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Callus Induction and Somatic Embryogenesis from Anther Cultures of *Hevea brasiliensis* Muell Arg.

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ABSTRACT

Hevea brasiliensis is an economically important perennial tree in southeast Asia. However, few studies have been conducted to evaluate the effects of sucrose and genotype on callus induction and plantlet regeneration from anther culture. Male flowers at the uninucleate stage were collected from clones numbered 2-nr, 6-nr, 1-tF and 1-em. An excised cluster of anthers was cultured on callus induction medium (CIM) consisting of Murashige and Skoog (MS) medium supplemented with 1 $mg.L^{-1}$ 2,4-diclorophenoxyacetic acid, 1 $mg.L^{-1}$ kinetin (KN), 1 $mg.L^{-1}$ α-naphthalene acetic acid (NAA) and sucrose at various concentrations ranging from 3 to 10%. The results revealed that the highest callus induction frequency (87.5%) was obtained from clone 1-em on CIM with 8% sucrose. Callus derived from clone 2-nr culturing on CIM supplemented with 5% sucrose gave the highest frequency and number of somatic embryo (SEm) formations. CIM supplemented with 5% sucrose and 5% coconut water caused a decrease in the percentage of callus induction (30.88%). The highest SEm formation at 20% was obtained when callus of clone 2-nr aged 8 wk was cultured on MS supplemented with 3% sucrose, 0.2 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ 6-benzyladenine, 3 $mg.L^{-1}$ KN and 0.05 $mg.L^{-1}$ gibberellic acid (GA_3). When calluses were cultured on CIM supplemented with 5% sucrose and maintained in darkness, 16% SEm formation was obtained. Calluses and SEms from the culture medium showed the same ploidy level as the mother plant after evaluation by flow cytometry.

Keywords: anther culture, callus induction, callus age, somatic embryogenesis, *Hevea brasiliensis*

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 (Srichuay and Te-chato, 2012). *In vitro* propagation of *Hevea* has played a very important role in the rapid multiplication of cultivars with desirable traits, true-to-type production, healthy and disease-free plants (Prammanee *et al*., 2011; Sirisom and Te-chato, 2012; Sirisom and Te-chato, 2013).

With *in vitro* culture, sucrose in a culture medium is commonly used as a carbon source and osmotic regulator for plant cell growth and development and this indicated the relationship between the concentration of sucrose and the percentage of callus induction subsequent to plant regeneration in anther culture of rice (Shahnewaz and Bari, 2004). Concentrations of sucrose had an effect on callus induction of *Stevia* (Pande

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and Khetmalas, 2012). In some species, high concentrations of sucrose (up to 3%) could stimulate callus induction, for example, *Muscari azureun* (Uranbey, 2011) and barley (Han *et al*., 2011). The addition of coconut water (CW) in the culture medium has also influenced successful development in cell culture in many plant species, such as *Phalaenopsis cornu-cervi* (Breda) Blume & Rchb. f. (Rittirat *et al*., 2012) and *Curcuma* (Jala, 2013).

The induction and regeneration of somatic embryo are sensitive to culture conditions, such as the composition of the culture medium and environment, the genotype and the explant source (Fuentes *et al*., 2000). Successful plantlet regeneration has been recorded using immature anthers in *Hevea* (Jayasree *et al*., 1999; Hua *et al*., 2010; Zhou *et al*., 2012), *Curcuma* (Kou *et al*., 2013) and *Coffea* (Silva *et al*., 2011). In order to propagate true-to-type clones, somatic embryogenesis appears to be a promising technique which can produce a large number of uniform plantlets in a short time (Ahmad *et al*., 2011; Singh *et al*., 2013). The age of the callus has been reported as playing a role in the promotion of plantlet regeneration in day-neutral strawberry (Nguyen *et al*., 2012). The plantlet regeneration rate in *Narigi crenulata* (Roxb) reflected the existence of peroxidase activity correlated with increasing callus age (Singh *et al*., 2011). Darkness has been reported to induce the high formation of somatic embryos in grapevine (Tangolar *et al*., 2008) and strawberry (Gerdakaneh and Zohori, 2013). However, somatic embryos of black grama were highly developed under light conditions (Osuna and Barrow, 2004).

In the current study, the anthers of rubber tree were used as explants for the induction of callus, followed by embryogenic callus formation subsequent to plantlet regeneration.

