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Rapid and efficient callus induction and plant regeneration from seeds of zoysiagrass (*Zoysia japonica* Steud.)

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Abstract

A rapid and efficient *in vitro* culture system has been established in zoysiagrass (*Zoysia japonica* Steud.). Embryos isolated from mature seeds were used to induce callus, which improved callus formation and shortened the culture period. A high concentration of 2,4-dichlorophenoxyacetic acid (5 mg L^{-1}) in a combination with low concentration of 6-benzyladenine (0.2 mg L^{-1}) was proved to be suitable for callus induction in zoysiagrass. Eight types of calli were observed; friable callus was the most regenerable type. Shoot regeneration efficiency was improved by using 1 mg L^{-1} of thidiazuron with 1-naphthaleneacetic acid (0.05 mg L^{-1}) and gibberellic acid (0.1 mg L^{-1}). Zoysiagrass shoots continued to grow in MS medium, and rooted in half-strength MS medium. With this system, it appears the duration of *in vitro* regeneration could be shortened to 16 weeks for zoysiagrass, i.e. from inoculation of embryos to regeneration of plantlets with shoots and roots.

Introduction

Zoysiagrass (*Zoysia japonica* Steud.) is a perennial warm-season grass which is indigenous in eastern Asia countries including Japan, China and Korea and used for forage and turf purposes (Engelke and Anderson 2003; Cai *et al.* 2005). In Japan zoysiagrass grasslands dominate most of the native grasslands being continuously grazed for years (Dhital *et al.* 2010). These grasslands are highly productive and can support a livestock population of a high density (Ito and Takatsuki 2005). Zoysiagrass utilizes the C_4 photosynthetic pathway (Carmo-Silva *et al.* 2009), which has higher rates of photosynthesis and higher water-use efficiency than C_3 pathway (Osborne and Freckleton 2009). The grassland is therefore noted for low maintenance requirement (Mao *et al.* 2009) and effortless management (Kitagawa *et al.* 2007). Zoysiagrass is also widely used as a sustainable turf grass in transitional and warm climatic regions throughout the world (Patton and Reicher 2007).

Forage breeding by traditional methods of hybridization and selection has been devoted to increase yields and improve the nutritional quality of forage. It also enhances the tolerance to abiotic and biotic stresses, and improves ecological environment. Breeding program of zoysiagrass for forage use has been carried out by using traditional breeding method in Japan (Sugihara *et al.* 1999). Traditional breeding methods such as

phenotypic evaluation, selection and hybridization still continue to be used. However, forage crop breeding programs have entered into the biotechnology era using molecular biology tools. Molecular breeding, namely the use of genomic and transgenic biotechnologies, is now applied to forage improvement programs (Yamada and Spangenberg 2009). Molecular markers have been developed on zoysiagrass (Yaneshita *et al.* 1997; Cai *et al.* 2004, 2005; Tsuruta *et al.* 2005; Li *et al.* 2009b).Transformation as one of important biotechnologies has also been developed in many forage grass species and it has produced abundant gene-modified plants, for example, reduction of lignin content to increase forage digestibility (Li *et al.* 2008). It is also expected an inspiring technique for genetic improvement of zoysiagrass as forage use.

To receive successful transformations on grass species, an efficient *in vitro* culture system is essential, since the identification and propagation of embryogenic calli are key factors affecting transformation frequency (Wang and Ge 2006). For zoysiagrass, while there have been reports of successful establishment of an *in vitro* culture system (Asano *et al.* 1996; Inokuma *et al.* 1996; Dhandapani *et al*. 2008), it was generally considered recalcitrant to the production of embryogenic callus and regeneration (Al-Khayri *et al*. 1989; Asano 1989; Asano *et al*. 1996; Dhandapani *et al*. 2008). The duration of more than 3 months for callus induction was mentioned in experiments using mature seeds of

zoysiagrass as explants (Asano *et al*. 1996; Inokuma *et al.* 1996; Toyama *et al*. 2003), which was longer than commonly 4-6 weeks of other grass species (Hu *et al*. 2005; Wang *et al*. 2001; Altpeter *et al*. 2000). An alternative method of using immature inflorescences and stem nodes was suggested in *Zoysia matrella* Merr. (Dhandapani *et al*. 2008), but it is season- and genotype-dependent. Zoysiagrass regeneration also was described as a tough task (Toyama *et al*. 2003). Only few papers focused on optimizing the regeneration medium and regeneration efficiency (Dhandapani *et al*. 2008; Li *et al.* 2009a).

The development of a rapid and efficient *in vitro* culture protocol is therefore of high importance for zoysiagrass genetic improvement. The objective of this study was to establish the *in vitro* culture system of zoysiagrass, which should be high efficient callus induction and shoot regeneration, and time-saving protocol. This *in vitro* system is expected to lay a foundation for transgenic molecular breeding of zoysiagrass.

Materials and methods

Mature embryo isolation

Mature seeds of a commercial cultivar of zoysiagrass 'Japanese lawngrass' (Snow Brand Co., Sapporo, Japan) with the ability of high germination rate were soaked in

distilled water for 2 days. Then the seeds were stirred in sodium hypochlorite (1% active chlorine) for 2 h for sterilization, followed by rinsing three times with sterile water. Glumes were removed and the embryos were cut off on a microscope platform. Each of twenty-five isolated embryos were placed on a separate callus-induction medium plate. Husked seeds (i.e. without glumes) and intact seeds were also incubated in callus induction medium as a means of comparison.

