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PLANT REGENERATION FROM LIQUID CALLUS CULTURES DERIVED FROM MATURE SEEDS OF PAMPAS GRASS [*CORTADERIA SELLOANA* (SCHULT. & SCHULT.F.) ASCH. & GRAEBN.]

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Abstract

An efficient system of plant regeneration from liquid culture of mature seed-derived callus was established in pampas grass [*Cortaderia selloana* (Schult. & Schult.f.) Asch. & Graebn.]. Calluses were induced from mature seeds on MS medium containing 30 g l^{-1} maltose, 8 g l^{-1} agar, and different concentrations (0, 1, 2, and 5 mg l^{-1}) of dicamba, NAA, picloram or 2,4-D. The highest callus induction was obtained on medium containing 5 mg l^{-1} picloram where 98% explants formed callus. Calluses were subcultured on MS medium containing 2 mg l^{-1} dicamba, NAA, picloram or 2,4-D and 30 g l^{-1} maltose for 3 months and the compact calluses obtained were transferred into the same corresponding liquid media. By using 2 month-old liquid-cultured calluses, effects of different concentrations of BAP (0, 0.1, 0.5, and 1 mg l^{-1}) and NAA (0, 0.1, 0.5, and 1 mg l^{-1}) on shoot regeneration were evaluated using MS medium with 30 g l^{-1} sucrose, 8 g l^{-1} agar, and 0.5 mg l^{-1} GA_3 supplemented to the basal medium. The highest frequency of shoot formation was obtained in callus induced by picloram on medium containing 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, where 92% of the calluses formed shoots. The calluses maintained high shoot regeneration ability (71-85%) and original ploidy level of diploid during the 8 months of subcultures, although drastic reduction in the regeneration ability (< 23%) and the appearance of tetraploid cells were observed after 12 months of subculture in liquid media.

Key words: micropropagation, ornamental grass, picloram

INTRODUCTION

Pampas grass [*Cortaderia selloana* (Schult. & Schult.f.) Asch. & Graebn.] is a dioecious plant native to Argentina, Brazil, and Uruguay (Connor and Edgar 1974). It has been cultivated worldwide as an ornamental perennial giant grass for landscaping and cut and dry flowers due to 20-30 cm long beautiful spikes with yellow, white or pink coloration (Madison 1992). It has also a great potential to become a biomass-energy crop due to high biomass yield with 3 m plant height, adaptability to a wide range of climates and low needs of input (Hornback 1994).

Robacker and Corley (1992) and Robacker (1995) previously reported efficient system for plant regeneration from immature inflorescence explants of *C. selloana* through direct or temporal callus intervening organogenesis. However, no detailed studies have been reported on the effects of different plant growth regulators (PGRs) on callus culture-mediated plant regeneration system from any kind induction and plant regeneration from other sources of explants including that of immature inflorescence. In some recalcitrant gramineous species, mature seeds were successfully used as a material for the induction of calluses with high plant regeneration ability (Seo et al. 2010, Zhang et al. 2012). Suspension culture system in liquid medium is also sometimes used efficiently for micropropaga-

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tion (Akutsu and Sato 2002, Shimizu et al. 2003) and genetic transformation (Ozawa and Takaiwa 2010) of such highly regenerable callus cultures because by this system, a large number of cells can be multiplied in short-term.

The aim of this study was to establish a highly efficient system for callus induction and liquid culture of callus from mature seeds of *C. selloana*, and to examine the stability of regeneration capacity of callus during long-term subcultures.

MATERIALS AND METHODS

Plant material

Mature seeds of *Cortaderia selloana* were collected at Kunming Botanical Garden, Yunnan, China and used for establishing an efficient plant regeneration system through callus induction.