MATERIALS AND METHODS

Plant material and culture conditions

The plant material consisted of immature male flower buds (1.2-1.5 mm in length) of *Hevea brasiliensis* Muell. Arg. (clones numbered 2-nr, 6-nr and 1-em) grown around the Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand and of clone 1-tF grown in its source area in Trang province, Thailand. Immature male flowers containing microspores at the uninucleate stage were collected and kept in a refrigerator at 4°C for 24 hr. The cold-hardened explants were washed in running tap water for 20-30 min. All explants were surface sterilized in 70% ethanol for 30 s and in 1.05% sodium hypochlorite for 20 min, followed by rinsing three times with sterilized distilled water.

The anthers (0.8-1.0 mm in length, Figure 2a) were excised from sterilized explants under a light microscope and cultured on Murashige and Skoog (MS) medium. The pH of the medium was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 $kg.cm^{-2}$ and 121°C for 15 min. The cultures were incubated at 26 ± 3°C in the dark for 8 wk.

Effect of sucrose and type of clone on callus induction

The anthers derived from clones 2-nr, 6-nr and 1-em were cultured on a callus induction medium (CIM) consisting of MS medium supplemented with 1 $mg.L^{-1}$,4-diclorophenoxyacetic acid (2,4-D), 1 $mg.L^{-1}$ kinetin (KN) and 1 $mg.L^{-1}$ α-naphthalene acetic acid (NAA). The medium was supplemented with different concentrations of sucrose at 3, 5, 8 and 10%. All cultures were maintained under the conditions specified above. The cultures were routinely subcultured at 4 wk intervals for 8 wk to induce callus. After 8 wk of culturing, the callus induction percentage was recorded.

Effect of coconut water on callus induction

Immature anthers of clone 2-nr were cultured on CIM supplemented with 5% sucrose and two concentrations of coconut water (0 and 5 %). The cultures were maintained under the same conditions described above. After 4 wk of culturing, the callus induction percentage was recorded.

Effect of callus age and type of clone on somatic embryo induction

Callus derived from anthers of clones 2-nr and 1-tF were subcultured onto the MS medium supplemented with 5% sucrose, 1 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ KN and 1 $mg.L^{-1}$ NAA at 4, 8 and 12 wk and transferred to a somatic embryogenesis medium (EIM) consisting of solidified MS medium supplemented with 3% sucrose, 0.2 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ BA, 3 $mg.L^{-1}$ KN and 0.05 $mg.L^{-1}$ GA_3 (adapted from Zhou *et al*., 2012).

The cultures were incubated at 26 ± 3°C under a 14 hr photoperiod of cool-white fluorescent lamps at an intensity of 12.5 µmol $m^{-2}.s^{-1}$ photosynthetic photon flux density. After 4 wk of culturing, somatic embryo induction and the number of somatic embryos per callus were recorded.

Effect of photoperiod on somatic embryo induction

Callus aged 8 wk derived from anther culture of clones 2-nr and 1-tF on the CIM supplemented with 5% sucrose were transferred to the same media and to EIM media. All cultures were maintained under two different sets of conditions—complete darkness and light/dark (14/10 hr), referred to as light conditions in the study. After 4 wk of culturing, the somatic embryo induction percentage and number of somatic embryos per callus were recorded.

Flow cytometry analysis

Well-developed SEs and callus derived from anther culture were used as starting material to analyze genetic instability through the ploidy level,

Approximately 0.5 cm^2 of tissue samples from control plants, callus and SEms were placed in 90 mm Petri dishes and soaked with 1 mL of an extraction buffer (50 M Tris-HCl, 0.5% polyvinylpyrrolidone, 0.01% Triton-X, 0.63% sodium sulfite, pH 7.5) for 5 min and then chopped with a sharp razor blade. After mixing the tissue well, buffer filtration was carried out through a 50 µm nylon mesh. The nuclear suspension was stained with 100 µL of 0.1% propidium iodide solution and incubated for at least 5 min at room temperature. The fluorescent intensities of each sample were measured by a flow cytometer (EPICS XL; Beckman Coulter; Tokyo, Japan), equipped with a 488 nm argon laser with a long path filter according to Ishigaki *et al*. (2009).