Callus induction

Different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 2, 5, 10 mg L^{-1}) and 6-benzyladenine (BA) (0, 0.02, 0.2, 2 mg L^{-1}) were supplemented in MS basal medium (Murashige and Skoog 1962) containing 30 g L^{-1} sucrose, 4 mg L^{-1} thiamine-HCl, and 100 mg L^{-1} α-ketoglutaric acid to induce callus. A combination of 2,4-D 1 mg L^{-1} and BA 2 mg L^{-1} was not included in the study, since the responding to callus induction auxin is generally regarded determinant and cytokinin plays an assistant role (Jimenez 2005). All media were solidified with 2 g L^{-1} gelrite, pH 5.8, and autoclaved. Each treatment included approximately 100 mature embryos with three replications. After 2 months under cultured conditions, embryos were subcultured once in the same media. Different callus types were classified and examined for regenerating

ability in regeneration medium. Callus was induced in darkness at $27 \pm 1°C$.

Plant regeneration

MS basal medium was supplemented with 30 g L^{-1} maltose, 1 mg L^{-1} thidiazuron (TDZ), 1-naphthaleneacetic acid (NAA) (0.05, 0.1 or 0.5 mg L^{-1}), and gibberellic acid (GA_3) (0 or 0.1 mg L^{-1}). GA_3 was filter sterilized and added in media after media cooled down after being removed from an autoclave. Friable-type calli, also known as embryogenic calli were divided into 5 mm pieces. Approximately 20 callus pieces were inoculated in each medium with three replications. The weight of callus pieces was measured before the inoculation. After two weeks of culture, shoots as well as bud primordia were observed. After counting the number of shoots, both shoots and bud primordia were subcultured in MS basal medium supplemented with 30 g L^{-1} maltose, which was labeled Re-free (M)medium. Two weeks later, shoots were counted and then transferred into half-strength (both macro- and micro-elements were reduced to half strength) MS medium supplemented with 30 g L^{-1} sucrose to examine whether the shoots had the capability to root. Culture for plant regeneration was kept in a 16h/8h light/dark photoperiod at $26 \pm 1°C$.

Results

Callus induction from mature embryos, husked seeds and intact seeds

Calli were formed from excised embryos earlier than those from husked seeds and intact seeds (Figure 1). Three days after inoculation, some tissues began to swell at the basal of shoot coleoptiles and around the hypocotyls, which was defined as callus. Callus appeared in husked seeds and intact seeds 4 and 6 days after inoculation, respectively. In addition, the efficiency of callus formation from embryos was significantly greater than those from husked seeds and intact seeds ($P = 0.001$). Almost all embryos formed callus (97.1%) at 18 days after callus formation except for those that were likely damaged by cutting or were inactive embryos. The rate of callus formation at four weeks under cultured conditions was 65.9 and 55.2% using husked seeds and intact seeds, respectively.

Callus induction medium and callus types

Two months after inoculation, eight types of calli were observed (Figure 2). These included Type a, shoot callus, which appeared only in medium containing 1 mg L^{-1} 2,4-D and were derived from mature embryos. Type b, bud callus, was observed

frequently in medium containing a relatively lower concentration of 2,4-D (1 or 2 mg L^{-1}) with a relatively higher concentration of BA (0.2 or 2 mg L^{-1}). Type c callus was white, tight, and dry. Type d callus was light yellow, friable and loose, and was similar to embryogenic callus (Asano *et al*. 1996). Type e callus was watery, transparent, sticky, and without definable structure. Type f callus was soft and transparent, but neither watery nor sticky. Soft callus type was initiated at the start of callus formation and other callus types were subsequently derived from it. Type g callus was characterized as callus covered by adventitious roots and were observed in 1 mg L^{-1} 2,4-D containing medium. Type h callus was brown and stopped growing after the first few days of culture, and subsequently died. The eight types of calli were transferred into the regeneration medium to investigate the regenerable capability. Friable callus, which was also embryogenic callus, was confirmed as the most regenerable callus type (Figure 2).

Calli formed in the different media were all tested, in the regeneration medium (detailed in next part) had the competence to regenerate. A range of 1.7–20% of calli was confirmed as regenerable. Calli induced in the medium 5 mg L^{-1} 2,4-D and BA 0.2 mg L^{-1} showed high (20%) regeneration ability ($P = 0.003$) (Figure 3), which was labeled MT4 medium, was suitable as callus induction medium for zoysiagrass.

Plant regeneration

After two weeks in culture, both shoots and bud primordia regenerated. In the present study, TDZ has been shown better plant regeneration response than other cytokinins, e.g. BA, kinetin and zeatin (data not shown). The regeneration efficiency was expressed as the percentage of callus pieces regenerated (either shoot [Figure 4b] or bud primordia [Figure 4a]) out of total callus pieces, which ranged from 42.1 to 59.6% ($P = 0.948$) (Table 1). The numbers of shoots were counted in different duration of the culture. In all the media, shoots as well as bud primordia formed after 2 weeks of the culture, then the number of shoots increased greatly as many bud primordia grew into shoots in 4 weeks; after 6 weeks of culture, it decreased as many shoots could not root and eventually died. The initiatory shoots were mostly slim, rosette and green-white in color. Such kind of shoots were generally formed significantly more ($P = 0.013$) in the media supplemented with GA_3 than those without GA_3. In the media without GA_3, bud primordia being capable of initiating shoots were dominant. Shoots and bud primordia were subcultured in Re-free(M) medium to allow the growth of shoots. After another two weeks in Re-free(M) medium, bud primordia developed to shoots. Most shoots were green-white in color. There were no differences ($P = 0.641$) in numbers of shoots among different regeneration media.

