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

R. Li[1], A. H. Bruneau[1] and R. Qu[1,2]

[1]Department of Crop Science, North Carolina State University, Raleigh, NC 27695-7620, USA; [2]Corresponding author, E-mail: rongda_qu@ncsu.edu

With 2 figures and 1 table

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Abstract

St Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is an important warm season turf and pasture grass. *In vitro* tissue culture of St Augustinegrass could serve as an important mean for its improvement through genetic transformation as well as induced somaclonal variation. To optimize tissue culture conditions for plant regeneration of St Augustinegrass, tissue culture responses of 11 explant tissues and four callus induction/subculture media have been examined. Embryogenic calli with regeneration potential were observed on cultures of early immature embryo [3 days after pollination (DAP)], immature embryo (7–14 DAP), and shoot base of young seedlings. The addition of benzyladenine (BA) in the callus induction/subculture medium enhances callus regeneration ability and does not harm callus induction for immature embryos. The best response came from 7 to 14 DAP immature embryo on MS medium containing 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 mg/l BA. The callus induction and regeneration rates were 97.7% and 47.6% respectively. However, BA supplement reduced callus formation and failed to enhance regeneration for young leaf bases. Scanning electron microscopy revealed that plant regeneration of St Augustinegrass is via somatic embryogenesis.

Key words: *Stenotaphrum secundatum* — benzyladenine — immature embryo — regeneration — scanning electron micrroscopy — somatic embryogenesis

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 cutivar 'Texas Common' were cultured on MS medium (Murashige and Skoog 1962) containing 1 mg/l 2,4-dichlorophenoxyacetic acid (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 mean 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, various explant tissues and culture media have been tested and the developmental pathway of its *in vitro* regeneration has been studied. The results are reported in this correspondence.

Materials and Methods

Plant materials: Cultivar 'Raleigh' (Busey et al. 1982) of St Augustinegrass, *Stenotaphrum secundatum* (Walt.) Kuntze, 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 s, 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 0.5–1 cm segments. Young inflorescences, 1.0–4.5 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 (DAP) when the endosperm was in a watery stage. Immature embryos were collected 7–14 DAP when the endosperm was in the milky stage. Young shoot bases and mesocotyls (0.7–1 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 benzyladenine (BA). The regeneration medium was MS medium supplemented with 1 mg/l BA (6-BA or 6-benzylaminopurine), 0.2 mg/l α-naphthaleneacetic acid (NAA), and 0.5 mg/l gibberellic acids (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 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 8-week-old were transferred onto the regeneration medium and maintained in a lighted culture chamber (CU-32L, Percival) with a 16-h photoperiod (140 $\mu mol/m^2/s$ cool white fluorescent irradiance) for 3 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 42–55 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 analysis of variance (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.

Scanning electron microscopy: Callus samples for scanning electron microscopy (SEM) were collected from culture media containing BA

