Plant Biotechnology 22(3), 245–248 (2005)

Tissue Culture Note

Plant regeneration from immature inflorescence of zoysiagrass (*Zoysia* spp.)

Anurug Poeaim[1], Yasushi Matsuda, Tatsuro Murata*

School of Agriculture, Kyushu Tokai University, Minamiasomura, Aso, Kumamoto 869-1404, Japan
* E-mail: tmurata@ktmail.ktokai-u.ac.jp Tel: +81-967-67-3912 Fax: +81-967-67-2659

Received April 4, 2005; accepted June 20, 2005 (Edited by S. Ogita)

Abstract Zoysiagrass displays good adaptability to its environment, and is utilized for golf courses and athletic fields. Immature inflorescences were obtained from 5 varieties of zoysiagrass, 'Miyako', 'Misato', 'Meyer', 'Yamato' and 'B14'. However, regenerated plants were only obtained in 'Miyako'. A relationship between the size of the inoculated inflorescences and the callus induction frequency was observed. A high frequency of callus formation was shown in earlier developmental stages shorter than 20 mm in the length of inflorescences in 'Miyako'. Plant regeneration was observed efficiently when the compact calli, which were formed in the callus induction medium (Linsmaier and Skoog 4 mg l^{-1} thiamine–HCl, 100 mg l^{-1} α-ketoglutaric acid, 2 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BA, 3% sucrose, 0.2% gelrite), were transferred to the regeneration medium (Linsmaier and Skoog 1 mg l^{-1} BA, 3% maltose, 0.5% gelrite) in 'Miyako'.

Key words: Immature inflorescence, plant regeneration, zoysiagrass.

Zoysiagrass (*Zoysia* spp.) is a warm season turfgrass; mainly originating in Asia, including Japan, and utilized for golf courses and athletic fields. In turfgrass, tissue culture *in vitro* has been initiated from a wide range of explants, such as mature and immature embryos, shoot tips and immature inflorescences. In many cases, plant regeneration in zoysiagrass has been reported from seed explants (Al-Khayri et al. 1989; Asano 1989; Asano et al. 1996; Poeaim et al. 2004). However, it is difficult to maintain the genotype of each variety for mainly outcrossing, when seeds are used to induce callus for explants. On the other hand, the regenerated plants derived from explants, such as immature inflorescence, usually maintain the genetic characteristics of the parent plants. Consequently, there are a number of reports concerning plant regeneration from immature inflorescence in many turfgrass species (Ahn et al. 1985, 1987; Artunduaga et al. 1988, 1989; Chaudhury and Qu 2000; Dale et al. 1981; Dale and Dalton 1983; Creemers-Molenaar et al. 1988). However, there are no reports concerning plant regeneration from callus induced from immature inflorescences of zoysiagrass. The present investigation has thus been undertaken to establish such a plant regeneration system from immature inflorescences of zoysiagrass.

Experiment I: In April and May 2003, immature inflorescences yet to emerge were obtained from 5 varieties of zoysiagrass, namely 'Miyako', 'Misato', 'Meyer', 'Yamato' and 'B14', grown in our university field. The immature inflorescences (10–50 mm in length), exposed by the removal of the surrounding leaves, were surface-sterilized in 70% ethanol for 1 min, and 3% sodium hypochlorite with 2 drops of tween® 20 for 10 min, and then rinsed twice with sterilized distilled water. The immature inflorescences were cultured on the basal LS media (Linsmaier and Skoog 1965) supplemented with 4 mg l^{-1} thiamine–HCl, 100 mg l^{-1} α-ketoglutaric acid, 3% sucrose, 0.2% gelrite and various combinations of 2,4-D (1 and 2 mg l^{-1}), BA (0.01 and 0.1 mg l^{-1}), casein hydrolysate (200 and 500 mg l^{-1}) and inositol (100 mg l^{-1}) (Medium No. 1–10 as shown in Table 1). Two months after the initiation of the culture, calli were transplanted to LS medium supplemented with 1 mg l^{-1} BA, 3% maltose and 0.5% gelrite for regeneration. These transplanted cultures were incubated at 28°C in a 16-h photoperiod. For histological observations, the collected materials were fixed in FAA (a mixture of 50% ethanol, formalin and acetic acid in proportion 90 : 5 : 5), following which 15 μm thick sections were taken by the ordinary paraffin method and stained with 1% Safranine O and 0.5% Fast green (Kokubu et al. 1982).