Following shoot growth in Re-free(M) medium, all shoots were subcultured in half-strength MS medium supplemented with 30 g L^{-1} sucrose for rooting. After 6 weeks, the shoots which were tough and dark green in color with developed roots were counted (Figure 4c). The number of such shoots developed from the regeneration media containing GA_3 were significantly greater ($P = 0.040$) than those without GA_3. The optimal medium contained 0.1 mg L^{-1} GA_3 and 0.05 mg L^{-1} NAA, in which 71.1 shoots per gram of callus developed (Table 1). However, 0.1 and 0.5 mg L^{-1} of NAA did not result in the development of significant numbers of shoots (Table 1). Hence, medium supplement 1 mg L^{-1} TDZ, 0.1 mg L^{-1} GA3 and 0.05 mg L^{-1} NAA, which was named TNG medium, was used as the regeneration medium for zoysiagrass.

The minimum period for zoysiagrass in *in vitro* regeneration was as little as 16 weeks (Figure 5), beginning with inoculation of mature embryos to regeneration of plantlets with shoots and roots (Figures 4d).

Discussion

Advances in the *in vitro* culture system have been enhanced to enable the production of embryogenic callus and convert callus into plant efficiently. It also allows to make the stable genetic engineered plants (Thorpe 2007), as embryogenic callus has high level of

activity of cell division and is recognized as one of the best target explants of monocotyledonous species (Cheng *et al*. 2004). In our study, rapid and efficient *in vitro* culture system was established in the important sustainable forage and turf species, *Zoysia japonica*.

Excising mature embryos from zoysiagrass seeds induced improved callus formation and shortened the culture period. In forage and turf grass species, most explants used for genetic transformation studies are calli derived from intact mature seeds (Smith *et al*. 2002; Aswath *et al*. 2005; Lee *et al*. 2006; Wu *et al*. 2007; Dong *et al*. 2008; Liu *et al*. 2008). Mature seeds were also used to induce callus formation on zoysiagrass (Asano *et al*. 1996; Inokuma *et al*. 1996) and *Z. sinica* Hancev (Li *et al.* 2006b). The required duration for callus induction was nearly 3 months in these studies, which is considered longer than the normal duration of 4-6 weeks of other turf and forage grasses species such as triploid bermudagrass (*Cynodon dactylon* [L.] Pers. × *C. transvaalensis* Burtt-Davy) (Hu *et al.* 2005)*,* tall fescue (*Festuca arundinacea* Schreb.) (Wang *et al.* 2001) and perennial ryegrass (*Lolium perenne* L.) (Altpeter *et al.* 2000)*.* By using mature embryos of zoysiagrass in the present study, 97.1% of embryos developed into calli just after 18 days of culture, which was greater than the frequencies of 50–70% in previous researches (Asano *et al.* 1996; Inokuma *et al.* 1996; Li *et al.* 2006b). Young

inflorescences, nodes of stems, and shoot tips were alternative explants to induce callus

(Dhandapani *et al.* 2008), but they were genotype or growth-season dependent. In fact,

Al-Khayri *et al.* (1989) used mature embryos in zoysiagrass *in vitro* culture; their results

also mentioned that 90 to 98% of the excised embryos from zoysiagrass seeds produced

callus. However, there appeared to be no discussion of the advantages of using mature

embryos in this research literature. Excising zoysiagrass mature embryos from seeds to

culture would eliminate some barriers. One possible barrier may be waxy glumes

contributing to the inhibition of germination and/or the promotion of dormancy by

reducing permeability to environmental elements (Yeam *et al*. 1988). Another barrier is

likely seed dormancy that would be released by excising mature embryos due to

desertion of dormancy-regulating hormones such as abscisic acid and small molecules

such as nitrogen-containing compounds in endosperm (Finkelstein *et al*. 2008). Mature

embryos have also been reported in transformations of some other grass species, e.g.

perennial ryegrass (Altpeter *et al*. 2000; Cao *et al*. 2006), darnel ryegrass (*Lolium*

temulentum L.) (Ge *et al*. 2007), tall fescue (Gao *et al*. 2008; Cao *et al*. 2006) and

buffelgrass (*Cenchrus ciliaris* L.) (Colomba *et al*. 2006).

   As certain auxin levels in combination with low levels of BA improve the rate of

callus induction, suggested by previous researchers working on barley (*Hordeum*

vulgare L.) (Sharma *et al*. 2005) and bermudagrass (*Cynodon dactylon* [L.] Pers.) (Chaudhury and Qu 2000), high concentration of 2,4-D (5 mg L^{-1}) with the addition of 0.2 mg L^{-1} BA was shown to produce good callus induction in zoysiagrass (Figure 3). Embryogenic calli were maintained in the subculturing medium(MT4-S), which contained a reduced concentration of 2,4-D (i.e. 2 mg L^{-1}), since it was shown that optimal embryogenic callus proliferation occurred at auxin concentrations lower than those required for optimum initiation of embryogenic callus (Ho and Vasil 1983; Jimenez 2005).

Eight types of calli were observed in zoysiagrass. Four of them were dry, friable, soft and watery types and four types were morphologically similar to types indicated by Toyama *et al*. (2003). Friable callus type, which was identified as the embryogenic callus by Asano *et al*. (1996), was the most regenerable callus type (Figure 2). Other four types such as shoot, bud, root and brown types were not characterized in previous researches; the usage of 1 m L^{-1} or 2 mg L^{-1} 2,4-D often resulted in these types. However, 2,4-D used in such concentrations for zoysiagrass callus induction was preferred by other researchers (Al-Khayri 1989; Toyama *et al*. 2003). The concentration of 5 mg L^{-1} 2,4-D was proved to be the most optimal in this study (Figure 3), probably for use of mature embryos.

