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Somatic Embryo (SE) Formation from Culturing Floral Explants of Rubber Tree (*Hevea brasiliensis* Muell. Arg.) and Assessment of Genetic Stability by RAPD and SSR Markers

Kanjanee Tongtape, Sompong Te-chato[*] and Sureerat Yenchon

Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla 90110, Thailand

([*]Corresponding author's e-mail: sompong.t@psu.ac.th)

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Abstract

Rubber tree is economically important rubber producing plant of Thailand. At present, a rubber tree plantation is susceptible to white root disease. Therefore, the use of rootstock from early introduce clone that proved to be resistant to white root disease could help sustain growing of rubber tree. Thus, the objectives of this research were to study the effects of plant growth regulators and different explants on somatic embryo (SE) induction of this rubber clone and assessment genetic stability. The results revealed that mix flower explant cultured on MS medium supplemented with 2.0 mg/L 6-benzyladenine (BA) and 1.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) provided callus subsequent somatic embryo (SE) formation at the highest frequency of 39.84 % and number of cotyledonary embryos (CEs) at 3.25 embryos /callus after 3 passages of subculture in the same culture medium (4 weeks/passage). SEs germinated into embryonic axis at 50 % and further development into shoot at 25 % after transfer to 0.25 mg/L GA_3 containing MS medium with the best concentrations of BA and 2,4-D for 4 weeks. The assessment gene stability by RAPD and SSR markers showed no variation between mother plant and *in vitro* plantlets. In this work, a novel explant source - the floral section of the rubber tree - was used for the first time in order to design an effective technique for *in vitro* somatic embryogenesis.

Keywords: Rubber tree, Types of explants, Plant growth regulators, Somatic embryogenesis, Genetic fidelity, RAPD, SSR

Introduction

Rubber tree is an economically important latex yielding crop. Natural rubber enterprises have been underpinning the socio-economic security in Thailand for a century [1]. Rubber latex is used as a raw material to produce several products such as rubber tyres, medical gloves, condoms, rubber bands, flexible tubing, etc. [2]. Nowadays, the products from rubber latex are a great demand. Therefore, the area of rubber plantation needs to be expanded in order to meet industry demand. The standard practice adopted in the commercial propagation of rubber clones is by budding selected clones onto seedling rootstocks and raised in polybags [3]. Generally, clone RRIM 600 is grown in 75 % of the rubber production area in Thailand and has been used for more than 60 years. However, this clone is highly susceptible to diseases caused by *Phytophthora* species [4]. The seedling of RRIM 600 mainly grown in Thailand is also sensitive to the white-root disease (*Rigidoporus microporus* (Fr.) Overeem) [5]. The disease causes economic lost not only for the yield but also persists on dead or living root debris for long time. It forms many white, flattened mycelial strands which grows and extends rapidly through the soil in the absence of any woody substrate [5-7]. Therefore, the use of resistant rootstock may solve this problem.

Currently, the amount of native rubber trees that resistant to white root disease in Thailand has been declined. Early introduced rubber clones (EIRpsu) were investigated in Prince of Songkla University (PSU) around the Faculty of Natural Resources and compared to other clones off-campus for their resistance to white root disease [5]. The results showed that EIRpsu I conferred resistant character the best. Hence, the propagation of this clone through tissue culture technique is necessary for multiplying the number of rootstocks. This technique has many advantages such as the large-scale production in a short period of time and production of true-to-type rubber tree. Micropropagation of rubber tree could be divided into 2 methods *i.e.* microcutting and somatic embryogenesis (SE). The SE is an efficient method for plant regeneration essential for mass propagation and crop improvement through transgenic approaches. In *Hevea*, EIRpsu I was reported to induce plantlet regeneration *via* somatic embryogenesis [8-10]. The most 2 possible sources

of explants with best results and practically used for embryogenesis are anther and integument. Anther produced callus, somatic embryo, and full plantlets [10]. On modified Murashige and Skoog (MS) medium supplemented with 3.0 % sucrose, 0.2 mg/L 1-naphthaleneacetic acid (NAA), 1.0 mg/L 6- benzyladenine (BA), 3.0 mg/L kinetin (KN), and 0.05 mg/L gibberellic acid (GA_3), somatic embryo induction was achieved. The somatic embryo induction rate was 20.0 % in those culture medium. In case of integument cultured a modified MS medium enriched with 5.0 - 6.0 % sucrose, 2.0 mg/L 2,4-D, 2.0 mg/L BA, and adjusted pH to 5.6 - 5.8 successfully promote embryogenic callus and plantlets regeneration from inner integument culture of immature fruit (8 weeks after pollination) [11]. To date, there is no report on the use of floral parts as initial explant for induction of callus and further development into somatic embryos. The present study is the first report to use floral explant, mainly corolla, for callus induction subsequent somatic embryo formation.