Statistical analysis

Experiments were performed in a complete randomized design. All experiments were replicated four times per treatment, with each replicate containing 10 explants. Mean values were analyzed using a one-way analysis of variance. Significant differences among treatments were detected using Duncan's multiple range test at the 0.01 level of probability.

RESULTS

Effects of sucrose and type of clone on callus induction

The immature anther cultured in CIM formed callus at all concentrations of sucrose. Callus induction from immature anther among the three clones of rubber tree gave different responses to different concentrations of sucrose (Figure 1). This indicated an interaction between the clones and the concentration of sucrose. Clone 1-em consumed a high concentration of sucrose, but a high percentage of callus formation from clone

2-nr was induced with a low concentration of sucrose in the medium. Clone 1-em had the highest callus induction (87.50%) on medium containing 8% sucrose (Figure 1). Sucrose at a concentration of 10% resulted in a low percentage of callus induction in clone 2-nr. However, callus derived from clone 2-nr cultured on CIM containing 5% sucrose was able to develop into somatic embryos (Figures 2d, 2e).

Callus induction from CIM supplemented with 5% sucrose for 4 wk promoted friable, moist, white callus (Figure 2b). After 8 wk, clone 2-nr that had been transferred to EIM was able to develop into somatic embryos (Figures 2c, 2f).

Effects of coconut water on callus induction

Coconut water had a reverse effect on callus induction. After 4 wk of culturing anthers on CIM containing 5% sucrose in the presence of coconut water at a concentration of 5%, the percentage of callus induction decreased to 30.88%. CIM supplemented with 5% sucrose without coconut water had a higher percentage (89.38%) of callus induction (Table 1).

Normally, CW has been reported to promote adventitious shoot formation in the tissue culture of many orchids (Asghar *et al*., 2011; Bodhipadma *et al*., 2012; Parab and Krishnan, 2012; Mukarlina *et al*., 2010) and in *Curcuma* (Jala and Bobhipadma, 2012). CW acts as cytokinin-like structure; thus, in the tissue culture of orchid, there is no need to add BA in the culture medium. The optimum concentration of CW has been reported to be 15%; however, its concentration depends on the species of the orchid (Asghar *et al*., 2011; Mukarlina *et al*., 2010). With perennial trees, there have been only a few reports on the use of CW in the promotion of adventitious shoot bud formation. In oil palm, the addition of 15% CW in MS medium produced altered shoots that were leafy with an inflorescent-like structure (Chemalee and Te-chato, 2008). A similar result was also found in

