CHAPTER V

Callus Level and Whole Plant Microculture Selection for More Salt Tolerant 'Seaside' Creeping Bentgrass

Abstract

More efficient methods for identifying salt tolerant turfgrasses are needed, since salt from irrigation is accumulating in soils. Also it would be desirable to irrigate turf or forage grasses with saline water where better quality water is not available or to save better quality water for salt sensitive crops or human use. In this study, putative salt tolerant Seaside creeping bentgrass cell lines were isolated via callus culture, plants were regenerated via somatic embryogenesis, and salt tolerance screening was repeated at the whole plant microculture (WPMC) level to verify the stability of salt tolerance traits by testing the root number, root area and shoot area of the WPMC regenerated plants by video image analysis. The putative salt tolerant plants selected from callus were unstable in terms of whole plant traits, as revealed in the subsequent WPMC screen. The tolerance mechanisms of salt tolerant regenerates requires further study over a number of seed generations.

Introduction

Creeping bentgrass (*Agrostis palustris* Huds.) is an outstanding cool season species used for golf greens, and bowling greens maintained at 0.2 to 0.3 inches cutting height. It is one of the most saline-tolerant of the cool season turfgrasses (Beard, 1973). Seaside is a creeping bentgrass cultivar widely planted near the seashore, but it can not survive on soil which contains 120 meq L^{-1} of $CaCl_2$ and NaCl (260 meq L^{-1} total salts in seawater)(Youngner et al., 1967). However, callus induction in creeping bentgrass has been achieved from mature embryos by incubating in MS media (Murashige and Skoog, 1962) with 2,4-D as a hormone source, and regenerated plants have been obtained on hormone free MS media (Krans, 1981; Zhong et al., 1991). However, there are no reports succeed to select salt tolerant whole plants via cell culture of this species (Torello, 1985). In vitro selection for salt tolerant cell lines has been conducted for tomato (Hassan and Wilkins, 1988), wheat (Mohmand and Nabors, 1990; Trivedi et al., 1991), barley (Ye et al., 1987), tobacco (Chandler et al., 1988, 1986; Nabors, 1983; Pua and Thorpe, 1986) and Kentucky bluegrass (Torello, 1985). Single step exposure of induced callus to the selective agent has been used to select a desirable genotype (Dyer et al., 1988; Gonzales and Widholm, 1985; Maliga el al., 1973). A whole plant microculture (WPMC) system has recently been developed which permits intact root growth observation through the culture medium and vessel (Pieper and Smith, 1988; Smith et al., 1990). In this report, in order to efficiently screen for potential cell-level salt tolerance, a single-step selective method from callus culture was used to isolate putative salt tolerant lines. Plantlets were regenerated and re-screened in a WPMC system to determine if cell-level selection resulted in plants with whole plant level tolerance.

The morphology of callus and plant regeneration was studied using scanning electron microscopy.

Materials and Methods

Salt screening at the callus level. Before dehusking, seeds of Seaside creeping bentgrass were surface sterilized with 70% ethanol for 2 min and immersed in 1.05 % NaOCL for 5 min, then rinsed in sterile distilled water 4 times. Twenty dehusked caryopses were cultured on 30 ml MS medium with 30 g L^{-1} sucrose, 8 g L^{-1} agar, and 0, 1, 5, or 10 mg L^{-1} 2,4-D in 15 x 100 mm disposable petri dishes sealed with Parafilm. There were 80 replications per treatment, and the experiment was repeated 1 time. All cultures were incubated in the dark at 24 ± 1°C for 4 wk in a growth chamber (Percival, Model I-37LL, Boone, IA). Callus area was then determined at this stage using video image analysis. Sealed petri dishes were placed on a clear glass stage above a diffused cool white fluorescent lamp and viewed at a distance of 31 cm through a 105-mm focal length Kiron lens with a Sony (Paramus, NJ) AVC-D1 CCD video camera rigidly mounted on a copy stand. Video images were captured with an Imaging Technology (Woburn, MA) FG-100-AT digitizer housed in an IBM PC/AT and operated with Image Pro imaging software (Media Cybernetics, Silver Springs, MD). Twelve samples were taken randomly from each treatment for measurement. Mean separation was analyzed by LSD (Student's t) at the 5% level of significance. Since the measurement was non-intrusive, the callus was subsequently separated from the initial explant and subcultured for use in maintenance and in regeneration experiments. The experiments were conducted twice with similar results; therefore, data from only one experiment is presented. After 4 wk, callus was transferred to 30 ml fresh MS medium