In nature, the genetic diversity and variability within a population are generated *via* recombination events. Factors such as environmental changes, crossing among related species, migration and population size influence genetic variability in different ways. The term 'somaclone' was referred to plants derived from any form of cell culture. In *in vitro*, the conditions of culture can induce mutation of regenerated plants derived from organ cultures, calli, protoplasts and somatic embryos; sometimes they can show phenotypic and genotypic variation [12,13]. However, it has been reported that culture environments, culture conditions, culture media, types of explants, successive transfer of culture, temperature, pH, plant growth regulators etc. cellular controls, resulted in genomic changes of *in vitro* regenerated plantlets [14-16]. The callus induction subsequent plant regeneration is a common way of generating somaclonal variation [17,18]. Recent studies revealed that cell or tissue cultures undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations) which are also expressed at biochemical or molecular levels [19,20]. Different molecular analytical techniques have been being used to point out somaclonal variation in plant tissue culture and regenerants of several plants. SSR (Simple sequence repeat) and RAPD (Randomly amplified polymorphic DNA) are widely used in studying genetic variability in regenerated plant from tissue culture such as arracacha [17], chili pepper [19], plum [20] stevia [21] and rubber tree [22,23].

Hence, the objectives of this study were to develop an efficient protocol for *in vitro* somatic embryogenesis from a new explant source, floral part, of rubber tree which has not been reported before and assess somaclonal variation of regenerated plantlets by Random Amplification of Polymorphic DNA (RAPD; technic for randomly amplified genomic DNA with random primers) and Simple Sequence Repeat (SSR; technic for amplification specific short tandem repeated of nucleotides by specific primers) or microsatellites markers which provided excellent targets and means of assessing genetic variation in tissue culture-derived materials. This protocol developed in this research study increases the number of rootstocks that were resistant to white root disease resulting in consistent quantity and quality of latex and being sufficient for use in the factory.

Materials and methods

Plant material

In this experiment, Young branches (one week after flushing) and young inflorescences of early introduced clone of rubber tree (EIRpsu I) grown naturally in front of Faculty of Natural Resources (FNR), Prince of Songkla University (PSU), Hatyai campus, Songkhla province, Thailand were collected during February to March, 2017 and used in this experiment. This plant is more than 70 years old and proved to be resistance to *Phytopthora* leaf fall and white root diseases [5]. Both explants were disinfested and prepared for callus induction through the method developed by different explants and different concentration of BA in combination with 2, 4-D [24]. Obtaining callus on MS medium with 1.5 mg/L 2,4-D and 2 mg/L BA was transferred to culture on the fresh medium with the same component 4 weeks intervals for maintenance and use in the next experiments.

Effect of types of explants on embryogenic callus (EC) formation and number of somatic embryos (SEs)

The callus derived from 4 different types of explants (longitudinal thin cell layer (lTCL), petal, single flower and mix flowers) obtained from previous study [24] were cultured on MS medium supplemented with the best concentrations of BA and 2, 4-D. The pH of the medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7 % agar and autoclaving at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were maintained at 28±2 °C under fluorescent bulbs at 15.0 $\mu mol/m^2/s$ for 14 h photoperiod. Subculture was carried out every 4 weeks for 12 weeks. After 12 weeks, the culture percentage of embryogenic callus formation and

numbers of SEs/explant were recorded and statistically compared among 4 different types of explants according to completely randomized design (CRD) with 5 replicates (each replicate consists of 10 explants). The data were statistically analyzed using ANOVA and means among the treatments were compared by Duncan's multiple range test (DMRT).