[1] Present Address: Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, 10520, Thailand

Abbreviations: BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; LS, Linsmaier and Skoog.

Copyright © 2005 The Japanese Society for Plant Cell and Molecular Biology

Table 1. Callus formation and plant regeneration from five varieties of immature inflorescences (Experiment I)

Medium No.	Calls induction media (mg l^{-1})				Variety: Callus formation (%)					Total no. of inflorescences inoculated	No. of inflorescences produced calli (%)
	2,4-D	BA	Casein hydrolysate	Inositol	Miyako	Misato	Meyer	Yamato	B14		
1	1	0.01			4/24[1](16.7)[3.4][2]	3/10(30.0)	3/8(37.5)	2/16(12.5)	1/8(12.5)	66	13 (19.7) [ab3]
2	1	0.1			4/22(18.2)	2/9(22.2)	0/7(0.0)	1/16(6.3)	2/7(28.6)	61	9 (14.8) [ab]
3	1	0.0	200		6/26(23.1)	1/10(10.0)	1/8(12.5)	4/18(22.2)	2/8(25.0)	70	14 (20.0) [ab]
4	1	0.01	500		2/22(9.1)	1/8(12.5)	1/7(14.3)	6/19(31.6)	2/7(28.6)	63	12 (19.0) [ab]
5	1	0.01		100	6/28(21.4)	2/9(22.2)	2/7(28.6)	2/14(14.3)	4/9(44.4)	67	16 (23.9) [ab]
6	2	0.01			4/24(16.7)	1/8(12.5)	2/10(20.0)	1/16(6.3)	2/9(22.2)	67	10 (14.9) [ab]
7	2	0.1			2/22(9.1)	1/8(12.5)	2/8(25.0)	1/18(5.6)	2/8(25.0)	64	8 (12.5) [b]
8	2	0.01	200		4/22(18.2)	2/8(25.0)	3/11(27.3)	4/17(23.5)	2/8(25.0)	66	15 (22.7) [ab]
9	2	0.01	500		4/24(16.7)	4/9(44.4)	2/8(25.0)	3/18(16.7)	4/9(44.4)	68	17 (25.0) [ab]
10	2	0.01		100	8/28(28.6)	5/10(50.0)	4/9(44.4)	5/16(31.3)	4/9(44.4)	72	26 (36.1) [a]
Mean %					44/242(17.8)	22/89(24.1)	20/83(23.5)	29/168(7.0)	25/82(30.0)	664	140 (20.9)

[1] No. of inflorescences produced calli/No. of inflorescences inoculated.
[2] [ ]: % of callus which formed shoots, no shoot formation observed in any other calli.
[3] Means indicated by the same letter (a and b) within a column are not significantly different at 5% level by Tukey test.

Immature inflorescences from 5 genotypes had induced callus on all media 4 to 5 weeks after the initiation of culture. No significant differences were detected among the callus induction medium used (see Table 1). Although most calli formed in these experiments were friable, translucent and fast-growing, some calli developed only roots and turned brown 1 month after the culture initiation. This type of callus has been typically described as a monocotyledonous plant (Felfoldi and Purmhauser 1992). The compact calli which were nodular and yellowish-white in color were produced only in 'Miyako'. Poeaim et al. (2004) reported that plant regeneration was observed after the compact calli derived from seeds of zoysiagrass were transferred to a regeneration medium.

Subsequently, calli isolated from explants were subcultured to the regeneration medium. Almost all of the calli turned brown and some survived calli developed only roots 1 month after subculture to the regeneration medium. In 'Miyako', regenerated shoots developed only from a single compact callus, which was induced on medium No. 1 (1 mg l^{-1} 2,4-D + 0.01 mg l^{-1} BA) (see Table 1).

Experiment II: In April and May 2004, the immature inflorescences were collected to examine the effects on the size of explants (inflorescences) on callus induction. Four categories divided by size of immature inflorescences namely, less than 20 mm, 20–30 mm, 30–40 mm and longer than 40 mm, were prepared from variety 'Miyako' and 'Belair' grown in the field. Sufficient number of inflorescences shorter than 20 mm in 'Belair' was not able to collect. Callus induction media (Medium No. 1–10 as shown in Table 1) and the regeneration medium was the same one in used Experiment I.