with 1 mg L^{-1} 2,4-D in 15 x 100 mm disposable petri dishes, and incubated under 24-h cool-white fluorescent lighting (36 µmol m^{-2} s^{-1}) at 26 ± 2°C. After 4 wk, green tissue was generated on the surface of the callus, 0.5 ± 0.1 g of this callus was exposed to 20 ml media with 0.1 mg L^{-1} 6-(r,r-dimethylallylamine) purine (2iP) and 0, 0.5%, 1.0%, or 1.5% Na_2SO_4 in a pyrex text tube, and incubated in the same environmental conditions as for subculture. After an additional 4 wk, regenerated plantlets were transplanted into soil:peat:vermiculite (1:1:1 v:v:v) mix in 170 cm^3 plastic pots, then acclimated in a high-humidity growth chamber. After 7-d, the transplants were transferred to a glasshouse under mist (5 sec at 10 min intervals) for 1 week, then to a greenhouse bench for further growth (salt free) for 9 wks (25^0C/20^0C day/night).

For scanning electron microscopy (SEM), callus was fixed in 2% (v:v) glutaraldehyde in 0.2M phosphate buffer (pH 7.2) for 2-h and washed in the same buffer 3 times. Specimens were dehydrated in an ethanol series (10%, 25%, 50%, 75%, 85%, 95% for 10-15 min at each level, then 2-3 times in a 100% ethanol solution at 10 min intervals), and dried to the critical point in CO_2 (Samdri-790, Tousimis Research Corporation, Rockville, MD). Specimens were coated with gold-palladium with a SPI sputter (Division of Structure Probe Inc., West Chester, PA) prior to examination with a scanning electron microscope (AmRay-1000A, Santa Clara, CA).

WPMC screening test. The same developmental stage of nodal stolon segments (2.5-3.0 cm) from seedlings, non-selected plants (regenerated from callus grown on Na_2SO_4-free medium), and selected plants (regenerated from callus grown on Na_2SO_4-containing saline stress medium) was harvested from the plants in the greenhouse. Nodal explants were sterilized in 70% ethanol for 10 sec, followed by 10 min in 1.05% NaOCL, then rinsed in sterile distilled water 4 times. Each explant was placed in a pyrex test tube containing 20 ml of media with 30 g L^{-1} sucrose,

1 mg L^{-1} indole acetic acid (IAA), 2.5 g L^{-1} gelrite, and either 0, 0.5%, 1.0%, or 1.5% of Na_2SO_4. Nodes from seedling plants were explanted only on salt free or 1.0% Na_2SO_4 medium. Nodes from selected plants from each putative salt tolerant plant were explanted on both salt free medium, and on medium with the same Na_2SO_4 concentration used during callus regeneration. Non-selected plants were explanted on all media formulations for comparison. Cultures were incubated for 4 wk under the same environmental conditions as used during plant regeneration. There were 20 replications per treatment, and the entire experiment was repeated once.

Evaluations of root number, root area (mm^2), and shoot area (mm^2) of each WPMC were made using video image analysis as previous described. Mean separation was analyzed by Student's t test, 5% level. Since the results of both experiments were similar, data from only one experiment is presented.