By usage of TDZ at 1 mg L^{-1} to regenerate zoysiagrass in this research, the regeneration efficiency was ranged from 42.1 to 59.6% (Table 1), which was generally higher than 13–59% in previous literature (Al-Khayri *et al*. 1989). Multiple shoots were induced by TDZ in many species, including African redwood (*Hagenia abyssinica* [Bruce] J.F. Gmel.) (Feyissa *et al*. 2005), witloof chicory (*Cichorium intybus* L.) (Yucesan *et al*. 2007), barley and wheat (*Triticum aestivum* L.) (Sharma *et al*. 2007). It also appeared to help regenerate shoots from callus in species in Gramineae (Shan *et al*. 2000; Gallo-Meagher *et al*. 2000). Whereas, TDZ is a type of substituted phenylurea (Shan *et al*. 2000), which inhibits shoot elongation (Bhagwat *et al*. 1996; Fasolo *et al*. 1989). Though TDZ induced shoot buds efficiently, it also reduced shoot growth (Sinha *et al*. 2000; Akasaka *et al*. 2000). Bud primordia of zoysiagrass formed in regeneration medium were therefore transferred onto Re-free(M) medium to grow into shoots.

Regenerating shoots from callus by combination of cytokinin and auxin in concentration lower than that of cytokinin was often recommended for use *in vitro* culture. Various combinations, including BA plus 2,4-D, kinetin plus 2,4-D, BA plus NAA, or BA plus IAA were applied to the culture of different explants (Holme *et al*. 1997; Yu *et al*. 2000; Tariq *et al*. 2008; Zambre *et al*. 2002). Our study also supported that a combination of 1 mg L^{-1} TDZ plus 0.05 mg L^{-1} NAA was the best combitation of

cytokinin and auxin. In addition, another plant growth regulator, GA_3, was proved to play a key role in regeneration of zoysiagrass (Table 1). It has been reported that GA_3 stimutates in plant regeneration (Fiuk and Rybczynski 2008) and it generally induce regeneration from callus by interacting with cytokinin and auxin (Li and Qu 2004; Li *et al*. 2006a). It seems that GA_3 stimulated zoysiagrass regeneration possibly by acting as a remedy of the slow growth of the species. Zoysiagrass is known to grow relatively slowly (Richardson and Boyd 2001; Patton *et al*. 2007), while GA_3 is noted for increasing seedling growth (Arabi and Jawhar 1999). It should be noted, however, that not all regenerated shoots of zoysiagrass grew to plantlets. Many shoots were green-white in color, rosette-shaped, and lacked root initiation and development. Shoots which developed roots were dark green, straight, and tough. The usage of GA_3 improved the development of the latter type of shoots.

Our success on *in vitro* culture system is expected to contribute to forage and turf molecular breeding in zoysiagrass. The following factors contributed to the success of this system; it is also expected to be adopted in some other grass species: First, using mature embryos rather than mature seeds will allow efficient callus induction and rapid *in vitro* culture duration in the species which have troubles of seed dormancy and slow growth. Second, the high level of auxin (2,4-D) in combination with a low level of

cytokinin (BA) will be beneficial for the embryogenic callus production. Third, cytokinin TDZ with lower concentration of auxin will regenerate plantlets efficiently. Further GA$_3$ will remedy the slow regeneration in the case of low-growing species such as zoysiagrass,.

Acknowledgements

References

Akasaka Y, Daimon H, Mii M (2000) Improved plant regeneration from cultured leaf segments in peanut (*Arachis hypogaea* L.) by limited exposure to thidiazuron. *Plant Sci* 156: 169-175.

Al-Khayri JM, Huang FH, Thompson LF, King JW (1989) Plant regeneration of

zoysiagrass from embryo-derived callus. *Crop Sci* 29: 1324-1325.

Altpeter F, Xu J, Ahmed S (2000) Generation of large numbers of independently

transformed fertile perennial ryegrass (*Lolium perenne* L.) plants of forage- and

turf-type cultivars. *Mol Breed* 6: 519-528.

Arabi M I, Jawhar M (1999) A rapid technique for determining common root rot

reaction in barley. *Plant Breed* 118: 278-280.

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* Steud.. *J Plant

Physiol* 149: 413-417.

Aswath CR, Kim SH, Mo SY, Kim DH (2005) Transgenic plants of creeping bent grass

harboring the stress inducible gene, *9-cis-epoxycarotenoid dioxygenase*, are highly

tolerant to drought and NaCl stress. *Plant Growth Regul* 47: 129-139.

Bhagwat B, Vieira LGE, Erickson LR (1996) Stimulation of *in vitro* shoot proliferation

from nodal explants of cassava by thidiazuron, benzyladenine and gibberellic acid.

Plant Cell Tissue Organ Cult 46: 1-7.

Cai H, Inoue M, Yuyama N, Nakayama S (2004) An AFLP-based linkage map of

zoysiagrass (*Zoysia japonica*). *Plant Breed* 123: 543-548.

Cai HW, Inoue M, Yuyama N, Takahashi W, Hirata M, Sasaki T (2005) Isolation,

characterization and mapping of simple sequence repeat markers in zoysiagrass

(*Zoysia* spp.). *Theor Appl Genet* 112: 158-166.

Cao MX, Huang JQ, He YL, Liu SJ, Wang CL, Jiang WZ, Wei ZM (2006)

Transformation of recalcitrant turfgrass cultivars through improvement of tissue

culture and selection regime. *Plant Cell Tissue Organ Cult* 85: 307-316.