The frequencies of callus induction within each size category of immature inflorescence are shown in Table 2. Comparing the callus induction frequency of both varieties, 'Miyako' was higher than 'Belair'. Both varieties also showed a tendency toward high frequency callus formation from the inflorescences of earlier developmental stages (<20 mm in 'Miyako', 20–30 mm in 'Belair'). In the case of 'Miyako', it was 72.5% in developmental stages shorter than 20 mm, while in 'Belair', it was 11.8% in developmental stages of 20–30 mm. The callus induction frequency decreased with the progression of the developmental stage of the inoculated inflorescences. The production of callus is influenced by the length and stage of the inflorescences (Ahn et al. 1985; Creemers-Molenaar et al. 1988; George and Eapen 1990). Our results correlate with those findings.

Most of the calli were friable, and the calli were induced from the base parts of the floret rather than a pistil organ such as an ovary and ovule (Figure 1A, B, C). In 'Miyako', a few compact calli were induced from the inflorescences of each developmental stages, <20 mm (4.5%), 20–30 mm (4.3%) and 30–40 mm (0.7%) in length (Table 2). The compact callus were found mainly to be small, round and with dense cytoplasm compared to friable callus, which were large, elongated, highly vacuolated structures (Figure 1D). The compact calli isolated from explants were transferred to the regeneration medium, and multiple shoots developed about 30 days after the transfer (Figure 1E, F, G). The shoots thus obtained were subsequently transferred to the LS medium without hormones for the further development of the roots (Figure 1H).

The frequency of shoot formation was calculated by the following formula: (No. of compact calli having