Effects of GA_3 on development of SEs from different explants

SEs at cotyledonary stage derived from previous experiment were transferred to culture on MS medium supplemented with GA_3 at different concentrations (0, 0.25, 0.50, 0.75 and 1.00 mg/L) and the best concentration of BA and 2, 4-D from previous experiment. The pH of the medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7 % agar and autoclaving at 1.05 kg/cm^2 and 121 °C for 15 min. The cultures were maintained at 28±2 °C under fluorescent bulbs at 15.0 µmol/m^2/s for a 14 h photoperiod. After 4 weeks of culture percentage of plant regeneration was compared using factorial from 2 factors (types of explants and different concentrations of GA_3) in CRD. The data were statistically analyzed using ANOVA and the means among the treatments and treatment combinations were compared according to DMRT.

Assessment of genetic stability

DNA extraction

Young fully expanded leaves from mother plant (MP) of rubber tree and young leaves from *in vitro* plantlets derived from different explants (S1-3 = petal, S4-6 = single flower and S7-9 = mix flowers) at 100 mg were used for DNA extraction according to the procedure modified [25]. In brief, the explants were ground to fine powder in liquid nitrogen and DNA was isolated using CTAB extraction buffer [2 % hexadecyltrimethyl-ammonium bromide (CTAB), 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl]. The plant extract mixtures were transferred to the microcentrifuge tube and incubated at 60 °C in water bath for 45 min, then centrifuged at 10,000 rpm for 45 min and transferred the supernatant to new clean microcentrifuge tube. Each tube was added with 500 µL of chloroform and the solution was mixed by inverting the tube, centrifuged at 12,000 rpm for 10 min and the upper aqueous phase (contains the DNA) were transferred to a clean microcentrifuge tube. The solution was added with 750 µL isopropanol and inverted the tube slowly for several times to precipitate the DNA. After precipitation, DNA pellet was washed with 70 % ethanol for 2 times and dried under room temperature. The quantity and quality of isolated DNA were determined by nanodrop spectrophotometer (BioDrop Ltd) before dissolving in TE buffer [20 mM Tris-HCl (pH 8.0) and 0.1 M EDTA (pH 8.0)] and stored at 4 °C for further polymerase chain reaction (PCR) analysis.

RAPD analysis

RAPD analysis was operated according to the method by [5,26]. Each amplification mixture of 25 µL contained 25 mM $MgCl_2$, 2.5 µL 10× *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer (OPAD01 and OPAD10), 1.5 units of *Taq* polymerase and 60 ng of template DNA. The thermal profile for RAPD-PCR was started at 95 °C for 30 s, followed by 41 cycles of 95 °C for 30 s, 37 °C for 1 min,72 °C for 2 min and finally 72 °C for 5 min. Amplification products were the separated by electrophoresis in 1.7 % (w/v) agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer at constant 100 V for 35 min. The gels were stained with ethidium bromide for 15 min, immersed in distilled water for 5 min and viewed under ultraviolet light with gel documentation. The amplification products of DNA were photographed and compared among the different sources of samples.

SSR analysis

Three SSR primer pairs (hmct5, hmac4 and hmtc1) were used for amplification of DNA following a protocol described by [25]. Each amplification mixture of 10 µL contained 2.5 mM $MgCl_2$, 2.5 µL 10× *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 unit of *Taq* polymerase 20 ng of template DNA. PCR amplifications were carried out on thermocycler (The MJ Mini cycler; Bio-Rad Ltd) using the following program: denaturation at 95 °C for 30 s; 34 cycles of 95 °C for 30 s, 52 °C for 60 s, 62 °C for 120 s and final elongation step at 72 °C for 5 min. The products of DNA were separated on 3 % (w/v) agarose gel in 0.5× TBE buffer at constant 100 V for 55 min. The gels were stained with ethidium bromide for 15 min, immersed in distilled water for 5 min and viewed under ultraviolet light with gel documentation. The amplification products of DNA were compared among the different sources of samples.