Callus induction and subculture

The seeds were surface-disinfected successively with 70% (v/v) ethanol for 1 min and 1% NaOCl solution containing a few drop of Tween-20 for 15 min. After rinsing 3 times with sterile distilled water, the seeds were cultured for callus induction on MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} maltose, 1 g l^{-1} casamino acids (Casamino Acids DAIGO, Nihon Pharmaceutical, Tokyo, Japan), 8 g l^{-1} agar, and either one of the following auxins: 3,6-dichloro-*o*-anisic acid (dicamba; Wako Pure Chemical Industries, Osaka, Japan), α-naphthaleneacetic acid (NAA; Sigma-Aldrich, St. Louis, MO, USA), 4-amino-3,5,6-trichloropicolinic acid (picloram; Sigma-Aldrich) or 2,4-dichlorophenoxyacetic acid (2,4-D; Wako Pure Chemical Industries) at different concentrations (0, 1, 2, 5, 8, and 10 mg l^{-1}). The pH of all variant of medium was adjusted to 5.8 before autoclaving at 121°C at 137 kPa for 15 min. The seeds were inoculated in plastic Petri dishes (100 × 20 mm) each containing 40 ml medium, and incubated at 25 ± 1°C in the dark. Twenty seeds (10 seeds per plate) were used for each treatment. Callus induction was observed after 5 weeks of culture and efficiency of callus induction was calculated as the ratio of number of callus-inducing seeds to total number of seeds cultured. Three callus lines derived from different seeds were selected from each of the 4 variants of callus induction medium with either dicamba, NAA, picloram, or 2,4-D at 5 mg and totally 12 lines were used for further experiments. In each callus line, compact callus clumps were divided into small pieces (ca. 3 mm in diameter) and 30 pieces of callus were transferred onto the same variants of the medium used for callus induction except that the concentration of these auxins was reduced from 5 to 2 mg l^{-1}. These calluses were subcultured on these callus maintenance media for 12 months by biweekly subcultures.

Establishment of liquid culture of callus

For induction of liquid cultures, 3 g fresh weight of compact callus, which had been subcultured on 4 types of callus maintenance medium with either dicamba, NAA, picloram or 2,4-D at 2 mg l^{-1} for 3 months, were transferred into 100 ml flasks, each containing 50 ml of liquid MS medium with 30 g l^{-1} maltose and 2 mg l^{-1} dicamba, NAA, picloram or 2,4-D. They were cultured at 25 ± 1°C in the dark on a gyratory shaker (100 rpm) for one year at 2 week intervals.

Plant regeneration from liquid-cultured callus

After 4, 8, and 12 months of subculture in liquid medium, calluses were transferred onto MS medium supplemented with 6-benzylaminopurine (BAP; Wako Pure Chemical Industries) at 0, 0.5, and 1 mg l^{-1}, NAA at 0, 0.1, and 0.5 mg l^{-1} and gibberellic acid (GA_3; Wako Pure Chemical Industries) at 0.5 mg l^{-1}. All variants of regeneration medium contained 30 g l^{-1} sucrose, 1 g l^{-1} casamino acids and 8 g l^{-1} agar in plastic Petri dishes each containing 40 ml medium. Ten callus pieces (ca. 3 mm) were placed on a single plate and three plates (total ca. 0.9 g fresh weight) were used for each treatment. The cultures were incubated at 25 ± 1°C under a 16 h photoperiod at 35 μmol m^{-2} s^{-1} with cool white fluorescent light. Frequency of shoot regeneration was calculated as the ratio of number of callus with regenerated shoots to total number of callus cultured on regeneration variants of the medium after 5 weeks of culture.

More than 20 regenerated shoots (ca. 2 cm in height) were transferred for rooting onto 8 g l^{-1} agar-solidified half-strength MS medium with 30 g l^{-1} sucrose, and cultured for ca. 1 month until they rooted and grew up to 4 cm long under the same temperature and light conditions as for shoot regeneration. Then, they were individually transferred into a 75 × 125 mm glass bottle containing 100 ml of the fresh medium with same composition and cultured for another month for further development of shoots and roots. Well-grown plantlets were transplanted to pots containing sterilized vermiculite and soil (Metro-Mix 350, Sun Gro Horticulture, Agawam, MA, USA) (2 : 1) in the growth chamber (BioTRON, Nippon Medical and Chemical Instruments, Osaka, Japan) at 22°C under 14 h photoperiod for acclimatization. After 3 weeks, they were transferred to a greenhouse for further growth.

