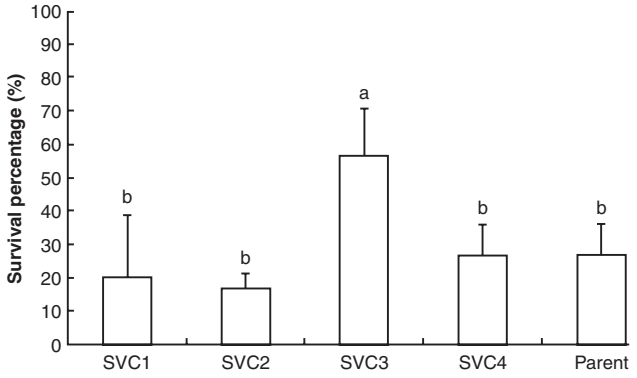

Fig. 4: Survival percentage of four somaclones a month after freezing treatment at −3ºC in the fourth round test. Columns represent means of three replicates, with 15 single-node cuttings per replicate, from the somaclones. *F*-test and least significant difference (LSD) tests were performed among the somaclones. Values followed by the same letter were not significantly different from each other according to the LSD (P = 0.05)

germplasm collected from various locations and somaclonal variants induced through tissue culture. In this project, germplasm collection Elm4 was consistently more freezing-tolerant than 'Raleigh' when different acclimation schemes were employed, while Co2, Ray and WS were comparable to 'Raleigh' in freezing tolerance (Figs 1 and 3). They are all in field trials at various locations for further evaluation of their cold hardiness as well as other traits.

Plant tissue culture often induces SV and has been used in breeding efforts to generate new crop cultivars (Jain 2001). To our knowledge, we are the first to evaluate SV for freezing tolerance in St. Augustinegrass and to apply the approach to St. Augustinegrass breeding (Li et al. 2009). The development of improved tissue culture techniques (Li et al. 2006) made it possible to regenerate nearly 8000 tissue culture plants, which created a potential genetic variation pool. Among them, Li et al. (2009) found 119 morphological somaclonal variants, most of which showed semi dwarf growth habit, and 19 had vigorous growth and demonstrated stability for this trait. In this study, SVC3 was identified as a somaclonal variant with improved freezing tolerance. The survival rate of SVC3 in our experiments (60%) was twice as high as its parent 'Raleigh' (29%) as noted in Fig. 4. In addition, the leaf blade of SVC3 is 52% longer and 14% wider than 'Raleigh' (Li et al. 2009), further supporting that it is indeed a somaclonal variant. Surprisingly, SVC3 is also more susceptible to large patch and grey leaf spot diseases in recent field tests (Reynolds et al. 2009) and probably cannot be directly developed to a new cultivar. It is not clear at this moment whether SVC3 is a single mutation which has pleiotropic effect, or it has multiple loci mutated, or other mechanisms are involved.

Various methods have been developed to predict, or correlate to, freezing tolerance in St. Augustinegrass, which include electrolyte leakage technique (Maier et al. 1994b) and differential thermal analysis (Philley et al. 1995). In our experiments, we measured survival rate 3 weeks after freezing. Cold-acclimation has been shown to be a crucial prerequisite for plants to survive freezing temperatures in nature as well as in laboratory tests (Fig. 1). However, natural acclimation is impossible to duplicate because acclimating conditions vary from year to year. To develop a more controllable procedure for acclimating St. Augustinegrass, we tested three acclimation conditions and found that a two-step process resulted in the best survival rate. In our experiments, the freezing test results from the two-step acclimation protocol and from the natural acclimation were consistent, demonstrating the reliability of this protocol in breeding efforts to develop freezing-tolerant St. Augustinegrass cultivars.

Numerous biochemical changes are induced during cold-acclimation that give plants the ability to survive freezing stress. A comparison of the two-step acclimation protocol described here with a single acclimation period at 3°C indicated that one or more metabolic conditions established at a warmer temperature must be in place before full acclimation at 3°C can occur. Cold-acclimation causes an increase in membrane stability (Cyril et al. 1998, Samala et al. 1998), and cold-regulated protein synthesis (Gatschet et al. 1996) among other biochemical changes. Such research is rare in St. Augustinegrass. In one report, it was observed that a slight increase in stolon sucrose levels occurred when 'Floratam' entered dormancy, but such an increase did not seem to improve freezing tolerance (Fry et al. 1991). 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 homologues of these genes from St. Augustinegrass may facilitate understand freezing tolerance in St. Augustinegrass at the molecular level.

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