Results and discussion

Effect of types of explants on EC formation and number of SEs

In this study, petal, single flower and mix flowers derived calli were achieved on MS medium supplemented with 2.0 mg/L BA and 1.5 mg/L 2,4-D. The callus grew rapidly on this medium. After culturing the callus on this medium for 12 weeks with subculture at 4 weeks intervals, EC were formed from all 3 types of floral explants except lTCL which failed to form EC. Floral EC provided SEs which developed from peripheral cells of the callus. The frequency of somatic embryogenesis and average number of SEs per EC increased when time of subculture increased. Among 3 different floral explants mix flowers gave the highest percentage of EC formation at 39.84 % and highest number of globular (**Figure 1a**), heart shaped- (**Figure 1b**) and cotyledonary staged-SEs (**Figure 1c**) at 7.43, 4.52 and 3.25 SEs per EC, respectively (**Table 1**), significantly different ($p < 0.01$) with another explants. Several factors such as the development stages, types of explants, plant growth regulators, basal culture medium composition, light intensity, etc. play important role in the induction of somatic embryogenesis in many plants including *Hevea* [24]. In the present study, MS medium supplemented with BA and 2,4-D could induce SEs and promoted them to develop into the mature stage (cotyledonary SEs). Similar result was also reported in plant regeneration from green budwood culture of *Hevea* but different concentrations required [28]. In green budwood culture, low concentration of BA at 0.5 mg/L and high concentration of 2,4-D at 2.0 mg/L required, plantlet regeneration in this study required high concentration of BA (2.0 mg/L) and slightly lower concentration of 2,4-D. This might be due to different amount of plant growth regulators and the respond to it.In addition, several researchers reported that MS medium supplemented with 0.06 mg/L NAA and 0.03 mg/L BA gave the suitable for SE induction and plant conversion rate in many plant species including *Canabis sativa* L. [16], *Cinchona officinalis* [18] and *Pinus koraiensis* [29]. The low level of auxin and cytokinin in the SE induction medium was sometimes responsible for the development of SE directly from the explants, implying that they did not develop through callus formation [30]. However, our results showed that SEs were induced indirectly through callus induction.

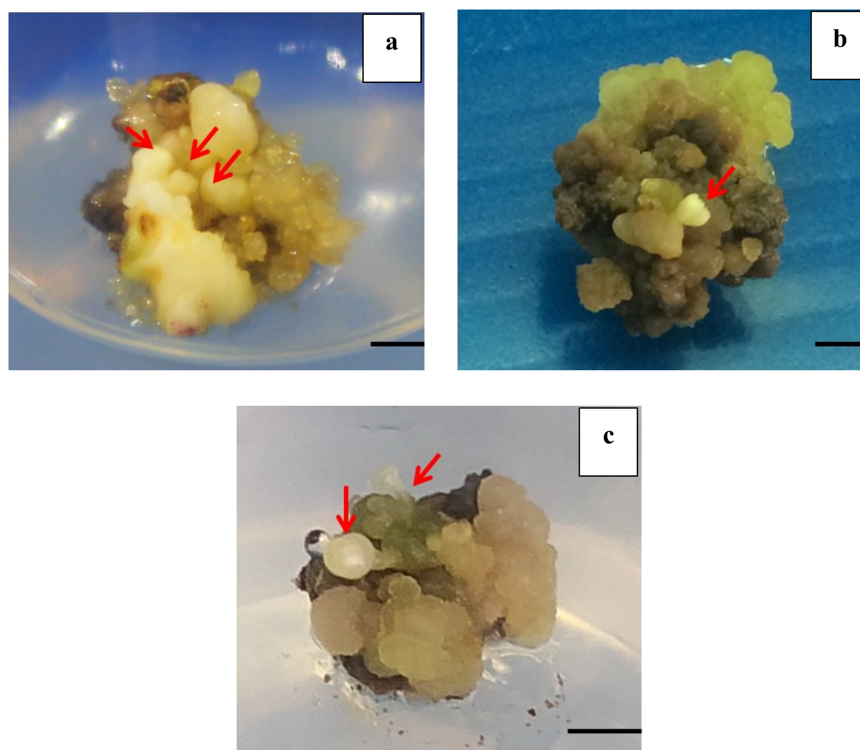

Figure 1 SE formation (red arrows) from callus of mixed flowers on MS medium supplemented with 2.0 mg/L BA and 1.5 mg/L 2,4-D after 12 weeks of culture, a) Globular embryo, b) Heart shaped embryo, c) Cotyledonary embryo (bars = 0.2 cm).