Flow cytometry (FCM) analysis

The ploidy levels of the calluses and leaves of control and regenerated plants were determined by FCM using Ploidy Analyzer (Partec, Münster, Germany). To release nuclei, about approximately 100 mg of calluses and 5 mm-square leaf segments were chopped with a razor blade in 0.2 ml of pretreatment solution (Solution A, Plant High Resolution DNA kit

type P, Partec) in a plastic Petri dish. After incubating the crude nuclear suspension for 5 min at room temperature, 2 ml of staining solution [100 mM Tris-HCl, 2 mg l^{-1} DAPI (4',6-diamidino-2-phenylindole dihydrochloride), 0.1% (v/v) Triton X-100 and 2 mM $MgCl_2$, pH 7.5] were added to the crude suspension and filtered through a 40 μm nylon mesh. After 1 min staining, the nuclear suspension was subjected to measurement of the relative nuclear DNA content on a linear scale histogram. Three independent tissue samples were prepared from each treatment and each sample was measured 3 times.

Data analysis

All experiments were independently replicated 3 times. The data obtained for callus induction frequency, callus growth rate, and shoot regeneration frequency were represented as the mean ± standard error (SE) and were subjected to an analysis of variance (ANOVA) using the SPSS statistical package (SPSS Statistics version 17.0, IBM, Armonk, NY, USA). Tukey's HSD test was subsequently performed to identify significant differences among the treatments, at a significance level of $p \leq 0.05$. The arcsine transformation was performed on all percentage data before statistical analysis.

RESULTS AND DISCUSSION

Effects of auxin on callus induction and growth in liquid medium

Seeds germinated 5-7 days after sowing and then callus formation occurred from zygotic embryo and basal part of small shoot (ca. 5 mm) within 2-3 weeks of culture on almost all variants of the medium except for PGR-free and 1-2 mg l^{-1} NAA supplemented media. On variants of the medium containing 1-2 mg l^{-1} NAA, seedlings had elongated to 5 cm after 2 weeks of culture and then calluses were produced at low frequency from the base of shoots after 4 weeks of culture. Almost 90% or higher callus induction rates were obtained on medium containing 2-8 mg l^{-1} picloram with the highest percentage (98%) at 5 mg l^{-1} and that containing 2-5 mg l^{-1} 2,4-D with the highest (93%) at 5 mg l^{-1}, respectively (Fig. 1). In many grasses, the most commonly used auxin for callus induction is picloram (Aguado-Santacruz et al. 2001, Sener et al. 2008) and 2,4-D (Yuan et al. 2009, Joyce et al. 2010). In the present study, callus cultured on agar-solidified variants of the medium supplemented with 2 mg l^{-1} picloram or 2,4-D grew at a 3-fold higher rate than that with 2 mg l^{-1} dicamba or NAA (Table 1). Most of the calluses induced from mature seeds with picloram or 2,4-D were white and compact and these characters were maintained during repeated subcultures (Fig. 2A).

The compact calluses could also grow as relatively compact cell clumps after transfer to liquid MS medium containing 2 mg l^{-1} picloram or 2,4-D. Growth rate of calluses in liquid variants of the medium containing picloram or 2,4-D was 3 times higher than that in variants of the medium containing dicamba or NAA on a fresh weight basis after 2 weeks of culture (Table 1). When 2 mg l^{-1} picloram or 2,4-D was supplemented to variants of the medium, callus cultured in liquid

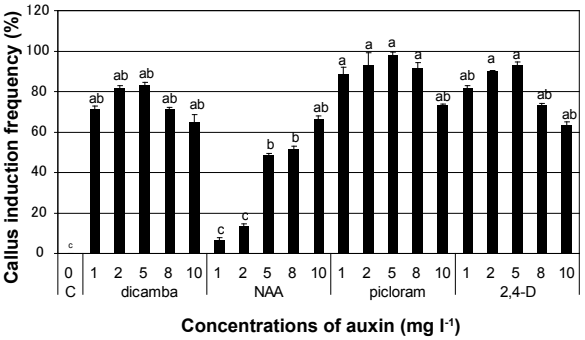

Fig. 1. Effect of type and concentration of auxins on callus induction from mature seeds of *C. selloana*.
Each value represents a mean ± SE of three independent experiments. Tukey's HSD test was performed to identify significant differences among the 21 treatments and the different letters indicate significant differences at $p < 0.05$.
C - PGRs-free medium.