Carmo-Silva AE, Keys AJ, Beale MH, Ward JL, Baker JM, Hawkins ND, Arrabaca MC,

Parry MAJ (2009) Drought stress increases the production of 5-hydroxynorvaline in

two C4 grasses. *Phytochemistry* 70: 664-671.

Chaudhury A, Qu R (2000) Somatic embryogenesis and plant regeneration of turf-type

bermudagrass: Effect of 6-benzyladenine in callus induction medium. *Plant Cell

Tissue Organ Cult* 60: 113-120.

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Invited review: Factors

influencing *Agrobacterium*-mediated transformation of monocotyledonous species.

In Vitro *Cell Dev Biol-Plant* 40: 31-45.

Colomba EL, Grunberg K, Griffa S, Ribotta A, Mroginski L, Biderbost E (2006) The

effect of genotype and culture medium on somatic embryogenesis and plant

regeneration from mature embryos of fourteen apomictic cultivars of buffel grass

(*Cenchrus ciliaris* L.). *Grass Forage Sci* 61: 2-8.

Dhandapani M, Hong SB, Aswath CR, Kim DH (2008) Regeneration of zoysia grass

(*Zoysia matrella* L. Merr.) cv. Konhee from young inflorescences and stem nodes. In

Vitro *Cell Dev Biol-Plant* 44: 8-13.

Dhital D, Yashiro Y, Ohtsuka T, Noda H, Shizu Y, Koizumi H (2010) Carbon dynamics

and budget in a *Zoysia japonica* grassland, central Japan. *J Plant Res* 123:519–530

Dong S, Shew HD, Tredway LP, Lu J, Sivamani E, Miller ES, Qu R (2008) Expression

of the bacteriophage T4 lysozyme gene in tall fescue confers resistance to gray leaf

spot and brown patch diseases. *Transgenic Res* 17: 47-57.

Engelke MC, Anderson S (2003) Zoysiagrasses (*Zoysia* spp.) In: Turfgrass Biology,

Genetics, and Breeding (Eds Casler MD, Duncan RR). John Wiley & Sons, Inc, New

Jersey, 271-286.

Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised

leaves of *in vitro* grown shoots of apple cultivars. *Plant Cell Tissue Organ Cult* 16:

75-87.

Feyissa T, Welander M, Negash L (2005) *In vitro* regeneration of *Hagenia abyssinica*

   (Bruce) J.F. Gmel. (Rosaceae) from leaf explants. *Plant Cell Rep* 24: 392-400.

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed

   dormancy. *Annu Rev Plant Biol* 59: 387-415.

Fiuk A, Rybczynski JJ (2008) Factors influencing efficiency of somatic embryogenesis

   of *Gentiana kurroo* (Royle) cell suspension. *Plant Biotechnol Rep* 2: 33-39.

Gallo-Meagher M, English RG, Abouzid A (2000) Thidiazuron stimulates shoot

   regeneration of sugarcane embryogenic callus. In Vitro *Cell Dev Biol-Plant* 36:

   37-40.

Gao C, Long D, Lenk I, Nielsen KK (2008) Comparative analysis of transgenic tall

   fescue (*Festuca arundinacea* Schreb.) plants obtained by *Agrobacterium*-mediated

   transformation and particle bombardment. *Plant Cell Rep* 27: 1601-1609.

Ge Y, Cheng X, Hopkins A, Wang ZY (2007) Generation of transgenic *Lolium*

   temulentum plants by *Agrobacterium tumefaciens*-mediated transformation. *Plant*

   Cell Rep 26: 783-789.

Ho WJ, Vasil IK (1983) Somatic embryogenesis in sugarcane *(Saccharum officinarum*

   L.) I. The morphology and physiology of callus formation and the ontogeny of

   somatic embryos. *Protoplasma* 118: 169-180.

Holme IB, Krogstrup P, Hansen J (1997) Embryogenic callus formation, growth and

regeneration in callus and suspension cultures of *Miscanthus× ogiformis* Honda

'Giganteus' as affected by proline. *Plant Cell Tissue Organ Cult* 50: 203-210.

Hu F, Zhang L, Wang X, Ding J, Wu D (2005) *Agrobacterium*-mediated transformed

transgenic triploid bermudagrass (*Cynodon dactylon × C. transvaalensis*) plants are

highly resistant to the glufosinate herbicide Liberty. *Plant Cell Tissue Organ Cult* 83:

13-19.

Inokuma C, Sugiura K, Cho C, Okawara R, Kaneko S (1996) Plant regeneration from

protoplasts of Japanese lawngrass. *Plant Cell Rep* 15: 737-741.

Ito TY, Takatsuki S (2005) Relationship between a high density of sika deer and

productivity of the short-grass (*Zoysia japonica*) community: a case study on

Kinkazan Island, northern Japan. *Ecol Res* 20: 573-579.

Jimenez VM (2005) Involvement of plant hormones and plant growth regulators on in

vitro somatic embryogenesis. *Plant Growth Regul* 47: 91-110.

Kitagawa M, Ikeda K, Nishida T, Yamamoto Y, Nashiki M, Hatanaka T (2007)

Establishment and growth of *Zoysia japonica* Steud. seedling transplanted on cattle

dung pats in a temperate pasture. *Jpn J Grassl Sci* 53: 102-108. (In Japanese with

English abstract.)