Results and Discussion

Salt screening at the callus level. Yellow callus was induced from the axes of embryos in 2,4-D treated media in 1 wk (Fig. 14). The explants on 1 mg L^{-1} 2,4-D medium showed significantly faster growth (63.1 mm^2) than those on 5 mg L^{-1} (34.6 mm^2) and on 10 mg L^{-1} (24.8 mm^2) after 4 wk. The seeds on 2,4-D free medium only germinated and did not produce callus. At the initial stage, only watery calli were observed; compact callus was enhanced after subculture for 1 or 2 passages. This type of compact callus demonstrated a high frequency of somatic embryogenesis on plant regeneration medium (Fig. 15a, 15b). Plantlets were regenerated from both the Na_2SO_4 free (153 plants) and stress (91 plants from 0.5%, 136 plants from 1.0%,

25 plants from 1.5%) regeneration medium within 5 months. The agar solidified Na_2SO_4 stress medium supplied a simple approach to select Na_2SO_4 tolerant plants in our experiment. Our selective method could avoid the cell suspension cultural problems of lost regenerative competence in the selected cell lines (Hassan and Wilkins, 1988).

WPMC screening test. Microculture plants could be generated on WPMC medium and the growth of roots and shoots were viewed through gelrite solidified medium (Fig. 16). The mean root number, root area and shoot area of WPMC plants derived from seedlings, or plants regenerated from callus grown on 0, 0.5%, 1.0% or 1.5% Na_2SO_4 stress media were exhibited in Table 4. The root number of seedling plants and non-selected plants grown on Na_2SO_4 free WPMC medium were significantly greater than selected plants. However, the number of roots was not related to root area and shoot area. The root area of seedling plants was markedly lower than selected plants; similarly, the shoot area of seedling plants was markedly lower than the 1.0% Na_2SO_4 selected plants when grown on Na_2SO_4 free WPMC medium. The non-selected plants showed significantly greater root area and shoot area than 0.5% Na_2SO_4 selected plants when grown on 0.5% Na_2SO_4 stress WPMC medium, but there were not significant differences in root area between the two treatments. The root number of seedling plants grown on 1.0% Na_2SO_4 stress WPMC medium was significantly greater than the non-selected and 1.0% Na_2SO_4 selected plants, but the root area of seedling plants was markedly lower than for non-selected plants and 1.0% Na_2SO_4 selected plants; the shoot area of 1.0% Na_2SO_4 selected plants was significantly greater than seedling plants. The seedling plants generated only short root systems and terminated extension growth on 1.0% Na_2SO_4 stress medium after 10 to 14 days. The growth of non-selected plants and 1.5% Na_2SO_4 selected plants showed no marked differences when

grown on 1.5% Na_2SO_4 stress WPMC medium. The root number of non-selected plants grown on Na_2SO_4 free WPMC medium was significantly greater than for plants grown on Na_2SO_4 stress WPMC medium. However, no marked differences in root area and shoot area were noted when plants were grown on Na_2SO_4 free and Na_2SO_4 stress WPMC medium. The Na_2SO_4 stress selected plants grown on both Na_2SO_4 free and Na_2SO_4 stress WPMC medium showed no marked differences on root number, root area, and shoot area, respectively. The putative salt tolerant regenerated plants from callus showed instability when vegetative characteristics were screened on WPMC prescreening system. The selected plants exhibited probably only salt adaptation at the callus level (Barkla and Blumwald, 1991; Ericson and Alfinito, 1984).

In conclusion, callus induction and plant regeneration via somatic embryogenesis was achieved from seed explants of Seaside creeping bentgrass. The putative salt tolerant plants selected from callus were unstable with regard to salt tolerance, but improved the ability of plants recovered from stress medium to grow under salt stressful conditions as revealed in the subsequent WPMC screen after compared to seedling plants. However, the root number may not be a valid criterion to verify salt tolerance stability compared to measuring the root area and shoot area in our WPMC screen system. The tolerance mechanisms of salt tolerant regenerates requires further study over a number of seed generations.