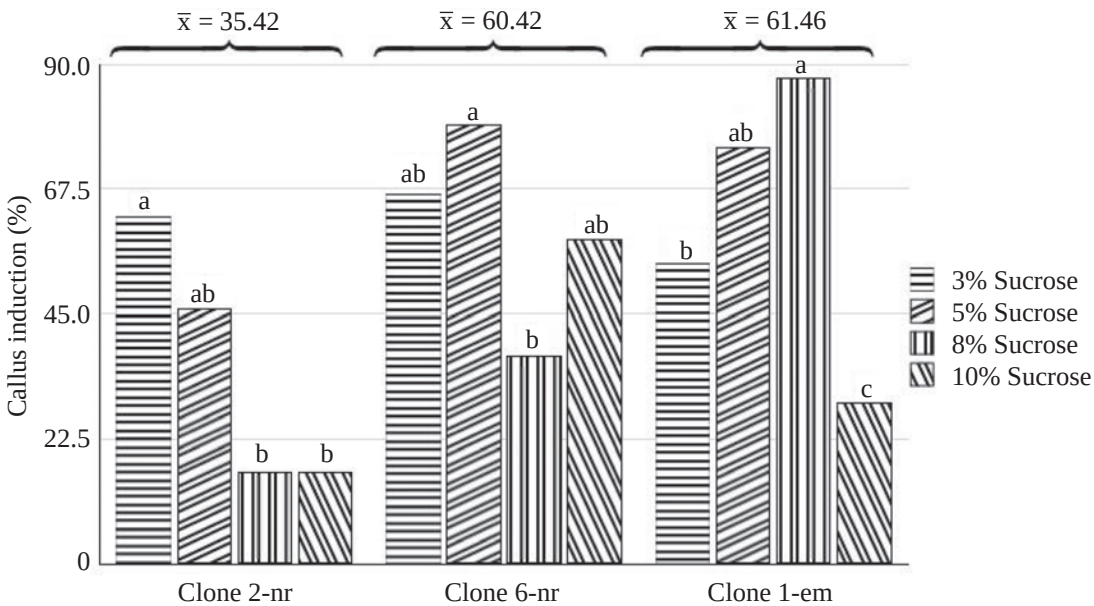


Figure 1 Influence of sucrose concentration and *Hevea brasiliensis* clone on callus induction from anther culturing on Murashige and Skoog medium supplemented with 1 mg.L^{-1} 2,4-diclorophenoxyacetic acid, 1 mg.L^{-1} kinetin and 1 mg.L^{-1} α-naphthalene acetic acid for 4 wk. Mean values followed by the same letter are not significantly different ($P \leq 0.01$).

Table 1 Effects of coconut water containing Murashige and Skoog medium supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-diclorophenoxyacetic acid, 1 mg.L^{-1} kinetin and 1 mg.L^{-1} α-naphthalene acetic acid on callus induction after 4 wk of culturing.

Coconut water (%)	Callus induction (% ± SD)
0	89.38±11.43[a]
5	30.88±20.82[b]
CV (%)	27.94

CV = Coefficient of Variation.
Mean values followed by the same letter within a column are not significantly different ($P \leq 0.01$).

the present study. Even a low concentration of CW (5%) caused a decrement in the callus formation percentage. A higher concentration than this might inhibit callus or SEm formation completely. From the present study, it is suggested that CW is not suitable for the tissue culture of rubber tree both in the callus induction and shoot regeneration phases.

Effects of callus age and genotype on somatic embryo induction

The frequency of somatic embryo induction in the two genotypes and ages of callus after 4 wk of culturing are shown in Table 2. Clone 2-nr had a high somatic embryo induction (20%) from callus aged 8 wk whereas the other clones and ages of callus produced no response. A similar result was also obtained in the number of SEms. Callus at age 8 wk of clone 2-nr gave approximately 4 SEms/callus. No SEms were obtained from the other clones for all ages of callus (Table 2). It is possible that early aged callus possesses a high number of meristematic cells and those cells divide or grow rapidly without any differentiation, while cells in older aged callus (12 wk) produce a large number of secondary metabolites, latex or isoprene. Therefore, the optimum age at which callus undergoes SEm differentiation was 8 wk.

Table 2 Effect of genotype and callus age on somatic embryo (SEm) induction on the Murashige and Skoog medium supplemented with 3% sucrose, 0.2 mg.L^{-1} α-naphthalene acetic acid, 1 mg.L^{-1} 6-benzyladenine, 3 mg.L^{-1} kinetin and 0.05 mg.L^{-1} GA_3.

Genotype/callus age (wk)	SEm (%)	Number of SEms/callus (±SD)
Clone 2-nr		
4	0.00	0.00[b]
8	20.00	4.18±3.80[a]
12	0.00	0.00[b]
Clone 1-tF		
4	0.00	0.00
8	0.00	0.00
12	0.00	0.00
CV(%)		146.57

CV = Coefficient of variation.
Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$)

This phenomenon was similar to that reported for *Miscanthus* x *giganteus* (Kim *et al*., 2010). It has been already reported that successful plantlet regeneration of rubber tree was genotype specific (Blanc *et al*., 2006; Zhou *et al*., 2010; Zhou *et al*., 2012). The present study also found that only clone 2-nr produced a response in callus subsequent to SEm formation. Many factors have been varied in attempts to affect plantlet regeneration including the concentration of sucrose, and plant growth regulators (PGRs) and various ages of callus, but SEm formation and plantlet regeneration were not obtained. Similar results were also reported in barley (Han *et al*., 2011), day-neutral strawberry (Nguyen *et al*. 2012) and black grama (Osuna and Barrow, 2004). In rubber tree, there are many factors affecting plant regeneration of which the concentration of sucrose, the use of different PGRs and various ages of callus are very important.

