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Evaluation of anther-derived somatic embryos in *Hevea brasiliensis* Muell Arg. by flow cytometry

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Abstract The influence of plant growth regulators on somatic embryogenesis in anther culture of rubber tree and evaluation of ploidy level of somatic embryos (SEs) by flow cytometry were investigated. Optimum somatic embryogenesis frequency at 64% and a number of somatic embryos per explant at 5.13 SEs were achieved on Murashige and Skoog (MS) containing 0.06 mg/l naphthaleneacetic acid (NAA), 0.03 mg/l benzyladenine (BA), 3% sucrose and 0.75% agar. These SEs from the medium showed the same ploidy level as mother plants after evaluation by flow cytometry.

Key words: *Hevea brasiliensis*, anther culture, somatic embryogenesis, flow cytometry

Introduction

Hevea brasiliensis Muell Arg., belonging to the Family Euphorbiaceae, is an economically important perennial tree grown in Thailand and Southeast Asia as the source of natural rubber. *H. brasiliensis* is still propagated by grafting clonal axillary buds onto unselected seedlings to maintain intraclonal heterogeneity for both vigour and productivity (Hua *et al*., 2010). Somatic embryogenesis is one of the powerful tissue culture techniques for mass propagation of elite *Hevea* clones. The utilization of this system also opens up new avenues for production of mass number of uniform rootstock and for molecular farming through genetic transformation. *Hevea* somatic embryogenesis was firstly developed in China and Malaysia, using the anther as initial mother tissue explants (Venkatachalam *et al*., 2007). In *Hevea*, plant development via somatic embryogenesis was achieved from many explants including inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al*., 2000; Montoro *et al*., 2000; Lardet *et al*., 2007) axillary buds (Mendanha *et*

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al., 1998) immature anther (Wang *et al*., 1984; Jayasree *et al*., 1999) unpolinated ovules (Kouassi *et al*., 2008) and root (Zhou *et al*., 2010). Anthers culture offers the possibility of homozygous haploid lines production. However, the long juvenile phase and the requirement of many generations in breeding program make this approach impractical. *In vitro* approaches to induce haploids in *Hevea* have only limited success in comparison with other plant species (Venkatachalam *et al*., 2007). The success of embryoids and plant development via somatic embryogenesis was achieved from anther wall-derived calli (Wang *et al*., 1984; Jayasree *et al*., 1999) which is somatic tissue. Thus, plantlets produced through this explants culture are diploid and the same as mother plant. However, assessment of those plantlets needs to be performed. The development of methods to assess the genetic stability of *in vitro* plants is highly valuable including chromosomal analysis, flow cytometry, and DNA fingerprinting by molecular markers. For micropropagation in *Hevea*, only chromosome analysis was reported for evaluation the genetic stability of anther-derived plantlets (Jayashree, 1999). So the aim of this study was to evaluate the ploidy level of somatic embryos (SEs) derived from anther culture in *H. brasiliensis* by flow cytometry, which simple, fast and efficient.

Materials and methods

Plant material and culture conditions

Early introduced clones of rubber tree grown naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were used as mother plants for the source of anther in the callus initiation experiment. The male flowers (3-3.5 mm in length; Fig. 1-a) from panicle inflorescence were collected and washed in running tap water for 10 minutes. The explants were surface sterilized in 70% ethanol for 30 seconds and in 20% sodium hypochlorite for 20 minutes, followed by three rinses with sterilized distilled water. The sterilized flowers were kept in 4°C for 24 hours. Immature anthers were excised and cultured on a callus induction medium (CIM) which was Murashige and Skoog (MS) medium supplemented with 1 mg/l 2, 4-dichlorophenoxybenzoic acid (2, 4-D), 1 mg/l kinetin (KN), 1 mg/l α-naphthaleneacetic acid (NAA) and 3% sucrose) (Te-chato *et al*., 2002). The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121 ℃ for 15 minutes. The cultures were maintained at 28±0.5^{o}C under fluorescent lamps at 12.5 μmol/m^2/s for a 14 hour photoperiod. The cultures were routinely subcultured at 4 week intervals to induce and proliferation of callus.