Table 1 Effect of types of explants on SE formation on MS medium supplemented with 2.0 mg/L BA and 1.5 mg/L 2,4-D subculture every 4 weeks for 12 weeks.

Kinds of explants	EC formation (%)	No. of SEs/EC		
		Globular embryos	Heart shaped embryos	Cotyledonary embryos
lTCL	0	0	0	0
Petal	28.46±1.46c	3.24±0.50b	2.18±0.38b	1.46±0.19b
Single flower	34.66±1.67b	3.48±0.42b	1.86±0.26b	1.25±0.12b
Mix flowers	39.84±1.82a	7.43±0.69a	4.52±0.75a	3.25±0.39a
F-test	**	**	**	**
C.V. (%)	14.25	12.64	10.18	18.74

** = significant different at $p \leq 0.01$; Means followed by the same letter within column are not significantly different according to DMRT.

Effects of types of explants and GA_3 on development of SE

In this study, SEs at cotyledonary stage were obtained on MS medium supplemented with 2.0 mg/L BA, 1.5 mg/L 2,4-D and 0.25 mg/L GA_3 after culture for 4 weeks. The results revealed that SEs from mix flower cultured on those PGRs containing medium gave the highest plant regeneration at 50 % (**Figure 2a**), significantly different with other SEs from another explants. Plant regeneration obtained from single flower and petal was 37.5 and 12.5 %, respectively (**Table 2**). GA_3 has been reported to facilitate germination of somatic embryos in *in vitro* conditions of many plant species [31-33]. However, the response to its concentration was quite different depended on initial cultured explant and plant species as well. In general, floral part and seed contain higher endogenous GA_3 concentration than leaf and internode. In pomegranate, [34] reported that early stage of small flower bud development contains higher concentration of GA_3. Thus, the suitable concentration of this PGR on germination of somatic embryo in pomegranate was relatively low (0.1 mg/L). In the presence study, a slightly higher concentration of GA_3 (0.25 mg/L) required for germination of somatic embryos from floral explants of rubber tree. Similar result was observed in *Quercus rubra* L. from immature cotyledon-derived embryogenic callus which required nearly the same concentration of BA and GA_3 for simultaneous development of both shoot and root at approximately 61 % [35]. In other plants, germination of somatic embryos from leaf and internode of *Wedelia calendulacea* Less [31] and *Podophyllum peltatum* L [32] required GA_3 at concentration of 0.5 and 1.0 mg/L, 2 and 4 times, respectively, higher than that used in the present study (0.25 mg/L). In genetically transformed avocado SEs, germination needed a very high concentration of GA_3 at 10 mg/L [34]. In addition, high concentrations GA_3 (34.6 mg/L) was reported to be necessary for plantlet regeneration from hypocotyl-derived callus through adventitious bud formation of *Spinacia oleracea* [35]. As mentioned earlier, plant species respond different concentrations of GA_3 due to different levels of PGR in those plants and explants. GA_3 have also been used in the elongation of regenerated shoots [31]. The effectiveness of GA_3 could be due to gene activation or synthesis of new gene-products for the completion of embryo development [36]. However, some SEs produced in this study only root (**Figure 2a**) or shoot (**Figure 2b**). It might be associated with meristem malfunction or asynchronous growth that requires an additional rooting or shooting step to recover complete plantlet as reported in some other species [37-39].

Table 2 Effects of types of explants and GA_3 on development of SEs on MS medium supplemented with 2.0 mg/L BA and 1.5 mg/L 2,4-D after 4 weeks of culture.

Types of explants	GA_3 (mg/L)	Complete plantlet (%)	Only shoot (%)
Petal	0	0	0
	0.25	12.5±0.51d	12.5±0.51b
	0.50	12.5±0.51d	12.5±0.51b
	0.75	0	0
	1.00	0	0
Single flower	0	0	0
	0.25	37.5±0.64b	12.5±0.51b
	0.50	25.0±0.72c	0
	0.75	25.0±0.72c	0
	1.00	12.5±0.51d	0

Types of explants	GA_3 (mg/L)	Complete plantlet (%)	Only shoot (%)
Mix flowers	0	0	0
	0.25	50.0±1.40a	25.0±0.72a
	0.50	25.0±0.72c	0
	0.75	12.5±0.51d	12.5±0.51b
	1.00	12.5±0.51d	0
F-test		**	**
C. V. (%)		17.58	34.90

** = significant different at P ≤ 0.01; Means followed by the same letter within column are not significantly different according to DMRT.