Effects of light on somatic embryo induction

Generally, the proliferation of callus derived from the anther of rubber tree was carried out under light illumination. After being subcultured for 8 wk, growth of the callus was sufficient to transfer to fresh medium of the same component after the first subculture embryogenic callus (EC) was formed. The calli were white and

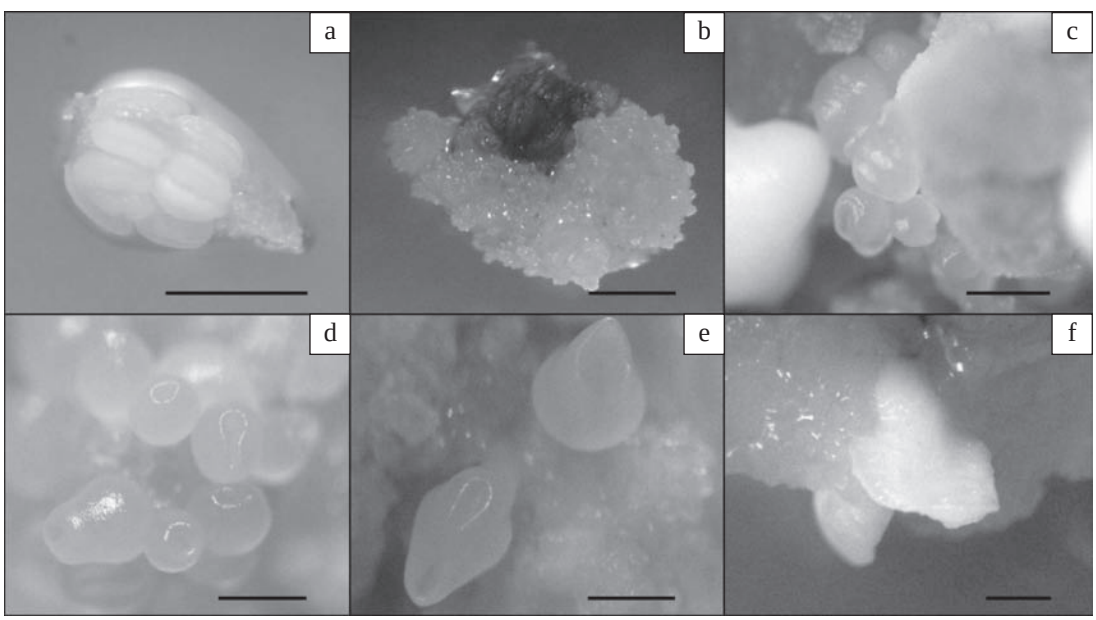


Figure 2 Development of somatic embryos (SEms) through somatic embryogenesis from anther culture of *Hevea brasiliensis*: (a) Freshly inoculated anther; (b) Callus induced after 4 wk of culturing on Murashige and Skoog (MS) medium supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-diclorophenoxyacetic acid (2,4-D), 1 mg.L^{-1} kinetin (KN) and 1 mg.L^{-1} α-naphthalene acetic acid (NAA); (c) SEms developed from 8-weeks-old callus on MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} 6-benzyladenine, 3 mg.L KN and 0.05 mg.L^{-1} GA_3 under light condition for 4 wk; (d) and (e) SEms at different stages of development from 8-weeks-old callus on MS medium supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN and 1 mg.L^{-1} NAA in the dark for 4 wk; (f) Cotyledonary stage embryos on MS medium supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN and 1 mg.L^{-1} NAA in the dark for 4 wk. (Scale bars = 1 mm).

shiny and contained a globular structure—the so-called proembryonic mass (PM)—in CIM under darkness conditions (Figure 2b) while compact, green calli with white, globular structure were found on EIM under light conditions (Figure 2c) after 8 wk of culturing. The development of SEm at 11.11% was found on EIM under light conditions whereas CIM induced SEm in darkness at 16%. The number of SEms with CIM under darkness produced a higher result (2.75 SEms/callus) than was obtained in EIM under conditions of light (1.2 SEms/callus) as shown in Table 3. Different developmental stages of SEm (Figures 2d, 2e and 2f) were found after 4 wk of culturing. This experiment suggests that darkness plays a substantial role in the development of SEms on the peripheral cells of embryogenic callus. Just after the induction of SEm formation, the callus should be transferred to EIM and maintained under light conditions to promote further development of the SEms (Figure 2c). Under darkness on EIM, there was no new SEm formation (Table 3). However, light conditions could promote further SEm formation with the percentage of SEm formation

Table 3 Effect of light on somatic embryo (SEm) induction from culturing anther-derived callus on callus induction medium (CIM) consisting of Murashige and Skoog (MS) medium supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-diclorophenoxyacetic acid, 1 mg.L^{-1} kinetin (KN) and 1 mg.L^{-1} α-naphthalene acetic acid (NAA); or on an embryogenesis medium (EIM) consisting of MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} 6-benzyladenine, 3 mg.L^{-1} KN and 0.05 mg.L^{-1} GA_3 for 4 wk.