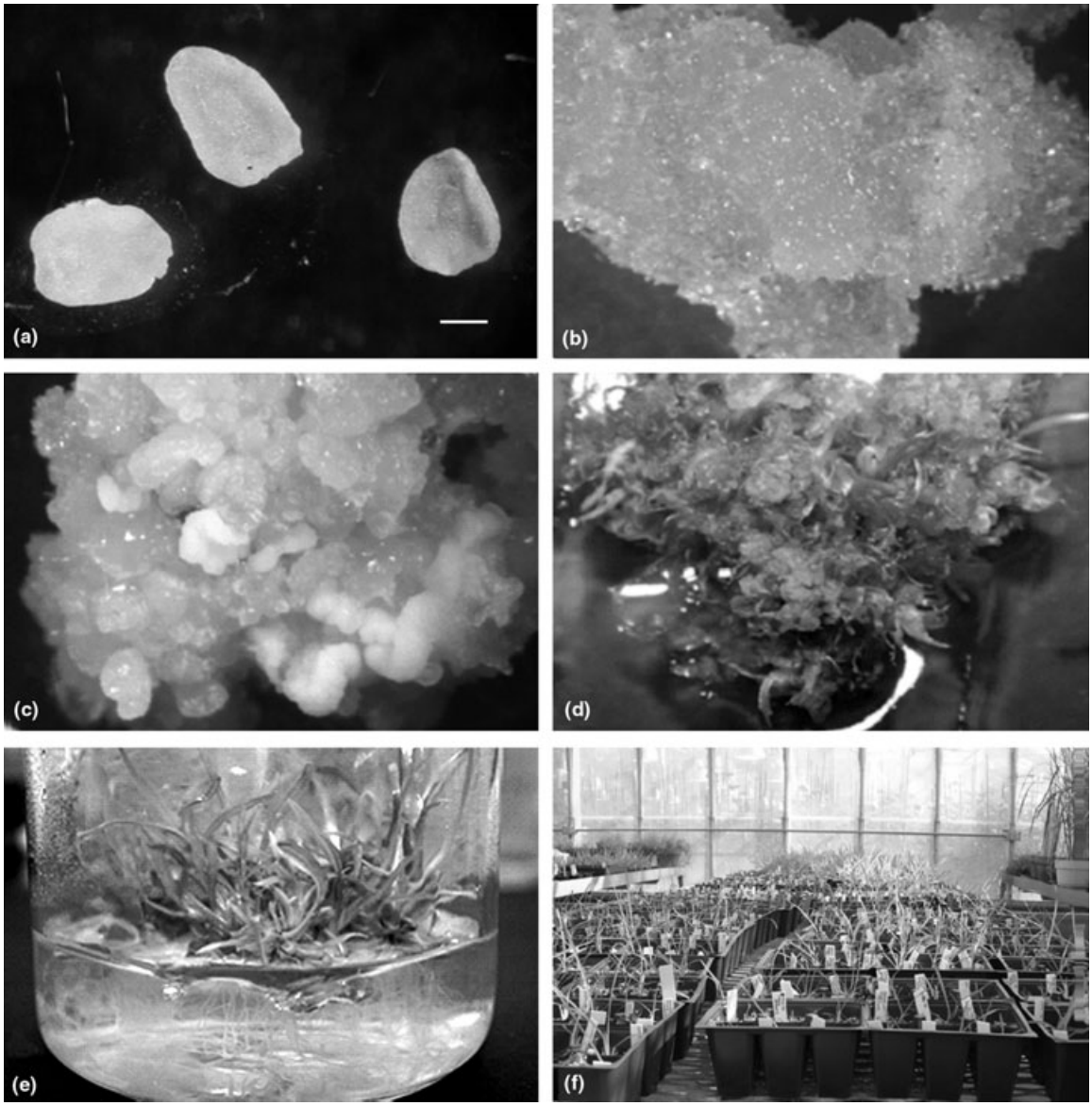

Fig. 1: Immature embryo culture of St Augustinegrass. (a) Cultured immature embryos. The bar in the figure represents 0.5 mm. (b) Non-embryogenic callus. (c) Embryogenic callus. (d) Shoot formation from embryogenic callus 3 weeks in the regeneration medium. (e) Regenerated shoots developed roots in the rooting medium. (f) Regenerated plantlets growing in a greenhouse

(0.01 mg/l) 2 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 hour, dehydrated in a graded cold ethanol series (30%, 50%, 70%, 95% and 100%) for 24 h each at 4°C, critical point dried in liquid carbon dioxide for 15 min, affixed to aluminium 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 (Altpeter and Posselt 2000, Chaudhury and Qu 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 facilitate later regeneration (Kuo and Smith 1993, Chaudhury and Qu 2000, Li and Qu 2004). As 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, scanning electron microscopy was employed 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 with 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. 1a), 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. 1b). In some cases, embryogenic calli were observed, which were pale white or yellowish, compact, granular and friable (Fig. 1c). 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.

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 1). In contrast, callus regeneration ability was highly significantly different among the medium, explant type and the interaction of the two (Table 1). The best result in the experiment came from the 7- to 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 1b). 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 1b).

Table 1: Statistical analysis of effects of media and explants in St Augustinegrass tissue culture. (a) ANOVA and (b) LSD test

(a) Source	d.f.	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 × 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 × E	6	188.8	3.9	0.0076

(b) Explant type	Medium treatment[1]	Callus induction rate (%)[2]	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]Medium treatments:
1. MS medium + 1.0 mg/l 2,4-D, culture for 4 weeks, subculture for an additional 4 weeks on the same medium.
2. MS medium + 1.0 mg/l 2,4-D, culture for 4 weeks, subculture for additional 4 weeks on MS medium with 0.5 mg/l 2,4-D and 0.25 mg/l kinetin (Kuo 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 4 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 for additional 4 weeks on the same medium.
[2]Values represent means of the three replicates of 55 early immature embryos, 44 immature embryos and 44 young shoot bases per replicate. Values followed by the same superscript letter within each column were not significantly different from each other at P = 0.05.