Effect of plant growth regulators on SEs formation

The immature anthers-derived calli on CIM under the conditions specified above for 8 weeks were used in this study. The calli (100 mgFW) were transferred to SEs induction medium which was MS medium supplemented with different concentrations of benzyladenine (BA) (0-1 mg/l) or 2, 4-D (0-1 mg/l) or 0.06 mg/l NAA and 0.03 mg/l BA. All culture media were supplemented with 3% sucrose. The pH of culture medium was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121 ℃ for 15 minutes. The cultures were maintained at 28±0.5^{o}C under fluorescent lamps at 12.5 µmol/m^2/s for a 14 hour photoperiod. The cultures were routinely subcultured at 4 week intervals to induce SEs formation. After cultured for 8 weeks, the percentage of SEs formation and the number of SEs per explant were recorded.

Flow cytometry analysis

Well-developed SEs was selected to determine ploidy level. Flow cytometry was performed to estimate the ploidy level of those SEs according to the method of Ishigaki *et al*. (2009). In brief, approximately 0.5 cm^2 of SE derived from anther culture or young leaf tissue from the mother plants were cut and placed on a 90-mm Petri dishes. These explant pieces were soaked with 1 ml of an extraction buffer (50 mol/l Tris–HCl, 0.5% polyvinylpyrrolidone, 0.01% Triton-X, 0.63% sodium sulfite, pH 7.5) for 5 minutes and chopped with a sharp razor blade. After filtering chopping tissue through 50 µm nylon mesh, 100 µl of 0.1% propidium iodide (PI) solution was added to the nuclear suspension and incubated for at least 5 min at room temperature to stain the nuclei. The fluorescent intensities of each nuclear suspension were measured by an EPICS XL, equipped with a 488 nm argon laser with a long path filter (Beckman Coulter, Tokyo, Japan).

Statistical analysis

Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.05 level of probability.

Results and discussions

Effect of plant growth regulators on SEs formation

Extensive experiments were carried out by several researchers to enhance the frequency of SEs induction and plant regeneration in *Hevea*. In this study, immature anther-derived calli were achieved from callus induction medium (CIM). Friable callus and fast growing were observed from CIM medium. After cultured the callus on SEs induction medium with different concentrations of plant growth regulator for 8 weeks, embryos were derived from calluses in all culture media. The frequency of somatic embryogenesis and average number of SEs per explant was increased when increasing the concentration of BA or 2, 4-D (Table 1). In the absence of growth regulators, callus failed to initiate SEs and died shortly after being cultured for 2 weeks (Fig. 1-b). However, SEs obtained from culture media containing BA or 2, 4-D were abnormal and ceased to develop at torpedo stage (Fig. 1c-d). Although MS medium containing NAA in combination with BA gave callus formation but those calluses could not develop into SEs (data not shown). Induction of SEs was found to be better in culture medium with 0.06 mg/l NAA and 0.03 mg/l BA. Percentage and number of SE induction were recorded to be 64.00±8.94 and 5.13±2.03 SEs/explants, respectively (Table 1). Those SEs could develop into the cotyledonary stage and tend to germinate into plantlet regeneration (Fig. 1e-f). Several factors such as the development stage, type of explant, plant growth regulators, basal medium composition, light intensity, etc. appear to play important role in the induction of somatic embryogenesis in many plants including *Hevea*. In the present study, modified MS medium supplemented with BA or 2, 4-D alone could induce SEs but those SEs did not developed into the mature SEs. Hua *et al*. (2010) observed that MS-based plant regeneration medium with 4.5 or 9.0 µM 2, 4-D gave the highest rate of plant regeneration through SEs from root culture of *Hevea* (CATAS 7-33-97 and CATAS 88-13) which was difference from our results. In addition, several researchers reported that ½ MS medium containing 0.06 mg/l NAA and 0.03 mg/l BA gave the suitable embryos induction and plant conversion in many plants including mangosteen, pawa (*Garcinia speciosa* Wall.) and somkhag (*G. atroviridis* Griff.) (Te-chato, 1997). Therefore, in this study, we tried to modify this medium for SEs induction in *Hevea* from anther-derived callus. The results showed that ½ MS medium containing 0.06 mg/l NAA and 0.03 mg/l BA gave the better SEs induction percentage and a number of SEs per explant than other culture media. The low level of auxin and cytokinin in the SE induction medium was sometimes responsible for the development of embryos directly from the explants, implying that they did not develop through callus formation

(Kouassi *et al*., 2008). However, our results showed that embryos were induced indirectly through callus induction.