Treatment	SEm formation (%)	No. of SEs/callus (±SD)
CIM		
Light conditions	0.00	0.00^{c}
Darkness	16.00	2.75±1.49^{a}
EIM		
Light conditions	11.11	1.2±0.45^{b}
Darkness	0.00	0.00^{c}
CV (%)		94.14

CV = Coefficient of variation.
Mean values followed by the same letter(s) within a column are not significantly different ($P \leq 0.01$).

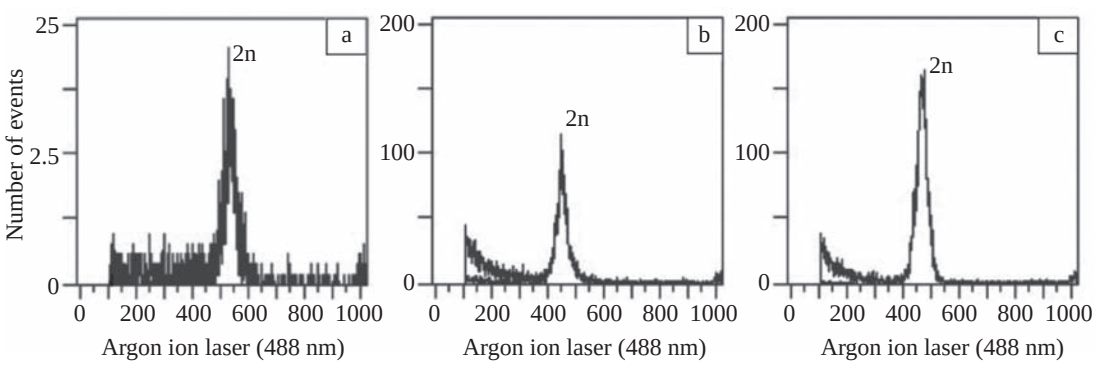


Figure 3 Histogram of fluorescence intensity of nuclei isolated from leaf sample of rubber tree: (a) Control treatment; (b) Embryogenic callus; (c) Somatic embryos developed on embryogenic callus.

and number of SEms/callus being 11.11% and 1.2 SEs/callus, respectively.

Flow cytometry analysis

Determination of the ploidy level of callus and SEms using a flow cytometry technique showed only one dominant peak at the same position which indicated the same ploidy level of embryogenic callus and SEm as in the control treatment (mothernal tree) (Figure 3). This phenomenon suggests that SEms derived from anther culture originated from somatic cells or tissues.

DISCUSSION

The concentration of sucrose plays an important role in callus formation in rubber tree tissue culture. Generally, 3% sucrose has been used in the culture medium to promote the growth and development of callus. This carbon source has been used to promote the percentage of callus production. A high percentage of maltose (6%) was reported to be important because it increased the callus induction of barley (Han *et al*., 2011). With *Ficus religiosa* L., a low concentration of sucrose decreased the percentage of callus induction (Siwach *et al*., 2011). Saikia *et al*. (2012) reported in *Aquilaria malaccensis* Lam. that 4% sucrose gave the best callus induction and growth. Different percentages of sucrose (1–6%) were reported to induce callus formation of *Stevia rebaudiana*; however, the highest response of callus induction was reported at 3% (Pande and Khetmalas, 2012). A high percentage of sugar (6%) has been reported to promote the growth of plantlets of *Calanthe* hybrids (Baque *et al*., 2011).

Coconut water containing complex compounds such as sugars, vitamins, minerals, amino acids and phytohormones was reported to enhance the proficiency of shoot formation (Yong *et al*., 2009; Molnar *et al*., 2011). Souza *et al*. (2013) reported that genotype and coconut water showed an interaction effect on the growth of plantlets in olive. In contrast, coconut water gave the lowest callus induction from anther culture of *Hevea* in the current study. In anther culture of rubber tree in the current study, 5% sucrose was required. In the medium, sucrose is the most common source of the carbon needed in callus formation and plantlet regeneration (Saikia *et al*., 2012). Moreover, sucrose act as an osmoticum and has been reported to play a positive role in SEm formation (Vahdati *et al*., 2008). A 4% sucrose content produced the highest callus induction and plantlet regeneration in rice (Shahnewaz and Bari, 2004) and *Aquilaria malaccensis* (Saikia *et al*., 2012). Ruminska *et al*. (2013) also reported that a low concentration of sucrose (1%) gave the lowest number of embryos whereas the highest number of embryos was produced with 5% sucrose.