Copyright © 2005 The Japanese Society for Plant Cell and Molecular Biology

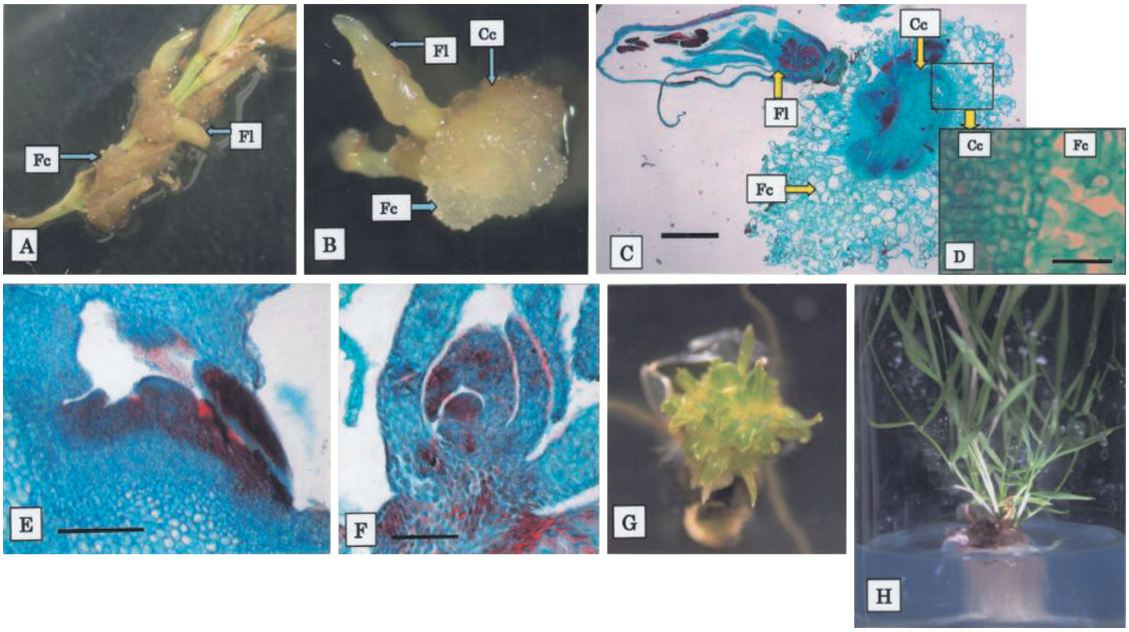

Figure 1. Callus induction and plant regeneration from the immature inflorescence of variety 'Miyako' which is an interspecific hybrid of *Z. japonica*×*Z. matrella.* (A) Callus induction from immature inflorescence; (B) Compact (Cc) and friable callus (Fc) induction from the base of the floret (Fl) in immature inflorescence 21 days after the culture initiation; (C, D) Longitudinal section of Figure 2B showing callus induction (C, Bar=0.5 mm; D, Bar=20 μm); (E) Longitudinal section of shoot initiation from compact callus 10 days after transferring to the regeneration medium (Bar=0.1 mm); (F) Longitudinal section of shoot regenerated from compact callus 14 days after transferring (Bar=0.1 mm); (G) Multiple shoots regenerated from compact callus 30 days after transferring; (H) Whole plantlets developed from multiple shoots after 30 days culture on the LS medium without hormones.

Table 2. Effects of the developmental stage (size) of immature inflorescences inoculated on callus induction (Experiment II)

Variety	Size of immature inflorescence (mm)	No. of inflorescenses inoculated	No. of inflorescences produced friable calli	No. of inflorescences produced compact calli	% of friable callus formation	% of compact callus formation
Miyako	<20	67	50	3	72.5 [a]	4.5 [a]
	20–30	209	154	9	66.8 [ab]	4.3 [a]
	30–40	275	162	2	58.8 [b]	0.7 [b]
	>40	111	43	0	31.4 [c]	0 [b]
Belair	20–30	86	9	0	11.8 [a]	0
	30–40	127	15	0	10.5 [a]	0
	>40	37	3	0	7.5 [a]	0

These value shows the total frequency of callus induction used for 10 kinds of callus induction media as shown in Table 1.
Within the same variety, means follow ed by different letters (a, b and c) indicate significant difference at 5% level by Tukey test.

formed shoots)/(No. of compact calli which were transferred to the regeneration medium)×100. This frequency was 80% (data not shown) from the compact calli cultured in the callus induction medium No. 7 (2 mg l^{-1} 2,4-D+0.1 mg l^{-1} BA, see Table 1). However, a low frequency of shoot formation was observed in the compact calli cultured in medium No. 3 (1 mg l^{-1} 2,4-D+0.01 mg l^{-1} BA+200 mg l^{-1} casein hydrolysate, see Table 1) and No. 8 (2 mg l^{-1} 2,4-D+0.01 mg l^{-1} BA+200 mg l^{-1} casein hydrolysate, see Table 1). The application of casein hydrolysate and inositol to the callus induction medium resulted in a high frequency of plant regeneration in other turfgrass (Wang et al. 2002). However, no clear relationship could be found between the frequency of plant regeneration and the medium condition in the present study.

A recurring theme in cereal tissue culture is varietal differences in the response of cultures. (Mikami and Kinoshita 1988; Powell and Caligari 1987; Tomes and Smith 1985; Ogawa et al. 1996, 1999; Cardona and Duncan 1997; Chaudhury and Qu 2000). In this study, the existence of varietal differences in plant regeneration from immature inflorescence was also observed, and regenerated plants were obtained only in the case of the 'Miyako'. In this study, plant regeneration was observed efficiently when the compact calli forming in the callus induction medium No. 7 (LS+4 mg l^{-1} thiamine–HCl, 100 mg l^{-1} α-ketoglutaric acid, 2 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BA, 3% sucrose, 0.2% gelrite) were transferred to the regeneration medium (LS+1 mg l^{-1} BA, 3% maltose,

Copyright © 2005 The Japanese Society for Plant Cell and Molecular Biology

0.5% gelrite) by using young immature inflorescences shorter than 20 mm in length. This is the first report used to obtain plant regeneration from the immature inflorescences of zoysiagrass. The method presented here will be useful in biotechnological approaches to improve zoysiagrass through *in vitro* induced artificial mutations using radiation such as X-ray, gamma-rays and ion beams.

