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Effect of seasonal collection on callus Induction, proliferation and somatic embryogenesis from anther cultures of *Hevea brasiliensis* Muell Arg

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Hevea brasiliensis **that is grown in Thailand and Southeast Asia is a very valuable source of natural rubber. However, few studies have been conducted to evaluate the effects of seasonal effect on plantlet regenera-tion through somatic embryogenesis from anther culture. Thus, this study discussed the effect of season on callus induction subsequent to plantlet regeneration from culturing of anther. Male flowers were aseptically opened and anthers were excised to culture on callus induction medium (CIM) which was MS supplemented with 5% sucrose, 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN, 1 mg.L^{-1} NAA. Somatic embryo (SE) developed on 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. This medium is so called embryo induction medium (EIM).Anther collectedfrom different season gave different result in callus induction, proliferation and somatic embryogenesis, healthy anther collected in February 15, 2013 gave the best result in callus induction (86.25%), proliferation (422 mgFW) and somatic embryogenesis (20%). Complete plantlets (15.63%)were obtained on MS medium supplemented with 5% sucrose, 0.5 mg.L^{-1} KN, 0.2 mg.L^{-1} IAA, 1 mg.L^{-1} BA, 0.3 mg.L^{-1} GA_3 and 0.2% phytagel. Seasonal collection plays influence rolein successful somatic embryogenesis in tissue culture of rubber tree.**

Key words: *Hevea brasiliensis*, rubber, antherculture, callus induction, seasonal collection, somatic embryogenesis.

INTRODUCTION

Hevea brasiliensis Muell. Arg. is one of the economic crop in Thailand and Southeast Asia as the source of natural rubber. *In vitro* propagation of *Hevea* has played a very important role in rapid multiplication of cultivars with desirable traits and production of true-to-type; healthy and disease-free plant somatic embryogenesis which appears

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to be a promising technique that produced mass number of uniform plantlets in a short time.

Somatic embryogenesis has been reported in many species. Among those species many factors influenced plantlet regeneration through that process. *Hevea* is one species which has been studied intensively (Dijkman, 1951; Venkatachalam et al., 2013). Beyond culture media, plant growth regulators and culture environments, time of explant collection, so called seasonal effect, is also important because it affect the success of regeneration processes. Season causes variations in temperature, photoperiod and rainfall so explant had a different health. Quality of explant gave varied result. Fei et al. (2000) reported that growing condition of inflorescences had a close relation with callus proliferation.The effects of climatic changes in Southern Thailand lead to flowering of rubber tree in rainy season due to long drought period in that season. Thus, flowering of rubber tree occurred both in summer and rainy season. In case of mangosteen, low fruit-quality was greatly influenced by the high crop lost because summer rainfall induces profusionof flowers (Apiratikorn et al., 2012). Climate change resembled season change response to collectibles food and development of plant. The effect of climate change is important in determining development of pollen in Shogun citrus (Chelong and Sdoodee, 2012). The possibility of development of rubber tree flower was affected by season change.

In the past, plantlet regeneration through somatic embryogenesis in many plant species was developed by many researchers. This technique was also successfully reported in rubber tree (Jayashreeet al., 1999; Huaet al., 2010; Zhou et al., 2010; Zhou et al., 2012). However, induction of somatic embryosdepended on genotype and explant source (Fuentes et al., 2000). So far, there are still no researches reported on the effect of season on growth and development of tissue culture of rubber tree.

In this study, seasonalcollection of explant as one of external factors influencing growth and developmentof anthers of rubber tree *in vitro* are described. Those development, including, induction and proliferation of callus followed by embryogenic callus formation subsequent to plantlet regeneration are examined.

MATERIALS AND METHODS

Plant material and culture conditions

Immature male flower (1.2-1.5 mm in length) from inflorescence of *H. brasiliensis*Muell. Arg. grown around Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand was used as plant material in this experiment. Immature male flowers containing microspores at the uninucleate stage were collected and kept in a refrigerator at temperature of 4°C for 24 h. The cold-hardened explants were washed in running tap water for 20-30 min. All explants were surface sterilized in 70% (v/v) ethanol for 30 s, followed by soaking in 1.05% sodium hypochlorite solution for 20 min, then rinsed thrice with sterilized distilled water.