Lee SH, Lee DG, Woo HS, Lee KW, Kim DH, Kwak SS, Kim JS, Kim H, Ahsan N,

Choi MS, Yang JK, Lee BH (2006) Production of transgenic orchardgrass via

Agrobacterium-mediated transformation of seed-derived callus tissues. *Plant Sci* 171:

408-414.

Li L, Fan X, Zhang J, Yan M, Bao M (2009a) Long-term cultured callus and the effect

factor of high-frequency plantlet regeneration and somatic embryogenesis

maintenance in *Zoysia japonica.* In Vitro *Cell Dev Biol —Plant* 45: 673-680.

Li L, Qu R (2004) Development of highly regenerable callus lines and biolistic

transformation of turf-type common bermudagrass [*Cynodon dactylon* (L.) Pers.].

Plant Cell Rep 22: 403-407.

Li M, Yuyama N, Hirata M, Han J, Wang Y, Cai H (2009b) Construction of a

high-density SSR marker-based linkage map of zoysiagrass (*Zoysia japonica* Steud.).

Euphytica 170: 327-338.

Li R, Bruneau Ah, Qu R (2006a) Improved plant regeneration and *in vitro* somatic

embryogenesis of St Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze).

Plant Breed 125: 52-56.

Li RF, Wei JH, Wang HZ, He J, Sun ZY (2006b) Development of highly regenerable

callus lines and *Agrobacterium*-mediated transformation of Chinese lawngrass

(*Zoysia sinica* Hance) with a cold inducible transcription factor, *CBF1*. *Plant Cell Tissue Organ Cult* 85: 297-305.

Li X, Weng JK, Chapple C (2008) Harnessing plant biomass for biofuels and biomaterials-Improvement of biomass through lignin modification. *Plant J* 54: 569-581.

Liu M, Yang J, Lu S, Guo Z, Lin X, Wu H (2008) Somatic embryogenesis and plant regeneration in centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.). In Vitro *Cell Dev Biol-Plant* 44: 100-104.

Mao PS, Xin Guo Wang XG, Wang YH, Han GJ (2009) Effect of storage temperature and duration on the vigor of zoysiagrass (*Zoysia japonica* Steud.) seed harvested at different maturity stages. *Grassl Sci* 55: 1-5.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473-497.

Osborne CP, Freckleton RP (2009) Ecological selection pressures for C4 photosynthesis in the grasses. *Proceedings of The Royal Society, Series B* 276: 1753-1760.

Patton AJ, Reicher ZJ (2007) Zoysiagrass species and genotypes differ in their winter injury and freeze tolerance. *Crop Sci* 47: 1619-1627.

Patton AJ, Volenec JJ, Reicher ZJ (2007) Stolon growth and dry matter partitioning

explain differences in zoysiagrass establishment rates. *Crop Sci* 47: 1237-1245.

Richardson MD, Boyd JW (2001) Establishing *Zoysia japonica* from sprigs: effects of topdressing and nitrogen fertility. *Hortscience* 36: 377-379.

Shan X, Li D, Qu R (2000) Thidiazuron promotes *in vitro* regeneration of wheat and barley. In Vitro *Cell Dev Biol-Plant* 36: 207-210.

Sharma VK, Hänsch R, Mendel RR, Schulze J (2005) Mature embryo axis-based high frequency somatic embryogenesis and plant regeneration from multiple cultivars of barley (*Hordeum vulgare* L.). *J Exp Bot* 56: 1913-1922.

Sharma VK, Hänsch R, Mendel RR, Schulze J (2007) Node-derived cultures with high-morphogenic competence in barley and wheat. *Plant Cell Tissue Organ Cult* 88: 21-33.

Sinha RK, Majumdar K, Sinha S (2000) *In vitro* differentiation and plant regeneration of *Albizia chinesis* (Osb.) Merr. In Vitro *Cell Dev Biol-Plant* 36: 370-373.

Smith RL, Grando MF, Li YY, Seib JC, Shatters RG (2002) Transformation of bahiagrass (*Paspalum notatum* Flugge). *Plant Cell Rep* 20: 1017-1021.

Sugihara S, Kobayashi M, Ebina M, Tsurumi Y, Ooya I, Nashiki M (1999) Review of researches concerning *Zoysia* in Japan. *Grassl Sci* 45:105-112. (In Japanese)

Tariq M, Ali G, Hadi F, Ahmad S, Ali N, Shah AA (2008) Callus induction and *in vitro*

plant regeneration of rice (*Oryza sativa* L.) under various conditions. *Pak J Biol Sci* 11: 255-259.

Thorpe TA (2007) History of plant tissue culture. *Mol Biotechnol* 37: 169-180.

Toyama K, Bae CH, Kang JG, Lim YP, Adachi T, Riu KZ, Song PS, Lee HY (2003) Production of herbicide-tolerant zoysiagrass by *Agrobacterium*-mediated transformation. *Mol Cells* 16: 19-27.

Tsuruta S, Hashiguchi M, Ebina M, Matsuo T, Yamamoto T, Kobayashi M, Takahara M, Nakagawa H, Akashi R (2005) Development and characterization of simple sequence repeat markers in *Zoysia japonica* Steud. *Grassl Sci* 51: 249-257.

Wang ZY, Ge Y (2006) Invited review: recent advances in genetic transformation of forage and turf grasses. In Vitro *Cell Dev Biol-Plant* 42: 1-18.

Wang ZY, Ye XD, Nagel J, Potrykus I, Spangenberg G (2001) Expression of a sulphur-rich sunflower albumin gene in transgenic tall fescue (*Festuca arundinacea* Schreb.) plants. *Plant Cell Reports* 20: 213-219.