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Table 4. Mean root number, root area and shoot area of whole plant microculture plants of creeping bentgrass Seaside derived from seedlings (seed), or plants regenerated from callus on 0, 0.5%, 1.0%, or 1.5% Na_2SO_4 stress media. Plants were grown at various concentrations of Na_2SO_4 for 4 wk in the whole plant microculture screen system. Data were collected by video image analysis.

Treatment of microcultured plants[Na_2SO_4(%)].	Source of explants for WPMC	Root number	Root area (mm^2)	Shoot area (mm^2)
0	Seed	7.9 b	178.9 ab	1061 abc
	0†	8.7 bcC‡	214.1 bcA	1160 abcdeA
	0.5	5.2 a	249.7 cde	1176 bcde
	1.0	5.3 a	268.4 de	1222 de
	1.5	5.0 a	247.3 cde	1158 abcde
0.5	0	5.3 aAB	272.7 eA	1246 deA
	0.5	5.0 a	218.2 bc	1025 ab
1.0	Seed	9.2 c	134.5 a	1021 a
	0	4.5 aA	222.2 bcdA	1154 abcdeA
	1.0	5.3 a	257.4 cde	1328 e
1.5	0	5.4 aB	262.2 deA	1182 cdeA
	1.5	4.8 a	223.0 bcd	1121 abcd

†Numbers indicate that plants regenerated from callus were the source of explantsfor WPMC and each munber is the percentage of Na_2SO_4 in the medium.

‡Means followed by the same lowercase (source of explants and treatment differences) and uppercase (treatment differences) letters are not significantly different at the 0.05 probability level by Student's t test.

Fig. 14. Callus (CA) was initiated from the axis of embryos of creeping bentgrass 'Seaside' seed (SE) incubation in darkness for 1 wk (X = 40, Bar = 0.1 mm).

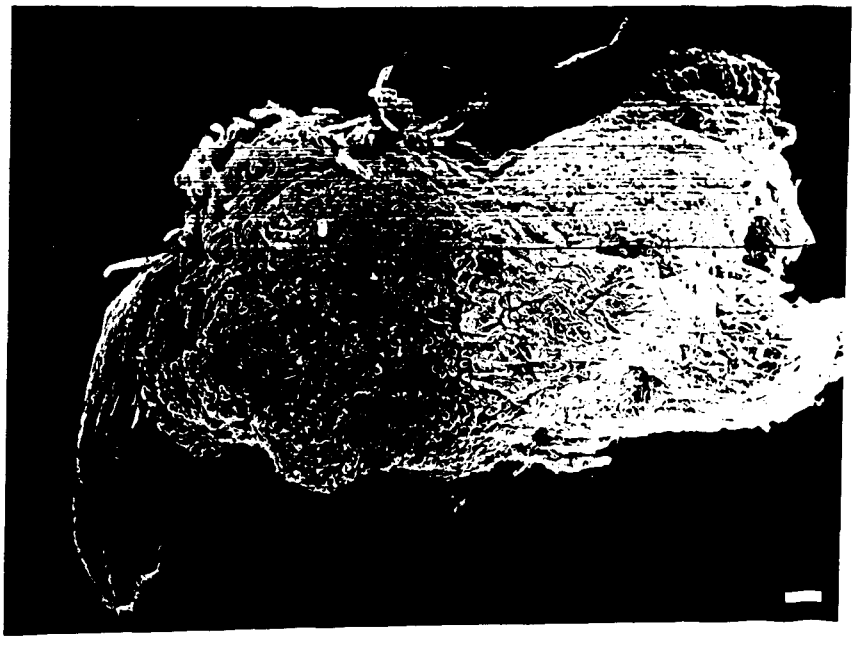

Fig. 15. Regenerated organs from callus derived from seeds of creeping bentgrass 'Seaside' after 8 wk in subculture medium. (a) regenerated leaf (LF)(X = 40, Bar = 0.1 mm). (b) regenerated root (RT)(X = 60, Bar = 0.1 mm).

