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BIOTIC AND ABIOTIC STRESS

In vitro selection of salinity tolerant variants from triploid bermudagrass (*Cynodon transvaalensis* × *C. dactylon*) and their physiological responses to salt and drought stress

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Abstract A protocol was established for in vitro selection of salinity tolerant somaclonal variations from suspension cultured calli of triploid bermudagrass cv. TifEagle. To induce somaclonal variations the calli were subcultured for 18 months and were then subject to three-round selections for salt-tolerant calli by placing on solid medium containing 0.3 M NaCl for 10 days followed by a recovery for 2 weeks. The surviving calli were regenerated on regeneration medium containing 0.1 M NaCl. Three somaclonal variant lines (2, 71, and 77) were obtained and analyzed. The selected somaclonal lines showed higher relative growth and less injury than TifEagle under salt stress, indicating that they increased salt tolerance. In addition, they had higher relative water content and lower electrolyte leakage than TifEagle after withholding irrigation, indicating that they also increased drought tolerance. The three somaclonal variant lines had higher proline content than TifEagle under normal growth condition. The line 71 had a higher K^+/Na^+ ratio, whereas the lines 2 and 77 had higher CAT activity under control and salt stress conditions, indicating that different mechanisms for salt tolerance might exist in these three lines.

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Abbreviations

BA	6-Benzyladenine
CAT	Catalase
DW	Dry weight
SOD	Superoxide dismutase
RWC	Relative water content

Introduction

Bermudagrass (*Cynodon dactylon*) is one of the most important warm-season turfgrass species in temperate and tropical regions. Common bermudagrass (*C. dactylon*) is a fertile tetraploid ($2n = 4x = 36$), whereas hybrid bermudagrass (*C. dactylon* × *Cynodon transvaalensis*) is a F_1 sterile triploid ($2n = 3x = 27$) and is propagated vegetatively. Tifeagle is a recently released triploid bermudagrass cultivar, which produces tighter and denser turf with excellent close mowing tolerance (Hanna and Elsner 1999) and is mainly used in greens of golf courses. As sterile triploids, conventional hybridization breeding procedures cannot be applied for their genetic improvement on tolerance to abiotic stresses, such as salinity and drought.

Callus induction and plant regeneration from young influorescences of common and hybrid bermudagrass was investigated by several groups (Ahn et al. 1985, 1987; Artunduaga et al. 1988, 1989; Chaudhury and Qu 2000; Li and Qu 2002; Lu et al. 2003a, b). Embryogenic callus has been induced from vegetative tissues, such as nodal segments of the triploid bermudagrass cultivars, Tifgreen (Chaudhury and Qu 2000; Li and Qu 2002, 2004), Tifway

(Qu and Chaudhury 2001), and TifEagle (Zhang et al. 2003; Lu et al. 2003a; Goldman et al. 2004; Hu et al. 2005). An efficient embryogenic callus suspension culture and regeneration system has been established in our group (Lu et al. 2006), which facilitates improvements on triploid bermudagrass through somaclonal variation and transformation.

During the establishment of tissue culture protocol of TifEagle, we noticed a high frequency of the somaclonal variation. Somaclonal variations in TifEagle have already been observed in our and other studies (Lu et al. 2003a, 2006; Goldman et al. 2004). Here we report the establishment of a procedure for selection of salt tolerant variants. The physiological responses of the stress tolerant variant lines were also studied.

Materials and methods

Plant material and embryogenic callus production

Triploid bermudagrass cultivar 'Tifeagle' (*C. dactylon* × *C. transvaalensis*) was planted in a greenhouse. Nodes of stolons were employed to induce callus according to the protocol described before (Lu et al. 2003a). Blade and leaf sheath were removed and the node segments were cut from stolons and sterilized with 70% ethanol for 30–60 s, and then soaked in 0.1% $HgCl_2$ solution for 5–6 min, followed by five rinses with the sterilized distilled water. The sterilized nodes were sliced into 5 mm segments, which were placed on callus induction medium-NB medium (N6 macro-elements and B5 micro-elements, EDTA-Fe) supplemented with Gamborg's vitamin, 6.6 mg/l dicambar, 0.6 mg/l BA, 0.5 g/l casein hydrolysate, 0.5 g/l glutamine, 0.5 g/l proline, 0.1 g/l inositol, 30 g/l sucrose, and 7 g/l agar, and incubated at 26 ± 2°C in the dark for embryogenic callus induction within 4 weeks. The embryogenic calli were subjected to suspension culture for 18 months according to the protocol described before (Lu et al. 2006).