Wu YY, Chen QJ, Cui XH, Chen H, Chen J, Wang XC (2007) Efficient regeneration and *Agrobacterium*-mediated stable transformation of perennial ryegrass. *Russ J Plant Physiol* 54: 524-529.

Yamada T, Spangenberg G (2009) Molecular breeding of forage and turf. Springer,

New York, 1-352.

Yaneshita M, Nagasawa R, Engelke MC, Sasakuma T (1997) Genetic variation and
interspecific hybridization among natural populations of zoysiagrasses detected by
RFLP analyses of chloroplast and nuclear DNA. *Genes Genet Syst* 72: 173-179.

Yeam DY, Mandava NB, Terry PH, Murray JJ, Portz HL (1988) Identification and
quantification of abscisic acid in zoysiagrass seeds and its inhibitory effect on
germination. *Crop Sci* 28: 317-321.

Yu TT, Skinner DZ, Liang GH, Trick HN, Huang B, Muthukrishnan S (2000)
Agrobacterium-mediated transformation of creeping bentgrass using GFP as a
reporter gene. *Hereditas* 133: 229-233.

Yucesan B, Turker AU, Gurel E (2007) TDZ-induced high frequency plant regeneration
through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). *Plant
Cell Tissue Organ Cult* 91: 243-250.

Zambre M, Chowdhury B, Kuo YH, Montagu MV, Angenon G, Lambein F (2002)
Prolific regeneration of fertile plants from green nodular callus induced from
meristematic tissues in *Lathyrus sativus* L. (grass pea). *Plant Sci* 163: 1107-1112.

Table 1 Regeneration efficiency which expressed as the percentage of callus pieces regenerated (either shoot or bud primordia) out of total callus pieces in 2 weeks regeneration culture and numbers of shoots per gram of callus counted at 2, 4 and 6 weeks of culture

TDZ (mg L^{-1})	NAA (mg L^{-1})	GA$_3$ (mg L^{-1})	Regeneration efficiency (%)	Numbers of shoots at different durations of culture		
				2 weeks	4 weeks	6 weeks
1	0	0	59.6 ± 31.0	9.7 ± 10.0	174.7 ± 138.6	37.2 ± 13.1
1	0	0.1	52.1 ± 20.3	14.3 ± 14.6	102.6 ± 57.6	59.6 ± 34.2
1	0.05	0	53.3 ± 19.6	9.5 ± 8.2	201.5 ± 108.9	31.6 ± 21.2
1	0.05	0.1	42.1 ± 4.0	25.9 ± 18.3	226.5 ± 78.6	71.1 ± 52.9
1	0.1	0	56.3 ± 16.5	1.8 ± 3.2	107.4 ± 35.9	17.6 ± 17.8
1	0.1	0.1	52.9 ± 24.2	29.0 ± 20.2	239.6 ± 155.0	33.5 ± 30.1
1	0.5	0	44.2 ± 9.5	12.9 ± 15.8	240.4 ± 237.7	20.1 ± 25.7
1	0.5	0.1	50.8 ± 17.5	44.1 ± 37.1	267.2 ± 43.5	45.5 ± 34.4

TDZ, thidiazuron; NAA, 1-naphthaleneacetic acid; GA$_3$, gibberellic acid.

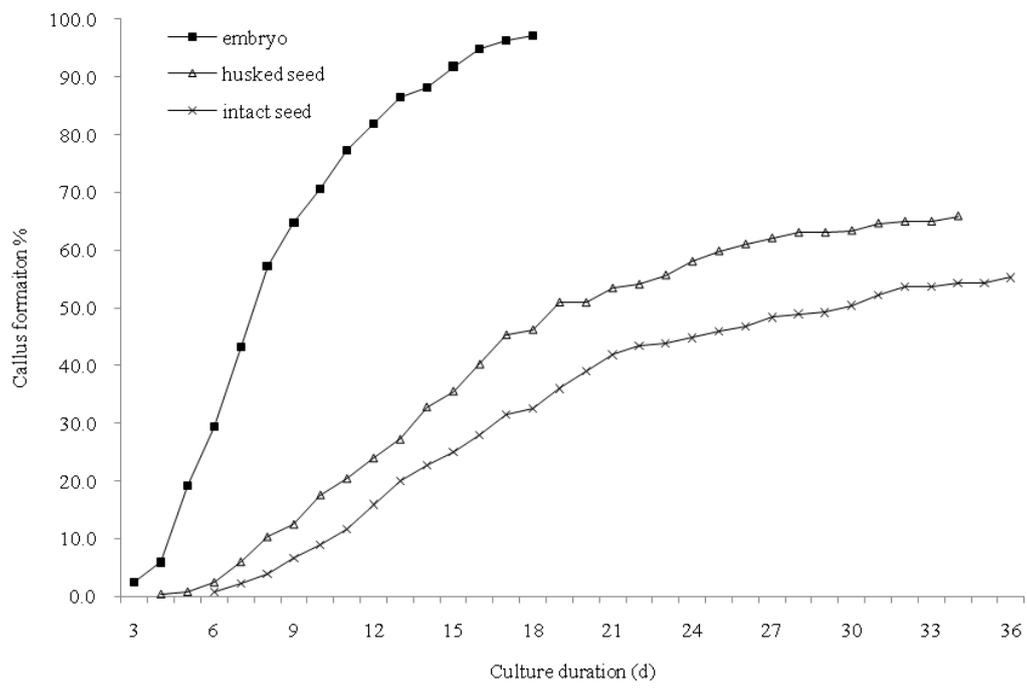Values are mean ± SD.