Table 1. Effect of auxin types on growth of mature seed-derived calluses of *C. selloana* in liquid medium.

Auxin (2 mg l^{-1})	Callus growth rate (fold)
Dicamba	1.6 ± 0.1 a
NAA	1.1 ± 0.2 a
Picloram	3.3 ± 0.2 b
2,4-D	3.1 ± 0.1 b

Data were expressed as the mean ± SE. Tukey's HSD test was performed to identify significant differences among the 4 treatments and the different letters indicate significant differences at $p < 0.05$.

Table 2. Effect of auxin and medium type on growth of calluses of *C. selloana.*

Auxin (2 mg l^{-1})	Callus growth rate (fold)	
	Liquid medium	Agar-solidified medium
Picloram	3.1 ± 0.2 a	1.1 ± 0.3 b
2,4-D	2.9 ± 0.5 a	1.2 ± 0.1 b

Data are expressed as the mean ± SE. Tukey's HSD test was performed to identify significant differences among the 4 treatments and the different letters indicate significant differences at $p < 0.05$.

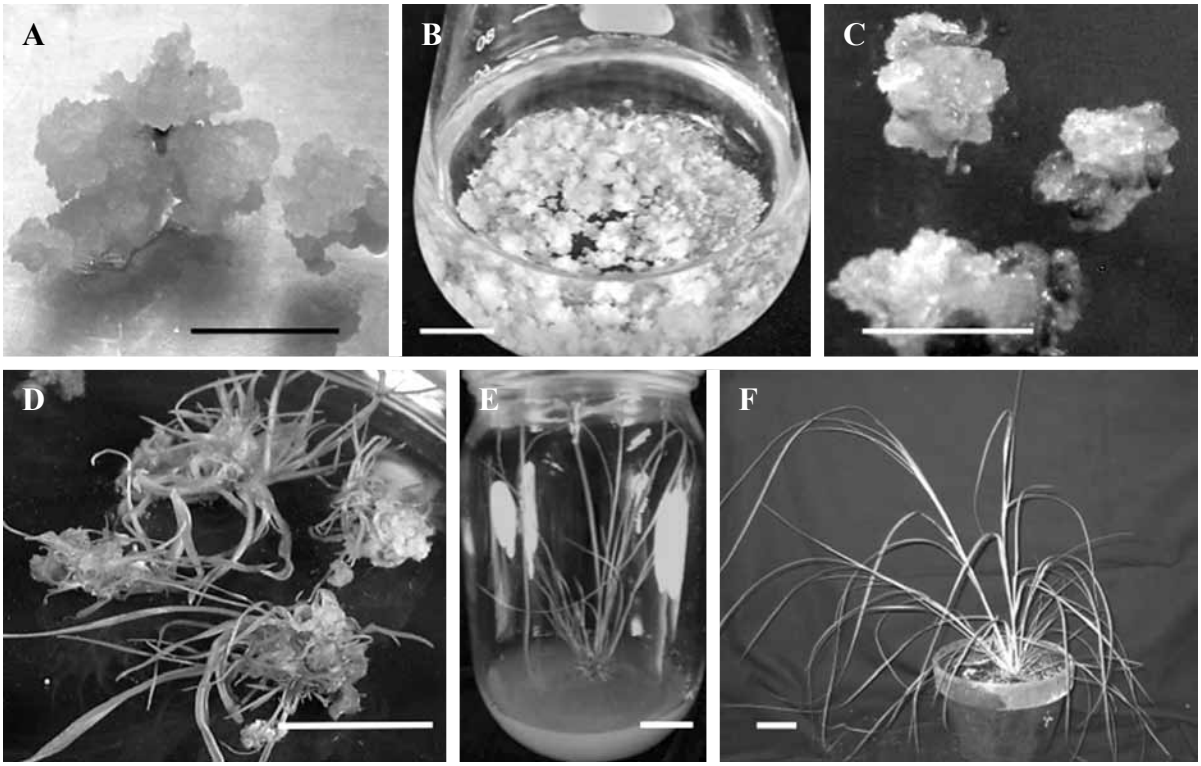

Fig. 2. Shoot regeneration from *C. selloana* callus. A) Callus induction from mature seeds of *C. selloana* after 2 months of culture under dark conditions on MS medium supplemented with 2 mg l^{-1} picloram (bar = 5 mm), B) Proliferated mature seed-derived calluses after 1 year of culture under dark conditions in liquid MS medium supplemented with 2 mg l^{-1} picloram. The calluses were subcultured every 2 weeks after initiation of culture (bar = 5 mm), C) Shoot induction from liquid cultured calluses after 10 days of culture under light condition on MS medium supplemented with 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (bar = 1 cm), D) Regenerated multi-shoots 1 month after transfer the liquid cultured calluses onto MS medium supplemented with 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (bar = 5 mm), E) A rooted plantlet 1 month after transfer the shoot onto PGR-free half-strength MS medium (bar = 3 mm), F) A plant with numerous branches established in a pot 3 months after the transfer of the plantlet shown in (E) to greenhouse conditions (bar = 5 cm).

medium (3 months on agar-solidified medium followed by 4 months in liquid medium) proliferated at 3-fold higher growth rate than that on agar-solidified medium (7 months on agar-solidified medium) (Table 2). Thus, we used liquid medium containing 2 mg l^{-1} picloram or 2,4-D for the subsequent experiments.