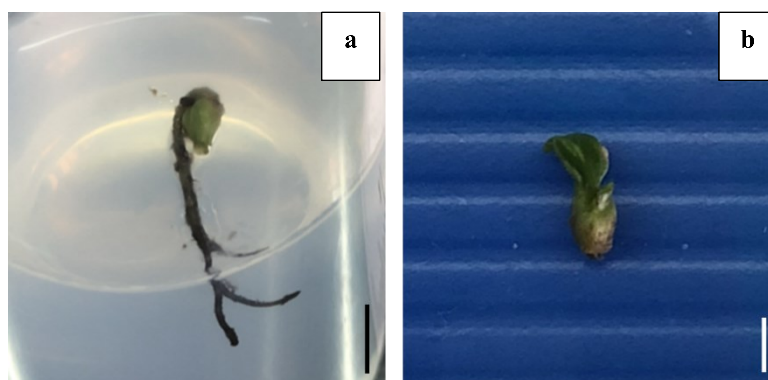

Figure 2 Plant regeneration from SE derived from mix flower on MS medium supplemented with 2.0 mg/L BA, 1.5 mg/L 2,4-D and 0.25 mg/L GA_3 after 4 weeks of culture, a) Plant regeneration (bar = 0.5 cm), b) Shoot (bar = 0.2 cm).

Assessment genetic fidelity

In plant propagation through tissue culture technique, the most crucial point is to retain genetic stability with respect to the mother plants (MP). However, it is known that *in vitro* culture techniques could induce genetic variation, namely somaclonal variation [40]. Somaclonal variation in tissue culture is a complex problem that needs several approaches to detect correctly [41]. It is considered as a problem for commercial micropropagation since it can negatively affect the production and reduce the uniformity of elite genotypes [42]. Recent studies revealed that cell or tissue cultures undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations) and these are also expressed at biochemical or molecular levels [18]. Different molecular analytical techniques have been being used to point out somaclonal variation in plant tissue culture and regenerants of several plants. RAPD and SSR are widely used in studying genetic variability in field grown plants [26,27] and regenerated plantlets from tissue culture [22].

In the present study, 2 out of 3 RAPD-primer tested (OPAD-01 and OPAD-10) could amplify and provided monomorphic patterns of DNA among *in vitro* rubber tree regenerated plantlets. The number of bands for each primer varied from 6 to 7 with average of 6.5 fragments per primer. The size of amplified products ranged from 300 to 1350 base pairs in size (**Figure 3**). Previous study [26] reported the assessment of genetic analysis in 53 early introduced clones of rubber tree using 8 RAPD primers (OPB-17, OPN-16, OPR-02, OPR-11, OPZ-04, OPAD-01, OPAD-10 and OPAD-12) and revealed that primer OPAD-01 gave polymorphisms of DNA profiles and could identify the genetic difference among those clones. Especially, Tjir1 clone had specific DNA profile at 700 bp. Thus, this primer was designed to use as marker for screening somaclonal variation in this present study and the result was clearly observed that no variation of DNA profiles found. Similar result was obtained with SSR marker analysis. All 3 primers of SSR; *hmac4, hmct1 and hmct5* could amplify DNA from all regenerants or somaclones and provide monomorphic patterns of DNA. The number of bands from each primer varied from 1 to 10 fragments per primer. The size of amplified products ranged from 200 to 300 bases pair in size (**Figure 4**). The result from above 2 molecular markers revealed that there was no somaclonal variation occurred among *in vitro* rubber tree regenerated plantlets derived from floral explants (**Figures 3** and **4**). The 3 SSR primers were reported to use in verification of genetic variation among early induce clones of rubber tree collected from different

areas in southern Thailand [26] and screening of rubber tree root stock and genetic background due to their polymorphisms [43]. All primers showed polymorphism among rubber clones tested. However, these primers provided monomorphic bands in this present study indicated that somaclones obtained by this technique are uniformity. Hence, these primers could be used to verify genetic uniformity of *in vitro* regenerated plantlets.