Table 1. Effect of MS medium supplemented with different concentrations of BA or 2, 4-D or NAA on SE formation from anther-derived callus of early introduced clones of rubber tree grown naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, for 8 weeks

Concentrations of PGR	**Somatic embryo formation (%)**	**Average number of somatic embryos/explant**
BA (mg/l)		
0	0c	0c
0.1	33.33 ±14.83b	1.67 ±1.09b
0.25	40.00 ±11.40b	2.00 ±1.48b
0.50	73.33 ±8.37a	3.67 ±1.30a
1.0	86.67 ±8.94a	4.33 ±3.67a
2,4-D (mg/l)		
0	0c	0c
0.25	48.00 ±11.40b	2.40 ±0.78b
0.50	56.00 ±13.04b	2.80 ±0.67b
0.75	52.00 ±8.94b	2.60 ±1.24b
1.0	84.00 ±10.95a	4.20 ±1.78a
0.06 mg/l NAA + 0.03 mg/l BA		
	64.00 ±8.94	5.13 ±2.03

Mean values followed by the same letter(s) within a column are not significantly different ($p < 0.05$) by DMRT; ± indicates standard deviation

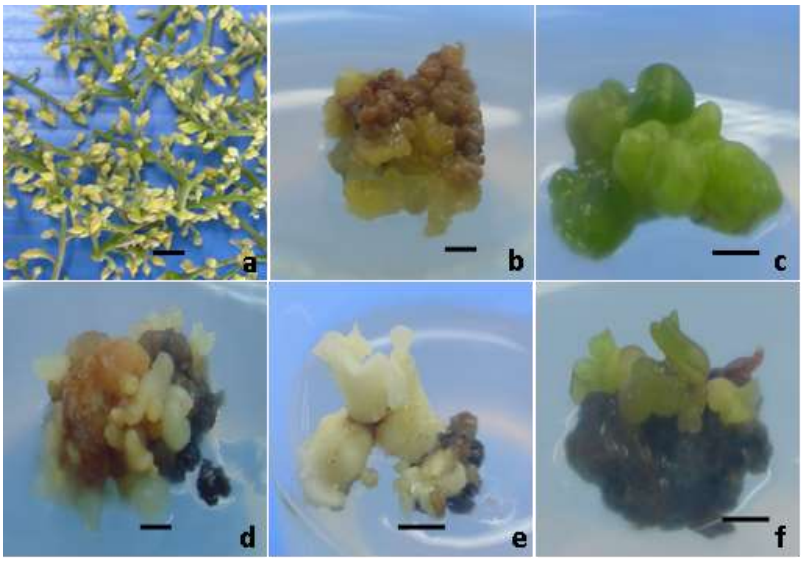
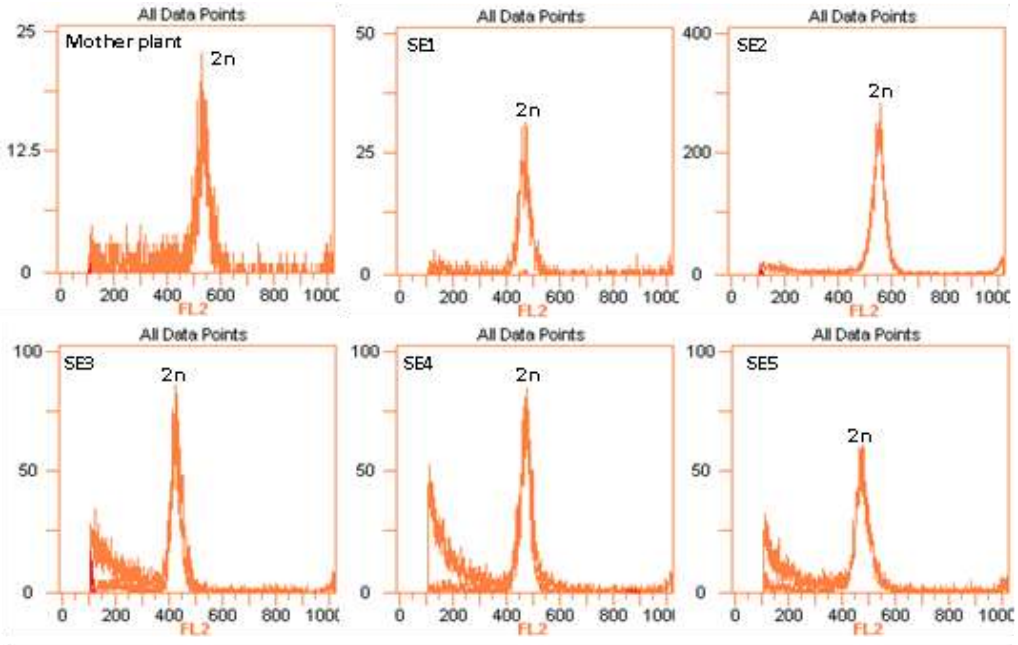