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. 1d). However, when placed at the rooting medium, the growth of shoots and roots was much accelerated (Fig. 1e). 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 (Fig. 1f) and in the field. 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 and 0.5 mg/l of

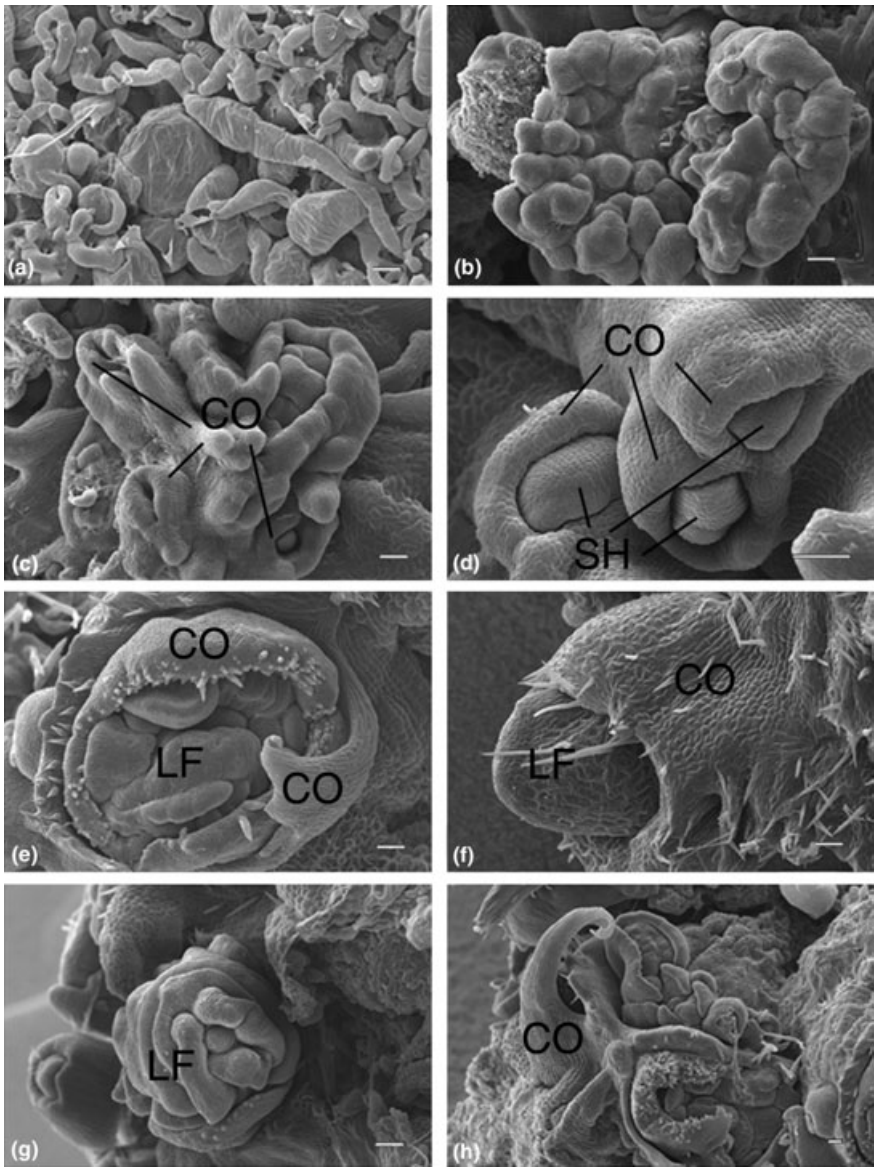

Fig. 2: Scanning electron micrographs of somatic embryogenesis of St Augustinegrass. Bar in each figure represents 100 μm. (a) Non-embryogenic callus showing long, tubular and loosely held cells on its surface. (b–e) Embryogenic calli 2 months on callus induction/subculture medium showing various stages 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–h) Embryogenic calli, 1 week in regeneration medium, showing shoot formation

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.

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 SEM examination (Fig. 2). 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. 2b); dense coleoptiles (CO) formed by circularization of the granular protuberances on compact structures (Fig. 2c); and early shoots (SH) often seen at the center of the developing, circular coleoptiles (Fig. 2c,d). They developed into one or more shoots (Fig. 2-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 (Fig. 2f–h).

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 (twofold to sixfold) over 0 and 0.01 mg/l. When compared with the previously reported medium treatment of St Augustinegrass tissue culture (Kuo and 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, tissue culture responses of various tissues of St Augustinegrass were investigated, 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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