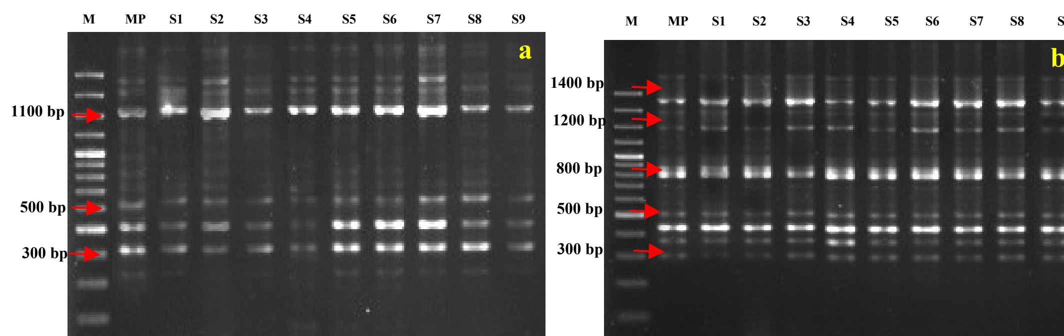

Figure 3 RAPD patterns of micropropagated plantlets compared to mother plant as amplified by primer a) OPAD-01 and b) OPAD-10. Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples (S1-3 = petal, S4-6 = single flower and S7-9 = mix flower).

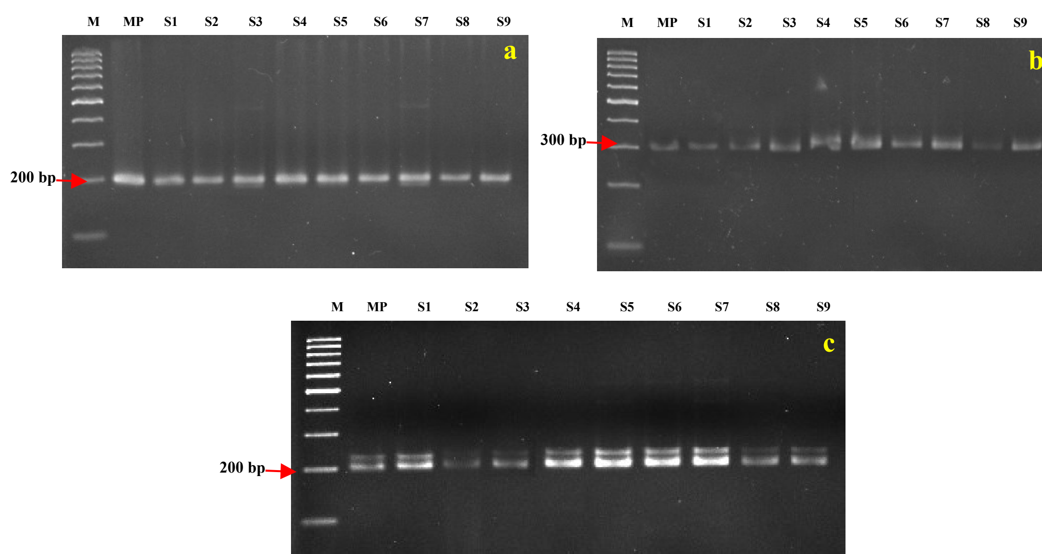

Figure 4 SSR patterns of micropropagated plantlets amplified by primer a) *hmac4,* b) *hmct1* and c) *hmct5*. Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples (S1-3 = petal, S4-6 = single flower and S7-9 = mix flower).

Conclusions

After 12 weeks of sub-culture the callus derived from mix flower gave the highest SE formation at 39.84 % and number of cotyledonary embryos (CE) at 3.25 embryos /explant. GA_3 at 0.25 mg/L with the best concentration of BA and 2,4-D containing MS medium gave the best result in plant regeneration at 50 % after culture for 4 weeks. Plantlets obtained by this procedure had the same profiles of DNA among each other and mother plant as revealed by 2 primers (OPAD01 and OPAD10) of RAPD and 3 primers (*hmac4, hmct1 and hmct5*) of SSR marker. It concludes that somaclones obtained from this protocol are uniformity (true-to-type).

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