Selection of salt tolerant callus and regenerated plantlet

The suspension-cultured calli were inoculated on a solid selection medium supplemented with 0.3 M NaCl and cultured for 10 days for selection of salt-tolerant calli. The calli were then transferred to a normal medium without NaCl and cultured for 15 days for recovery growth. The surviving calli were again transferred to the selection medium for the second round selection (the same procedure as the first). After three round selections, the surviving calli were transferred to a normal medium and cultured for additional 2 weeks. Finally, the surviving calli were transferred to a regeneration medium for plantlet regeneration. The regeneration medium contained NB basal components and was supplemented with Gamborg's vitamin, 0.6 mg/l BA, 0.5 g/l casein hydrolysate, 0.5 g/l glutamine, 0.1 g/l inositol, and 30 g/l sucrose, and 7 g/l agar (Lu et al. 2006), plus 0.1 M NaCl. The plated calli were illuminated with a 16 h photoperiod (80 μmol/m^2/s) at 26 ± 2°C. The regenerated plantlets were transferred to a new regeneration medium without any phytohormone for rooting.

Plant growth and experimental design

The regenerated plants were transplanted to 5-cm diameter plastic pots containing mixture of peat and perlite (3:1, *v/v*) and grown in a growth chamber, with a 16 h photoperiod (200 μmol/m^2/s) at 26 ± 2°C, for 2 weeks. The pot plants were then transferred to a greenhouse at temperature of 20–30°C under natural light for 2-months growth. Plants were then transplanted into pots (15 × 15 cm) in a greenhouse under natural light with routine management by daily irrigation, weekly mowing and biweekly fertilizing with 0.3% solution of N-P-K fertilizer (15-15-15). Partial stonlons were detached from the pot plants and planted in a new pot for proliferation vegetatively in the greenhouse.

The sods with similar area produced in the pots were transplanted into new pots, and were grown for 1 month in the greenhouse with routine management. After 2 weeks post mowing, the plants were placed in a completely randomized design and were exposed salinity or drought stress in the greenhouse under natural light. The experiment consisted of four plant lines and two treatments, irrigation versus salinity or drought stress. Each sample for measurements was from the plants in one pot. Four pots were used as replication for each of plant lines. The presented data were the means of four independent measurements.

Salinity treatments and salt tolerance measurements

For measurement of tolerance to salt stress-induced damage, the bemudagrass plants after mowed for 2 weeks were subjected to salinity treatment by irrigating 50 ml of 0.25 M NaCl solution once every 2-day and irrigating 50 ml of water on the other day. Those plants irrigated by 50 ml of water daily were used as control. The treatment would allow the soil to accumulate elevated levels of salt gradually. The shoots were harvested at 12 days post salt treatment for analysis of proline, K^+, Na^+, and enzyme activity.

For measurement of growth, the plants were mowed and then were subjected to salinity treatment by irrigating 50 ml of 0.15 M NaCl solution once every 2-day and irrigating 50 ml of water on the other day. Those plants

irrigated by 50 ml of water daily were used as control. Plants were not damaged at 0.15 M NaCl, but their growth was inhibited. After treated for 12 days, the shoots were harvested and dried at 105°C for 1 h, followed at 80°C for 12 h, to obtain dry weight. The relative growth was calculated as percentage of dry weight under the salt stress over that under water treatment.