The anthers at length of 0.8-1.0 mm (Figure 2a) were excised from sterilized young male flowers under light microscope and cultured on MS medium. The pH of 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°C for 15 min. The cultures were incubated at 26±3°C in the dark for 8 weeks orunder 14 h photoperiod with light supplied by cool-white fluorescent lamps at intensity of 12.5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Effect of seasonal collection of explants on callus induction

The inflorescences of rubber containing immature male flowers were collected at three times; 22 Jun, 2012 (Rainy season), 15 February, 2013 (Summer season) and 31 July, 2013 (late Rainy season). Anthers from right stage of pollen development were aseptically excised according to the method described above. The anthers were cultured on MS supplemented with 5% sucrose, 1 mg.L^{-1}2,4-diclorophenoxyacetic acid (2,4-D) (Fluka), 1 mg.L^{-1}kinetin (KN) (Fluka), 1 mg.L^{-1}α-naphthalene acetic acid (NAA) (Fluka), so called CIM. All cultures were maintained under the conditions as specified above. The cultures were routinely subcultured at 4 week intervals for 8 weeks to induce callus. After 8 weeks of being cultured, callus induction percentage was recorded.

Effect of seasonal collection of explants on callus proliferation

The calli induced from anthers at two different periods as mentioned earlier (22 Jun, 2012 and 15 Feb, 2013) were transferred to culture on MS supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1}6-benzyladenine (BA) (Fluka), 1 mg.L^{-1}thidiazuron (TDZ) (Sigma). The cultures were maintained under the same conditions as describe above. After 4 weeks of being cultured, fresh weight of calli was recorded and statistically compared between the two different periods.Proliferation rate of the callus was determined by the ratio of increment in fresh weight divided by initialfresh weight as the following equation:

$$\text{Proliferation rate of callus} = \frac{\text{Fresh weight at } t_t - \text{Initial fresh weight}}{\text{Initial fresh weight}}$$

Effect of TDZ and coconut water (CW) on callus proliferation

Calli derived from anther culture were transferred to MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA and 1 or 2 mg.L^{-1} TDZ or CW. The cultures were maintained under the same conditions as described above. After 4 weeks of being cultured, fresh weight of calliwas recorded and statistically compared among different types and concentrations of BA, TDZ and CW. Proliferation rate of the callus was determined by the same method as described above.

Effect of gelling agents on SE induction

Eight-week-old calli derived from anther were cultured on embryogenic callus (EC) induction medium (EIM) which was 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}gibberellic acid (GA_3) (Fluka). The culture medium was solidified with two different types of gelling agent, phytagel and agar.The concentrations of those two gelling agents were 0.2% for phytagel and 0.75% for agar.After 4 weeks of culture, somatic embryo (SE) induction percentage and a number of somatic embryos per callus were recorded and statistically compared. EC together with SEs were transferred to MS medium supplemented with 0.2 mg.L^{-1} IAA, 0.5 mg.L^{-1} KN, 0.3 mg.L^{-1} GA