Fig. 1. Somatic embryogenesis derived from anther culture of early introduced clones of rubber at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand. (a) male flowers, (b) browning callus on MS medium without plant growth regulators, (c) greenish color SEs on MS medium with 1 mg/l BA, (d) yellowish white color SEs on MS medium with 1 mg/l 2, 4-D, (e and f) well developed SEs regenerated from browning callus on MS medium with 0.06 mg/l NAA and 0.03 mg/l BA after culturing for 8 weeks (bar = 0.5cm).

Flow cytometry analysis

Flow cytometry was employed to estimate the ploidy level of regenerated plantlets of SEs due to the accuracy, efficiency of numbers and convenience of the approach when compared to chromosome counting (Ishigaki *et al*., 2009). Flow cytometry is regarded as the most accurate tool for ploidy estimation, whereas traditional cytological analysis takes more time and labor intensive (Gu *et al*., 2005; Yang *et al*., 2006). In our study, determination the ploidy level of those SEs by flow cytometry technique, showed the same peak as mother plant (Fig. 2). Previous cytological observation in *Hevea* indicated that regenerated plantlets induced from anther culture were diploid (Wang *et al*., 1984; Jayasree *et al*., 1999), suggesting that callus might be formed from parenchyma cells at the cut end and apex and in the septum and wall. The pollen grains in the anther disintegrated and died shortly after being cultured (Wang *et al*., 1984). Genetic performances also proved that anther SEs could originate from somatic cells or tissues of the anther.



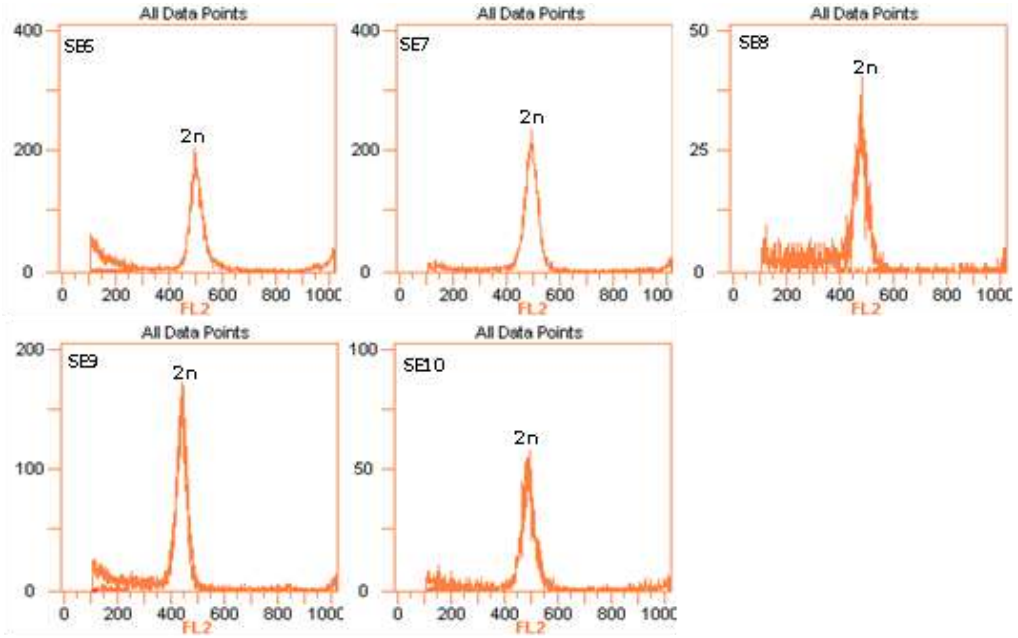


Fig. 2. Fluorescent intensity measures of somatic embryos derived from anther culture on MS medium with 0.06 mg/l NAA and 0.03 mg/l BA for 8 weeks by flow cytometry. SE1-SE10 were the samples of SEs from number 1 to 10.

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