Furthermore, genotype promoted callus induction. The highest callus frequency were formed from clone 1-em, followed by clones 6-nr and 2-nr, respectively, which gave frequencies of callus formation of 61.46, 60.42 and 35.42 %, respectively (Figure 1). A similar result was obtained in South Brazilian maize (Grando *et al*.,2013), where different genotypes gave different responses in embryogenic callus induction; genotypes Hs1 and Hs2 had a frequency of callus induction higher than from genotypes Hs3, Hs4 and Hs5. In barley, the genotype Zaoshu 3 gave the highest percentage of callus induction, whereas another 10 genotypes had significantly lower callus formation (Han *et al*., 2011). Sugarcane genotype S-2003-us-127 regenerated on callus formation media 3 but genotype S-2003-us-371 showed no regeneration, indicating that genotype can play a key role in callus formation and plant regeneration (Anjum *et al*., 2012).

Callus age is another important factor in somatic embryogenesis and SEm induction. With strawberry, callus aged 2 mth was best for subculturing to induce plantlet regeneration at the

highest rate of 16%. However, plant regeneration was not obtained from callus aged either 1 or 3 mth (Nguyen *et al*., 2012). In sugarcane, 28-day-old callus produced the maximum frequencies and numbers of shoot formation (Anjum *et al*., 2012). Long term callus (older than 18 mth) of *Asparagus officinalis* L. has also been reported to give a high number of shoot primordial formations at 89% (Pontaroli and Camadro, 2005). The highest regeneration frequency of wheat was 95% from 26-day-old callus in genotype Chakwal-97, whereas 48-day-old callus gave the lowest regeneration at 13% (Raja *et al*., 2009).

With regard to the impact of photoperiod on SEm formation,Tangolar *et al*. (2008) reported that two types of grapevine and various concentrations of auxin responded differently to light and dark conditions. *Yalova incisi* gave the best result in SEm induction under darkness whereas a high frequency of SEms was formed from 41 B rootstock under light conditions. Light irradiation may result in an increase in cytokinins, thus promoting shoot formation in horserdish (Saitou *et al*., 1992). Photoperiod also increased the efficiency of SEm induction on EIM in rubber tree in the present study.

The germination of somatic embryos from callus aged 8 wk induced on MS medium showed the same ploidy level as the mothernal plant. In most cereal crops, plants regenerated from anther culture were haploid (Dunwell, 2010) but some were diploid such as Latvian wheat, where plants regenerated from anther culture were reported to be 34 % diploid and 64% mixaploid (Grauda *et al*., 2010). With anther culture of bamboo, 3% of regenerated plants had the same ploidy level (haxaploid) as the seeding plant (Qiao *et al*., 2013). Many researchers reported the ploidy level in *Hevea* regenerated from plantlets derived from anther culture as being diploid (Wang *et al*., 1984; Jayasree *et al*., 1999; Sirisom and Te-chato, 2013). The present study showed clear evidence from flow cytometry that 100% of the regenerated plantlets from anther callous were diploid—the same as the mother plant. This suggests that callus might be induced from other somatic tissues of the anther—for example, the anther wall or tapetum which consists of diploid cells rather than pollen.

CONCLUSION

Callus from rubber tree clone 2-nr can be induced from anthers on MS medium supplemented with 1 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ KN, 1 $mg.L^{-1}$ NAA and sucrose at various concentrations ranging from 3 to 10%. Somatic embryos were induced on MS medium supplemented with 1 $mg.L^{-1}$ 2,4-D, 1 $mg.L^{-1}$ KN, 1 $mg.L^{-1}$ NAA and 5% sucrose. The cultures were maintained in darkness. Four key factors affecting successful somatic embryo induction from anther culture in rubber tree are: genotype, age of callus, concentration of sucrose and light conditions.

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