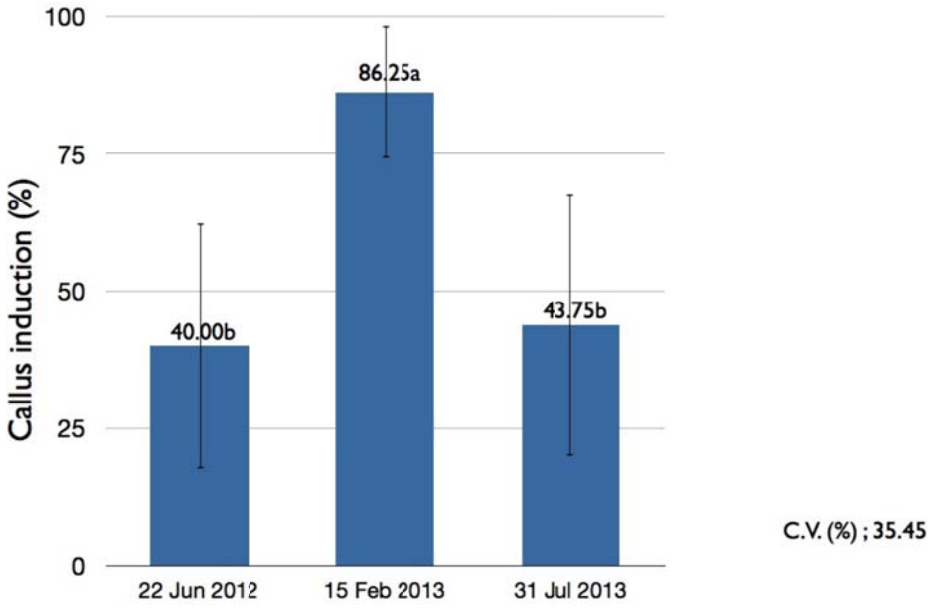

Figure 1. Effect of seasonal collection on callus induction from anther culture on MS medium supplemented with 1 mg.L^{-1} 2,4-D, 1 mg.L^{-1} KN and 1 mg.L^{-1} NAA for 4 weeks ($P < 0.01$).

and 1.0 mg.L^{-1} BAand cultured for further 4 weeks. Germination of SEs was recorded and statistically compared.

Statistical analysis

All experiments were performed in a completely randomized design (CRD). Each consisted of four replicates per treatment andten explants were performed in each replication. Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected usingleast significant difference(LSD) at the 0.01 level of probability.

RESULTS

Effect of seasonal collection of explant on callus induction

Collection period of explantsof rubber tree affect callus induction because photoperiod, temperature and rainfall affect photosynthetic rate of this plant (Sdoodee and Rongsawat, 2012). Inappropriate conditions of those environments made low productionof photosynthesis leading to the reduction growth rates of meristematic cells. Inflorescences collected in some seasonsresponded at low frequency in callus induction rate (Figure 1). Collection of inflorescence in15 February, 2012, which was the first season of flowering, showed the highest callus induction. The highest percentage callus formation was recorded to be 86.25%; significantly different ($P<0.01$) with the second season of flowering. Callus induction percentage from anther and from inflorescence collectedat second season of flowering (22 Jun, 2012 and 31 Jul, 2013) was 40.00 and 43.75%. Structures of callus induced from anther collected from difference seasons were different. Anther collected in 31 Jul, 2013 gave compact, hard and white callus (Figure 2c), whereas callus from anther collected from the first season of flower-ing was friable, watery and white in color (Figure 2b).

Effect of seasonal collection of explants on callus proliferation

Callus obtained from culturing anther collected from both seasons could proliferate on MS medium supplemented with 3% sucrose, 0.2 mg.L^{-1} NAA, 1 mg.L^{-1} BA, 1 mg.L^{-1} TDZ. However, callus from anthers collectedin15 Feb, 2013 gave the highest fresh weight of callus at 422 mg after being cultured for 4 weeks (Table 1). Proliferation of callus induced from anthers collected in 22 Jun, 2012 was far lower than that obtained innormal season of flowering.

Effect of TDZ and CW on callus proliferation

For proliferation of callus from anther culture, the result shows that 1 mg.L^{-1} TDZ gave the highest fresh weigh at 576 mg after being cultured for 4 weeks (Table 2). A 15% CW and 2 mg.L^{-1} TDZ containing medium reduced proliferation rate of callus. High concentration of cytokinin also resulted in low proliferation rate of callus. Among cytokinins tested, CW at concentration of 15% gave the