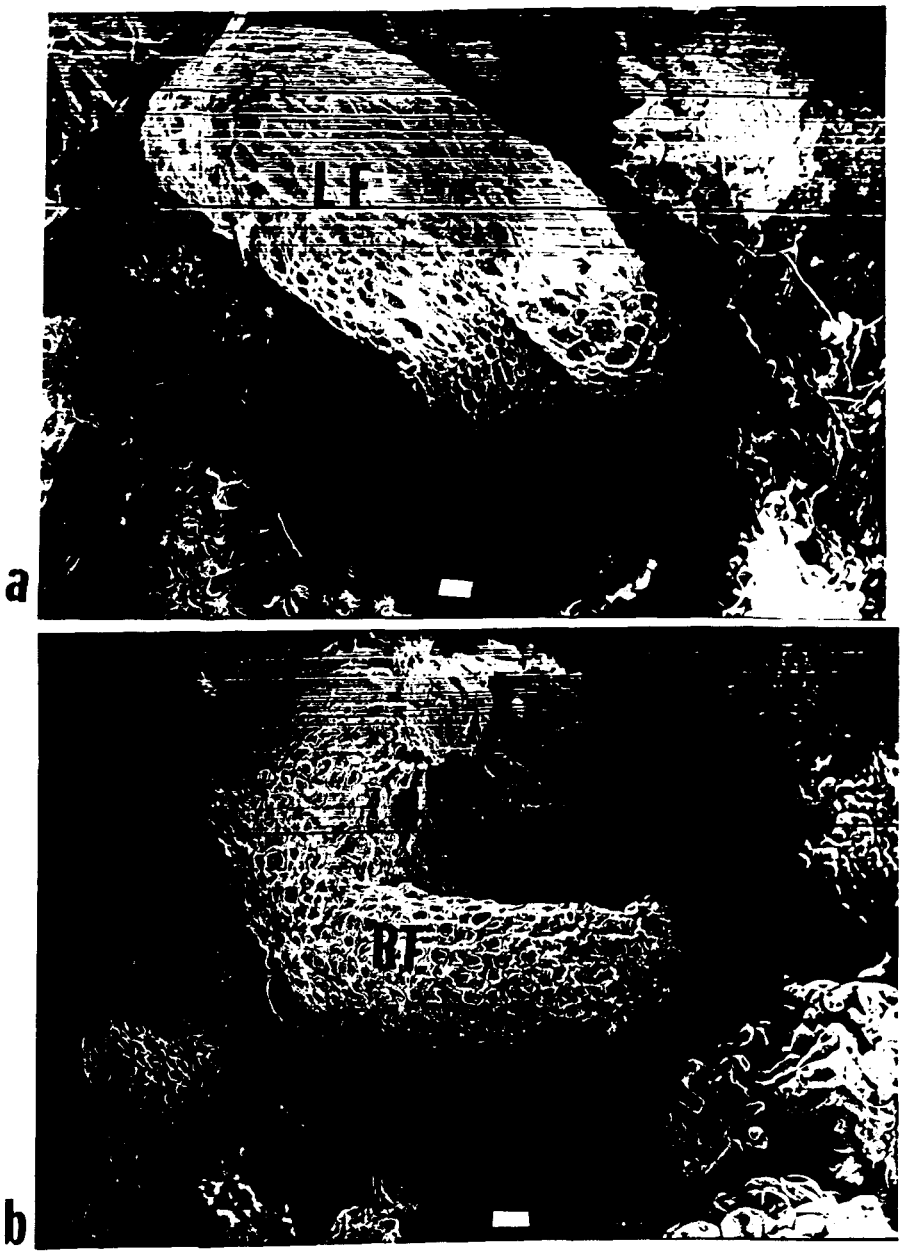
a
LF
b
BF

Fig. 16. The growth of roots and shoots of plants grown in the whole plant microculture system was viewed through gelrite solidified medium.