Determination of proline, Na^+ and K^+

Fresh shoots were harvested at day 12 following salt treatment with 50 ml of 0.25 M NaCl solution and dried at 105°C for 1 h, followed at 80°C for 12 h. The dried samples were powdered in a mortar with a pestle, and used for determination of Na^+, K^+ and proline. A 100 mg samples were extracted in 1M HCl for 3 h. The solutions were analyzed for K^+ and Na^+ content by atomic absorption spectrometry (Qian et al. 2000). For measurement of praline, 50 mg dried powder samples were extracted at least 1 h in 3 ml of 80% ethanol. After filtrated, the filtrate was incubated in a boiling water bath to evaporate the ethanol, and distilled water was then added to make the final volume to 10 ml. The solution was shaken for 10 min after addition of Permutit, and then was filtrated. Into 2.5 ml of filtrate, 2.5 ml of acetic acid glycial and 2.5 ml ninhydrin solution (2.5 g ninhydrin dissolved in 60 ml of acetic acid glycial and 40 ml of 6 M phosphoric acid) were added. The mixture was incubated in a boiling water bath for 1 h, and then was extracted with 2.5 ml of benzene by shaking them vigorously for 5 min. The phases were allowed to separate, and the benzene phase was used to determine the absorbance at 515 nm (Troll and Lindsley 1955). Proline concentration was calculated as to the standard curve.

Determination of superoxide dismutase and catalase activity

Fresh shoots (0.1 g) were ground in a mortar with a pestle in 3 ml of 50 mM phosphate buffer (pH 7.8) at 4°C. The homogenate was centrifuged at 13,000×*g* for 15 min. The supernatant was recovered for determination of superoxide dismutase (SOD) and catalase (CAT) activity as we described before (Zhou et al. 2005). For determination of SOD activity, the 3-ml reaction solution contained 13 μM methionine, 63 μM *ρ*-nitro blue tetrazolium chloride (NBT), 1.3 μM riboflavin, 50 mM phosphate buffer (pH 7.8), and the enzyme extract. The reaction solution was incubated for 10 min under fluorescent light with 80 μmol/m^2/s. Absorbance was determined at 560 nm by a spectrophotometer (Model UV-2010, Hitachi, Japan). One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of NBT by 50%. For determination of CAT activity, the 3-ml reaction solution contained 15 mM H_2O_2, 50 mM phosphate buffer (pH 7.0), 50 μl of the enzyme extract. The reaction was initiated by adding the enzyme extract, and the decreased absorbance of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) within 1 min at 240 nm was recorded. One unit of CAT activity was defined as the amount of enzyme required for catalyzing the conversion of 1 μmol H_2O_2 into water per min. Protein content in the enzyme extracts was determined according to the method of Bradford (1976).

Drought treatment and measurement of ion leakage and relative water content (RWC)

Plants were grown in pots (10 × 10 cm) with routine management by daily irrigation, weekly mowing and fertilizing with a 15-15-15 (N-P-K) fertilizer in a greenhouse under natural light. Plants were subjected to drought stress by withholding irrigation. TifEagle became wilting in 4 days. Ion leakage and RWC were determined at day 5 after withholding irrigation as described before (Lu et al. 2003b). For measurement of ion leakage, leaf samples were rinsed with distilled water and immersed in 10 ml of distilled water for 12 h. The conductivity of the solution (R1) was measured using a conductivity meter (Model DDS-11A, Shanghai Leici Instrument Inc., Shanghai, China). Samples were then heated in boiling water for 20 min, and then cooled to room temperature. The conductivity of killed tissues (R2) was again measured. Ion leakage was calculated as the ratio of R1–R2. For measurement of RWC, fresh leaves were weighed (W_f) and rinsed in water overnight until the weight of the leaves became constant. The water-saturated leaves were weighed (W_S) and then dried for 24 h at 80°C for determination of the dry weigh (W_d). RWC was calculated by the formula: $RWC = (W_f - W_d)/(W_S - W_d) \times 100\%$.

Statistical analysis

All data were subjected to analysis of variances according to the model for completely randomized or a split plot design using a SPSS program (SPSS Inc.). Differences among treatment means were evaluated by the least significant difference (LSD) test at a 0.05 probability level when the *F*-test showed a significant ($P \leq 0.05$) effect.