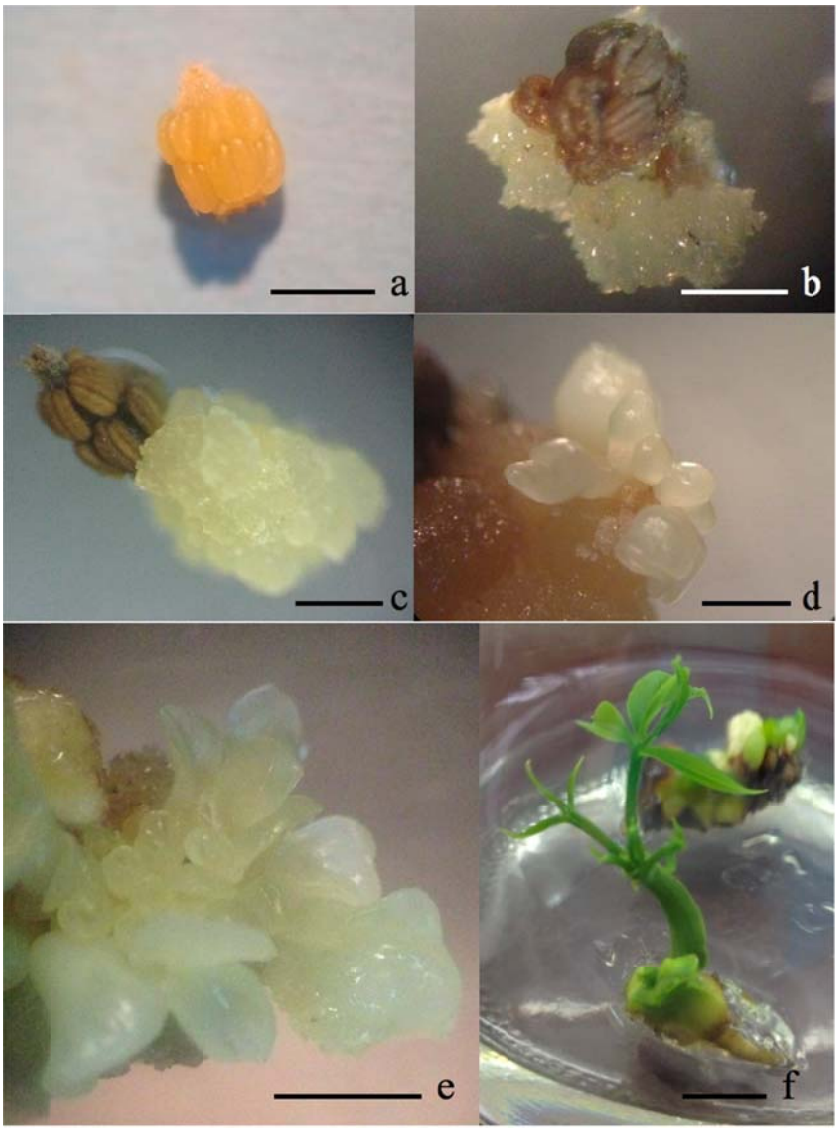

Figure 2. Development of plantlet via somatic embryogenesis from anther culture of *Heveabrasiliensis*.**a.** Anther. **b.** Friable and moist, callus induced from 15 Feb, 2013 collecting anther. **c.** Compact and hard callus induced from 31 Jul, 2013 collecting anther. **d.** SEs developed from 8-weeks-old callus on MS medium supplemented with 0.75% agar. **e.** SEs developed from 8-weeks-old callus on MS medium supplemented with 0.2% phytagel. **f.** Complete plantlet regenerated on MS medium supplemented with 5% sucrose, 0.5 $mg.L^{-1}$ KN, 0.2 $mg.L^{-1}$ IAA, 1 mg.L BA, 0.3 $mg.L^{-1}$ GA_3 and 0.2% phytagel (a, b, c, d and e bar: 1 mm; f bar : 5 mm).

Table 1. Effect of seasonal collection on callus proliferation culture on MS medium with 0.2 $mg.L^{-1}$ NAA, 1 $mg.L^{-1}$ BA, 1 $mg.L^{-1}$ TDZ, and 1 $mg.L^{-1}$ $AgNO_3$ for 4 weeks.

Date	NAA/BA/TDZ	Initial fresh weight (mg)	FW callus (mg)±SD	Proliferation rate of callus
22 Jun, 2012	0.2/1/1	50	151±38[b]	2.03
15 Feb, 2013	0.2/1/1	50	422±87[a]	7.45
F-test			**	
C.V. (%)			22.69	

Mean values followed by the same letter(s) within a column are not significantly different (P < 0.01).

Table 2. Effect of PGRs containing MS medium with 1 mg.L^{-1} $AgNO_3$ on callus proliferation after 4 weeks of culture.

NAA	BA	TDZ	CW	FW callus (mg)±SD
0.2	1			249 ± 118^{b}
0.2	1	1		576 ± 222^{a}
0.2	1	2		393 ± 118^{ab}
0.2	1		15	227 ± 80^{b}
F-test				**
C.V.(%)				30.48

Mean values followed by the same letter(s) within a column are not significantly different (P < 0.01).