Figure legends

Figure 1 Callus formed from mature embryos, husked seeds and intact seeds of zoysiagrass.

Figure 2 Eight callus types of zoysiagrass. (a) shoot, (b) bud, (c) dry callus, (d) friable callus, (e) watery callus, (f) soft callus, (g) callus rooting, (h) brown callus. The frequency of regeneration (%) is in the brackets.
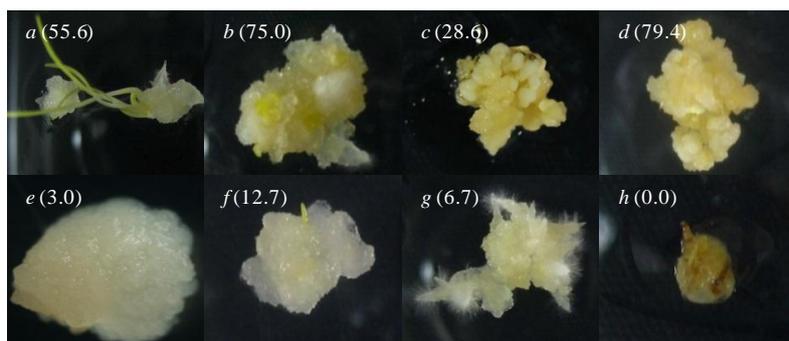
Figure 3 Percentage of regenerable callus of zoysiagrass in different media.

Figure 4 Regeneration of plantlet in zoysiagrass. (a) bud primordia; (b) shoots which were slim, rosette and green-white in color; (c) shoots which were tough and dark green in color; (d) plantlet in half-strength MS medium.
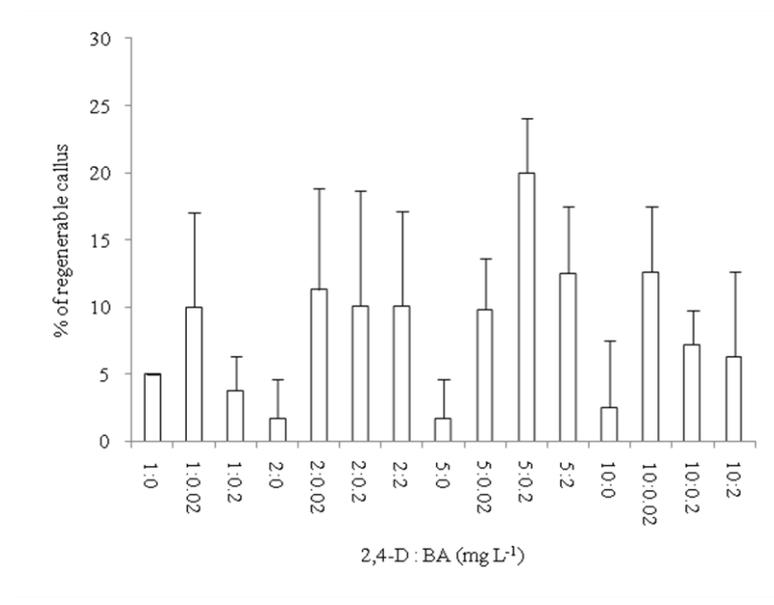
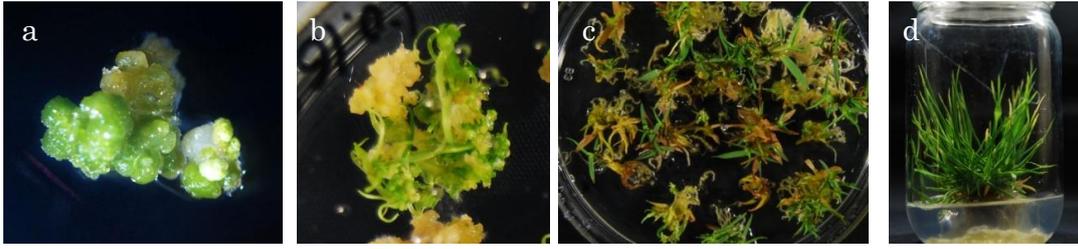**Figure 5** Procedure of *in vitro* culture system in zoysiagrass. Friable calli were maintained in MT4-S medium.
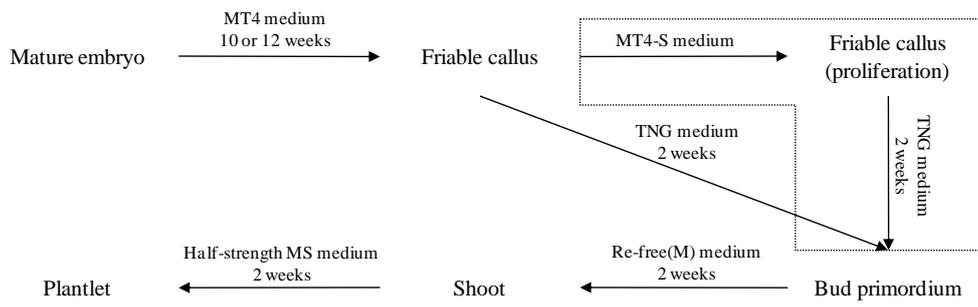
Wang et al. / Figure 1

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Mature embryo → (MT4 medium, 10 or 12 weeks) → Friable callus → (MT4-S medium) → Friable callus (proliferation)

Friable callus → (TNG medium, 2 weeks) → Bud primordium

Friable callus (proliferation) → (TNG medium, 2 weeks) → Bud primordium

Bud primordium → (Re-free(M) medium, 2 weeks) → Shoot → (Half-strength MS medium, 2 weeks) → Plantlet

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