Effect of BAP and NAA on shoot regeneration from liquid cultured calluses

After 4 months of liquid culture, the calluses (Fig. 2B) were used for examining the effect of BAP and NAA on shoot regeneration. Calluses turned yellow to green after 10 days of transfer (Fig. 2C), and regenerated multiple-shoots on all tested variants of medium after 2 weeks of culture (Fig. 2D), even in the absence of PGRs, although the frequency was low (Fig. 3). The two types of calluses induced by picloram and 2,4-D, respectively showed the same tendency of shoot regeneration ability in response to PGR concentrations, and the highest shoot regeneration frequencies, obtained after 5 weeks of culture on medium containing 0.5 mg

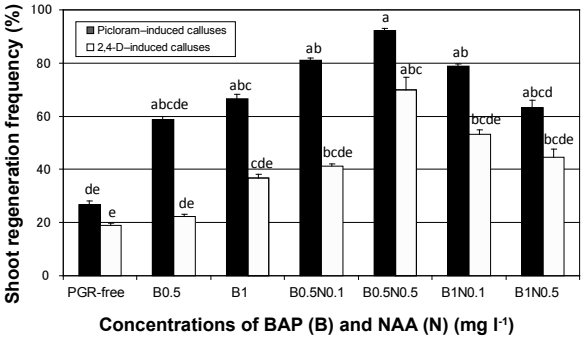

Fig. 3. Effects of BAP and NAA on shoot regeneration frequency of *C. selloana* liquid cultured calluses.
Each value represents a mean ± SE of 3 independent experiments. Tukey's HSD test was performed to identify significant differences among the 14 treatments and the different letters indicate significant differences at $p < 0.05$.

l^{-1} BAP and 0.5 mg l^{-1} NAA were 92.1% in picloram-induced calluses and 70.8% in 2,4-D-induced ones.

Table 3. Changes in growth rate, ploidy level and shoot regeneration ability during the long term subcultures of callus maintained in liquid medium with picloram.

Subculture period (month)	Callus line	Callus growth rate (fold)	Ploidy level	Shoot regeneration frequency (%) **
4	1	3.2 ± 0.1 a	diploid	85.4 ± 1.9 a
	2	3.3 ± 0.1 a	diploid	81.3 ± 3.8 ab
	3	3.6 ± 0.1 a	diploid	83.3 ± 3.3 ab
8	1	3.0 ± 0.1 a	diploid	75.0 ± 3.4 bc
	2	3.1 ± 0.2 a	diploid	70.8 ± 5.2 c
	3	3.0 ± 0.2 a	diploid	77.0 ± 4.6 ab
12	1	3.2 ± 0.3 a	diploid	22.9 ± 2.6 d
	2	3.2 ± 0.4 a	diploid	20.8 ± 2.1 d
	3	3.4 ± 0.3 a	diploid, tetraploid	8.3 ± 2.8 e

Data were expressed as the mean ± SE. Tukey's HSD test was performed to identify significant differences among the 9 treatments and the different letters indicate significant differences at $p < 0.05$.