References

Ahn BJ, Huang FH, King JW (1985) Plant regeneration through somatic embryogenesis in common bermudagrass tissue culture. *Crop Sci* 25: 1107–1109

Ahn BJ, Huang FH, King JW (1987) Regeneration of bermudagrass cultivars and evidence of somatic embryogenesis. *Crop Sci* 27: 594–597

Al-Khayri JM, Huang FH, Thomson LF, King JW (1989) Plant regeneration of Zoysiagrass from embryo-derived callus. *Crop Sci* 29: 1324–1325

Artunduaga IR, Taliaferro CM, Johnson BL (1988) Effect of auxin concentration and growth of embryogenic callus from young inflorescence explants of old world bluestem (*Bothriochloa* spp.) and bermuda (*Cynodon* spp.) grasses. *Plant Cell Tiss Org Cult* 12: 13–19

Artunduaga IR, Taliaferro CM, Johnson BB (1989) Induction and growth of callus from immature inflorescences of "Zebra" bermudagrass as affected by casein hydrolysate and 2,4-D concentration. *In vitro Cell Devel Bio* 25: 753–756

Asano Y (1989) Somatic embryogenesis and protoplast culture in Japanese lawngrass (*Zoysia japonica*). *Plant Cell Rep* 8: 141–143

Asano Y, Katsumoto H, Inokuma C, Kaneko S, Ito Y, Fujiie A (1996) Cytokinin and thiamine requirements and stimulative effects of riboflavin and α-ketoglutaric acid on embryogenic callus induction from the seeds of *Zoysia japonica* Stued. *J Plant Physiol* 149: 413–417

Cardona CA, Duncan RR (1997) Callus induction and high efficiency plant regeneration via somatic embryogenesis in paspalum. *Crop Sci* 37: 1297–1302

Creemers-Molenaar J, Loeffen JPM, Vandervalk P (1988) The effect of 2,4-dichlorophenoxyacetic acid and donor plant environment on plant regeneration from immature inflorescence derived callus of *Lolium perenne* L. and *Lolium multiflorum* L. *Plant Sci* 57: 165–172

Chaudhury A, Qu R (2000) Somatic embryogenesis and plant regeneration of turf-type bermudagrass: Effect of 6-benzyladenine in callus induction medium. *Plant Cell Tiss Org Cult* 60: 113–120

Dale PJ, Thomas E, Brettell RIS (1981) Embryogenesis from cultured immature inflorescences and nodes of *Lolium multiflorum. Plant Cell Tiss Org Cult* 1: 47–55

Dale PJ, Dalton SJ (1983) Immature inflorescence culture in *Lolium, Festuca, Phleum* and *Dactylis. Z Pflanzenphysiol* 111: 39–45

Felfoldi K, Purnhauser L (1992) Induction of regenerating callus cultures from immature embryos of 44 wheat and 3 triticale cultivars. *Cereal Research Communications* 20: 273–277

George L, Eapen S (1990) High frequency plant-regeneration through direct shoot development and somatic embryogenesis from immature inflorescence cultures of finger millet (*Eleusine coracana* Gaertn). *Euphytica* 48: 269–274

Kokubu T, Murata T, Endo F (1982) Anatomical observation on the fertilization and embryogenesis in sweet potato, *Ipomoea batatas* (L.) Lam. *Jpn J Breed* 32: 239–246

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiol Plant* 18: 100–127

Mikami T, Kinoshita T (1988) Genotype effects on the callus formation from different explants of rice, *Oryza sativa* L. *Plant Cell Tiss Org Cult* 12: 311–314

Ogawa T, Fukuoka H, Ohkawa Y (1996) Effects of reduced nitrogen and sucrose concentration on varietal differences in rice cell culture. *Breed Sci* 46: 179–184

Ogawa T, Fukuoka H, Yano H, Ohkawa Y (1999) Relstionships between nitrite reductase activity and genotype-dependent callus growth in rice cell cultures. *Plant Cell Rep* 18: 576–581

Poeaim A, Matsuda Y, Inoue T, Shigeyasu T, Murata T (2004) Optimization of callus induction and plant regeneration from seed explants of *Zoysia* species. *J Jpn Soc Turfgrass Sci* 31: 3–10

Powell W, Caligari PDS (1987) The *in vitro* genetics of barley (*Hordium vulgare* L.) detection and analysis of reciprocal diffences for culture response to 2,4-dichlorophenoxyactic acid. *Heredity* 59: 293–299

Tomes DT, Smith OS (1985) The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. *Theor Appl Genet* 70: 505–509

Wang Y, Ruemmele BA, Chandlee JM, Sullivan WM, Knapp JE, Kausch AP (2002) Embryogenic callus induction and plant regeneration media for bentgrasses and bluegrass. *In vitro Cell Dev Biol* 38: 460–467

Copyright © 2005 The Japanese Society for Plant Cell and Molecular Biology