Results

In vitro selection of salt tolerant calli and regenerating plantlets

During the establishment of embryogenic callus suspension culture in TifEagle, the regenerating plantlets showed so-

maclonal variations (Lu et al. 2006). In order to induce somaclonal variations, the calli were subcultured for 18 months. The calli were then placed on a solid selection medium supplemented with 0.3 M NaCl for 10-days selection. Most of the calli became brown and died. Only 10% of the calli survived after the selected calli were transferred to a new medium without NaCl for recovery growth for 2 weeks. The living calli were further subjected to the second round selection on the selection medium containing 0.3 M NaCl for 10 days. Half of the calli survived after the selected calli were transferred onto a new medium without NaCl for 2 weeks in recovery growth. Seventy percentage of the calli survived after the third round selection as above. Finally, the surviving calli were transferred to a regeneration medium supplemented with 0.1 M NaCl (Fig. 1a–c). One third of the calli could be regenerated. Most of the regenerating plants, such as line 77, exhibited similar morphological characteristics to TifEagle, whereas partial plants decreased growth under normal condition and decreased leaf width, such as in line 71.

Salt tolerance of somaclonal variants

Leaf firing usually occurred when turfgrass plants were subjected to salt treatment (Qian et al. 2000; Marcum 1999). In order to screen salt tolerant lines from the regenerated plants, plants were irrigated with 50 ml of 0.25 M NaCl solution once every 2 days, by which plants were allowed to have 1 day for adaptation to the gradually-elevated accumulation of salt in the soil. Leaf firing initially occurred in TifEagle at day 3 after salt treatment, and increased gradually with treatment time. Among the tested 23 somaclonal variant lines, three lines (lines 2, 71, 77) showed less injury caused by salt stress. At day 10 after salt stress, 22, 18 and 8% of leaf area showed leaf firing in lines 2, 71, and 77, respectively, in comparison to 42% in TifEagle. The leaf firing symptom in line 77 and its parent cultivar, TifEagle, is shown in Fig. 1. Line 77 maintained green and had less injury after 0.25 M NaCl treatment in comparison to TifEagle (Fig. 1d).

Relative growth is another indicator of salt tolerance (Munns 2002). In order to measure the relative growth of plants without damage, NaCl concentration was reduced to 0.15 M as salinity treatment. The somaclonal variant lines had lower dry weight of shoots than TifEagle under normal condition (Fig. 2a), indicating that their growth was reduced. Under salt stress of 0.15 M NaCl, lines 2 and 77 had similar or slightly higher dry weight of shoots than TifEagle, whereas the dry weight of line 71 was still lower than TifEagle (Fig. 2a). The calculated relative growth of TifEagle was 66.5% (Fig. 2b), indicating that its growth was greatly reduced by the salt stress. The relative growth in lines 2, 71, and 77 was at a level of close to or higher than 100%, indicating that their growth was not inhibited by salt stress (line 2) or even slightly improved (lines 71