Effects of the period of subcultures in liquid medium on growth rate, ploidy level, and shoot regeneration ability of calluses

During the subcultures for 12 months, calluses showed a similar morphology to, and no significant change in growth rate, from the original calluses, but reduction in shoot regeneration ability was found with increased subcultures (Table 3). Although 4 and 8 month-old calluses maintained relatively high shoot regeneration ability at 81.3-85.4 and 70.8-77.0%, respectively, 12 month-old calluses showed drastic reduction of shoot regeneration ability to 8.3-22.9% (Table 3). Results of FCM analysis revealed that no change in ploidy level of the calluses from the original plants was observed till 8 months in comparison with the control plants (Fig. 4A,B). However, 12-month old liquid-cultured calluses showed appearance of cells with tetraploid level (Fig. 4C) in one of the callus lines (line # 3), which had the lowest shoot regeneration ability (8.3%) (Table 3). These results show that the mature seed-derived callus could be maintained in liquid medium containing 2 mg l^{-1} picloram for at least 8 months without losing shoot regeneration ability and affecting ploidy level. In sugar beet, decrease in regeneration ability of calluses was closely related with genic DNA methylation (Maury et al. 2012). In barley, decrease in shoot regeneration ability of calluses was associated with chromosomal aberrations (Singh 1986). Calluses with higher ploidy levels also showed reduced plant regeneration ability in *Pisum sativum* (Ochatt et al. 2000) and *Dianthus acicularis* (Shiba and Mii 2005). At present, the mechanism involved in the drastic reduction of shoot regeneration ability of *C. selloana* calluses observed after 8 months of liquid culture is not clear and needs to be elucidated in future studies.

Characterization of regenerated plants

The shoots easy rooted after transfer onto PGRs-free half-strength MS medium and grew into healthy plantlets (ca. 10 cm in height with ca. 7 leaves) after 2 months of culture (Fig. 2E). The regenerated plantlets continued to grow normally after acclimatization and transferring to the greenhouse (Fig. 2F). No morphological differences were observed between control

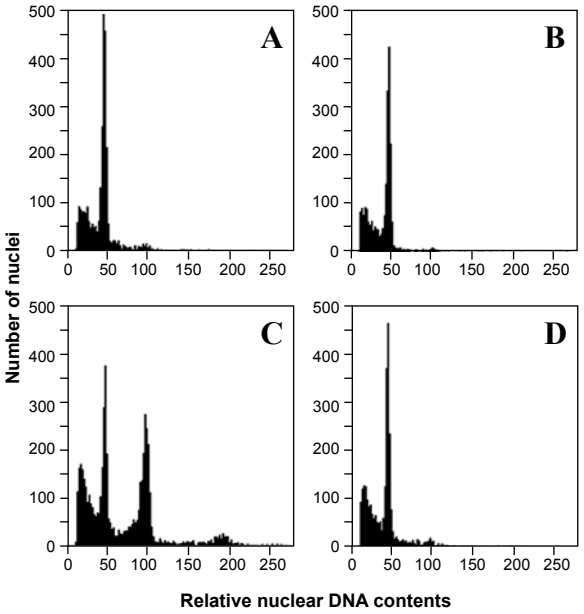

Fig. 4. Flow cytometry (FCM) analysis of calluses and regenerated plant of *C. selloana*. A) Leaf of control plant, B) Callus after 8 months of subculture in liquid medium containing 2 mg l^{-1} picloram, C) Callus after 12 months of subculture in liquid medium showing the appearance of tetraploid level of cells, D) Leaf of regenerated plant from callus after 4 months of subculture in liquid medium.

seedling plants and regenerated plants. Moreover, no variations in ploidy level were observed in the control plants and regenerated plants (Fig. 4A, D). These results suggest that ploidy level did not change in the plant regeneration process.

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