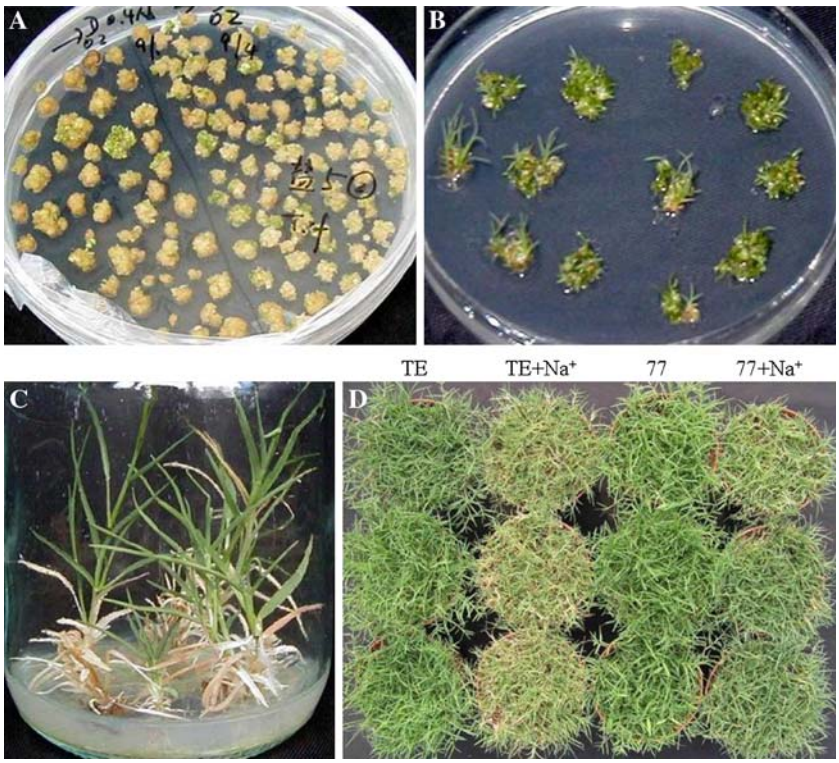

Fig. 1 In vitro selection for salt tolerant plants of triploid bermudagrass cv. TifEagle. The survived calli selected in 0.25 M NaCl were placed on a regeneration medium contained 0.1 M NaCl and were illuminated for regeneration (**a**). The greening buds were transferred to a new medium containing 0.1 M NaCl (**b**). Regenerated plantlets were placed on a rooting medium (**c**). One salt tolerant regenerated line and TifEagle were treated with 0.25 M NaCl solution every 2-day for salt tolerance test (**d**). *TE*: TifEagle; *77*: line 77; $+Na^+$: NaCl treatment

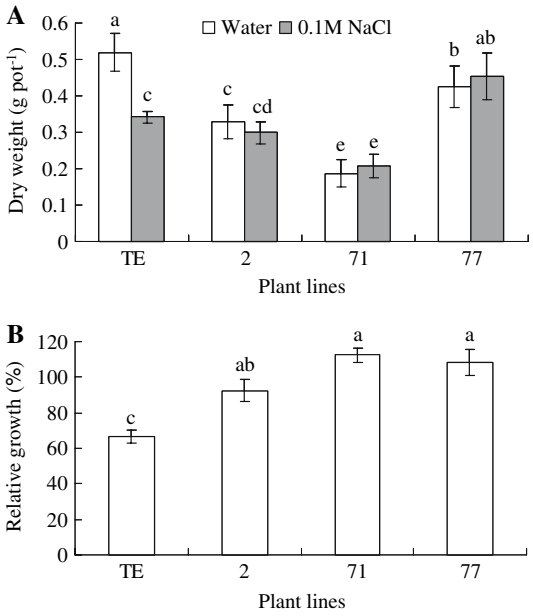

Fig. 2 Salt tolerance of the somaclonal variant lines in comparison to TifEagle (*TE*). The dry weights of shoots were obtained after plants were treated for 12 days by irrigating water as a control, or 0.15 M NaCl solution every 2-day as salt stress (**a**). The relative growth (**b**) was calculated as the percentage of dry weight under salt stress to the dry weight under normal condition. *Different letters* indicate statistically difference at $P < 0.05$ level according to Fisher's LSD ($\alpha = 0.05$) test

and 77) (Fig. 2b). The above leaf firing and relative growth data results indicated that lines 2, 71 and 77 increased salt tolerance. In order to investigate the mechanism of salt tolerance, praline, K^+/Na^+ ratio, and antioxidant enzymes were further comparatively determined in the three variant lines and TifEagle after treatment with 0.25 M NaCl solution.

Comparison of salt tolerant lines with TifEagle in proline content

Proline content was very low (14.1 μg/g DW) in TifEagle under control condition, while that was increased by 2.9–11.1 folds in the three somaclonal variant lines. Proline accumulation occurred in all of the plants after salt treatment. In TifEagle proline accumulation increased 57.4 folds, while in the somaclonal variant lines proline accumulation increased only 2.9–20.7 folds (Fig. 3).

K^+, Na^+ content comparison of the salt tolerant lines with TifEagle

The contents of Na^+ and K^+ were at 1.7–2.5 mg/g DW and 18.2–24.9 mg/g DW in the control plants, respectively. After the salt treatment for 12 days, Na^+ increased to 15.8–18.4 mg/g DW, and K^+ decreased to 12.4–17.5 mg/g DW. The calculated K^+/Na^+ ratio ranged at 7.4–14.8 in the control plants, but it was 0.74–1.01 in the salt-stressed plants (Fig. 4). The line 71 had higher levels of K^+ and a higher ratio of K^+/Na^+ than TifEagle under both the control and salt stress conditions. The contents of K^+ and Na^+ and K^+/Na^+ ratio in lines 2 and 77 were not significantly different from those in TifEagle under both the control and salt stress conditions (Fig. 4).

Antioxidant enzymes activity

The line 77 had a higher SOD activity than TifEagle under the control condition. SOD activity in the other two lines was not different significantly from TifEagle under the control and salt stress conditions (Fig. 5a). Lines 2 and 77 had a higher CAT activity than TifEagle under both control and salt stress conditions, whereas line 71 was not different from TifEagle in CAT activity under both the control and salt stress conditions (Fig. 5b).

Drought tolerance of the salt tolerant lines

The somaclonal variant lines were tested for drought tolerance by measurement of RWC and ion leakage after withholding irrigation. All of the three lines had higher RWC and lower ion leakage than TifEagle at day 5 after the plants were not irrigated, indicating that these lines developed an increased drought tolerance in comparison to TifEagle (Fig. 6).

Discussion

In vitro selection is an efficient, rapid and low cost breeding method. By using this method, several lines of *Brassica napus* with improved resistance to *Sclerotinia sclerotiorum* have been selected (Liu et al. 2005). Salt-tolerant plantlets of sugar beet (*Beta vulgaris*) were selected on the medium containing NaCl after the multiple bud clumps were γ-irradiated (Yang et al. 2004). Somaclonal variation occurs in the triploid bermudagrass (Goldman et al. 2004; Lu et al. 2003a, 2006). Somaclonal variation during tissue culture in triploid bermudagrass is a common phenomenon. In this paper, somaclonal variations were induced by long-time (18 months) subculture of embryogenic calli, from which salt-tolerant calli were selected on the medium containing NaCl. Three salt tolerant lines were identified from the regenerated plants. The growth of line 77 is slightly higher than that of TifEagle under the salt stress, indicating its potential application. The three lines were tolerant to the drought stress. Similarly, alfalfa lines originally in vitro selected as tolerant to

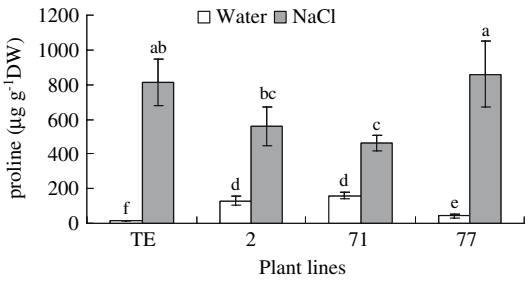

Fig. 3 Proline contents of the salt tolerant lines in comparison to TifEagle (*TE*) under control and salt stress. Proline was measured at day 12 after the plants were irrigated with 0.25 M of NaCl solution every 2-day or water as a control. *Different letters* indicate statistically difference at $P < 0.05$ level according to Fisher's LSD ($\alpha = 0.05$) test

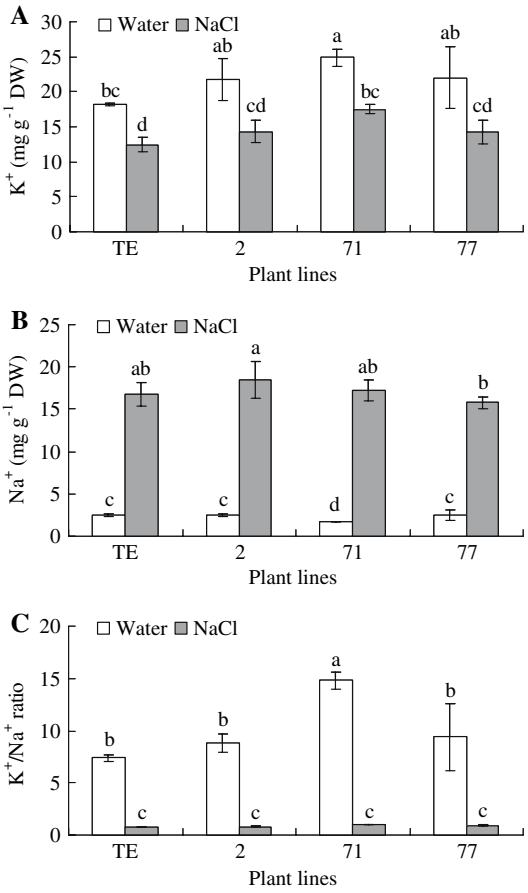

Fig. 4 K^+ (**a**), Na^+ (**b**), and K^+/Na^+ ratio (**c**) of the salt tolerant lines in comparison to TifEagle (*TE*) under control and salt stress. Na^+ and K^+ contents in shoots were measured at day 12 after the plants were irrigated with 0.25 M NaCl solution every 2-day or water as a control. *Different letters* indicate statistically difference at $P < 0.05$ level according to Fisher's LSD ($\alpha = 0.05$) test

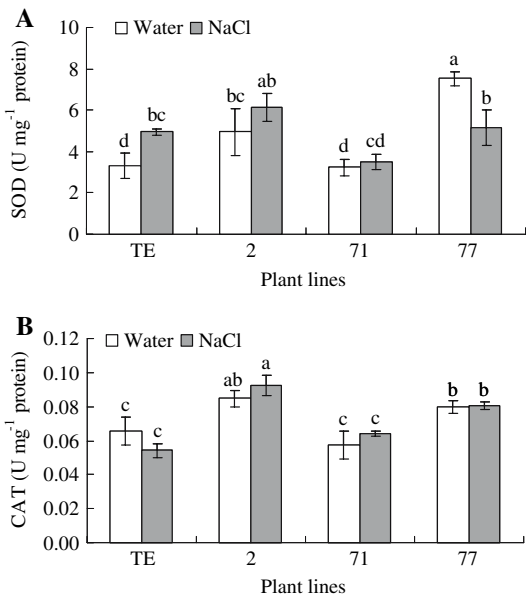

Fig. 5 SOD (**a**) and CAT (**b**) activities of the salt tolerant lines in comparison to TifEagle (*TE*) under control and salt stress. Enzyme activities were measured at day 12 after the plants were irrigated with 0.25 M of NaCl solution every 2-day or water as a control. *Different letters* indicate statistically difference at $P < 0.05$ level according to Fisher's LSD ($\alpha = 0.05$) test

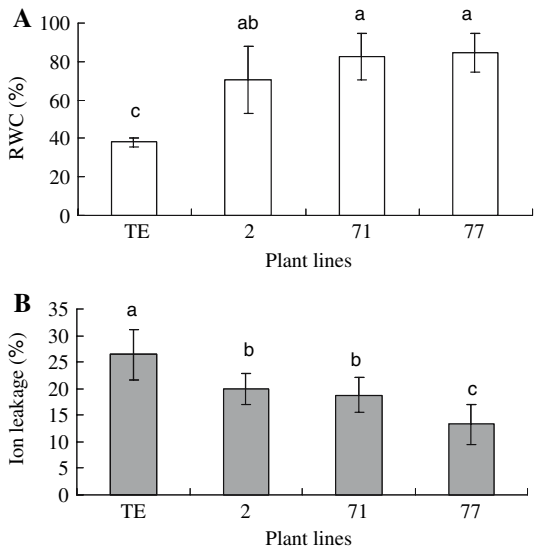

Fig. 6 RWC and ion leakage of the salt tolerant lines in comparison to TifEagle (*TE*) under withholding irrigation. RWC (**a**) and ion leakage (**b**) were measured at day 5 after withholding irrigation. *Different letters* indicate statistically difference at $P < 0.05$ level according to Fisher's LSD ($\alpha = 0.05$) test

osmotic stress have a higher salt tolerance (Djilianov et al. 2003). Some common mechanisms of salt and drought tolerance exist in plants, such as osmotic adaptation, anti-oxidant defense system, signal transduction, and regulation of genes expression (Munns 2002; Zhu 2001; Bartels and Sunkar 2005). The results of this study demonstrate that in vitro selection is a useful protocol for selection of abiotic stress tolerance in triploid bermudagrass.

Increasing salt tolerance has two main types of mechanisms: minimizing the entry of salt into the plants and minimizing the concentration of salt in the cytoplasm (Munns 2002). Plants may restrict uptake of ions like Na^+ and Cl^- or take up only selective ions to maintain a higher K^+/Na^+ ratio when they are exposed to salt stress (Maathuis and Amtmann 1999). Higher level of K^+ and lower level of Na^+ are maintained in the callus of salt-tolerant cultivar than in the sensitive cultivar of rice (Basu et al. 2002). The higher levels of K^+ and K^+/Na^+ ratio in line 71 are associated with its higher tolerance to salt stress. Higher K^+/Na^+ in shoots contributed to the salt tolerance in Kentucky bluegrass cultivars (Qian et al. 2001). The K^+/Na^+ in the lines 2 and 77 are not significantly different from that in TifEagle, indicating that they might have other mechanisms for salt tolerance.

Proline is the most common organic compatible solute in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole. In vitro selected salt tolerant callus lines of maize have a higher proline content (Voetherg and Sharp 1991). All of the salt tolerant lines of triploid bermudagrass selected in this study have a higher level of proline than TifEagle under normal condition. The high levels of proline may improve the osmotic adaptation and protect the plants against the salt or drought-induced damages. Proline is accumulated significantly under salt or drought stress (Matthioni et al. 1997) and is suggested as an indicator of stress tolerance (Dubey and Rani 1989). Less proline is accumulated in salt tolerant cultivars than in sensitive cultivars under salt stress in some crops, such as sunflower (Mutlu and Bozcuk 2005), rice (Vaidyanathan et al. 2003), wheat (Colmer et al. 1995) and turfgrasses (Marcum 1999; Qian et al. 2001). The significant increase in proline content under salt stress might indicate the physiological responses of plants to the stress-induced injury.

Antioxidant defense system is positively associated with the salt tolerance in plants. SOD dismutes oxygen radicals into H_2O_2 (Bowler et al. 1992). H_2O_2 is still a reactive oxygen species and toxic to cells. It is metabolized by catalase or ascorbate-peroxidase in plants (Noctor and Foyer 1998). The salt-tolerant cultivar of rice has higher CAT activity than sensitive cultivar under normal growth conditions, and CAT activity is increased in either salt-tolerant or sensitive cultivars under salt stress (Vaidyanathan et al. 2003). There is no difference in SOD and CAT activities between salt tolerant and sensitive cultivars in mulberry, but activities of these two enzymes are increased to a higher level in salt tolerant cultivar than in sensitive cultivar under salt stress (Sudhakar et al. 2001). In this experiment, the line 77 has higher SOD activity than TifEagle under control condition, whereas lines 2 and 77 have higher CAT activity under control and salt stress conditions. The higher SOD and CAT activities in these salt tolerant lines would scavenge active oxygen radicals more effectively and thereby protect them against salt or drought stress-induced oxidative damages.

In conclusion, three lines of triploid bermudagrass with increased salt and drought tolerance have been obtained through in vitro selection. The results demonstrate the effectiveness of this in vitro selection protocol in triploid bermudagrass improvements. In comparison to TifEagle, all of the three lines have a higher level of proline. The line 71 has a higher K^+/Na^+ ratio, while the lines 2 and 77 have a higher CAT activity under control and salt stress conditions, indicating different mechanisms for salt tolerance might exist in these three selected lines.

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