Table 3. Effect of Phytagel and Agar on SE induction from 8 weeks old callus culture on 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 for 4 weeks.

Solidify type	% of SEs Induction	No. of SEs per explant
Phytagel (0.2%)	20	7.63 ± 5.57^{a}
Agar (0.75%)	15	2.17 ± 0.75^{b}
F-test		*
C.V.(%)		73.52

Mean values followed by the same letter(s) within a column are not significantly different (P < 0.05).

lowest proliferation rate of callus. Lata et al. (2013) reported that high concentration of TDZ decrease percentage of shoot formation in stavia. However, a low concentration of TDZ was reported to give the best result in callus proliferation in many plants (Guo et al., 2011).

Effect of phytagel and agar on SE induction

Gelling agents play important role in SE formation. In this study, phytagel increased SE induction rate (20%) from anther-derived callus leading to the highest number of SE formation at 7.63 SEs per explant (Table 3, Figure 2e). Phytagelis pure synthetic agar used at lower concentration for solidifying culture media. So, it seems that there is no impurified substances which hampered growth and development of plant tissue. Complete plantlet (Figure 2f) was obtained at 15.63% after SE was transferred to culture on 0.2% phytagel containing MS medium in the presence of 0.2 mg.L^{-1} IAA, 0.5 mg.L^{-1} KN, 0.3 mg.L^{-1} GA and 1.0 mg.L^{-1} BA for 4 weeks.

DISCUSSION

The quality, growth and development of pollens frompollen megaspore mother cells (PMC), of anther from donor plantisthe key factoraffecting callus induction and further development into plantlet. An important factor controlling quality of anther should be raised under optimum condition. In this study, we reported the relationship between the time of collection explant and the percentage of callus induction. Normal season of flowering, in February, illustrated the most suitable for callus induction in terms of growth and development. Photosynthesis is one parameter indicated quality of anther due to accumulation of photosynthates. This evident is highly impact by the suitable sunshine or radiation duration (PAR: 400-700) (Sdoodee and Rongsawat, 2012). However, long period of sunshine in a day stimulate the formation of ethylene that reduced the embryogenic callus induction in buffalograss (Feiet al., 2000). In addition, seasonal collection of explants has been reported to influence upon shoot formation in nodal culture of oak.Collecting the explants in May was the best time for the highest percentage of shoot forming explant and multiplication rate (Kartsonas and Papafotiou, 2007). A high frequency of sprouting from nodal cutting mulberry was observed in summer at 83.3% (Chitra and Padmaja, 2002). Contrary result was obtained in two pepper genotypes, Kekova cultivar gave the highest embryogenic response in summer season while 8 cultivars of Sera Demre gave a good response in winter (Ercan et al., 2006).In anther culture of flax, it also reported that collecting of explants in summer season gave the best result due to the active or meristematic activity of tissue in those explants (Krause et al., 2003).

Meristematic activity ofinitial cells is important for callus induction just after culture in culture conditions. Callus

induction from anther collecting from the first seasonal flowering gave higher proliferation rate than the second seasonal flowering (Table 1). Climate change affected the embryogenic callus growth rate due to a good quality of explants as describe above (Feiet al., 2000). Growth conditions of donor plant underappropriated photoperiod and temperature promoted a high quality of anther, like those reports in pepper (Ercan et al., 2006). Moreover, shoot was rapidly developed and proliferated from a good quality of mulberry anther (Chitra and Padmaja, 2002).

Plant growth regulators (PGRs) containing media promoted callus growth and proliferation under *in vitro* conditions. Besides types of PGR its concentration was also important in callus culture. In case of rubber tree callus, TDZ at 1 $mg.L^{-1}$ gave better result in growth rate than 2$mg.L^{-1}$.Moreover, low concentration (0.2-0.6 $mg.L^{-1}$) of TDZ was reported to be the best for callus induction and combination of TDZ with NAA promoted shoot regeneration in *Astragalus cariensis* (Erisen et al., 2011).

In case of SE induction, phytagel promoted the formation ofSE fromculturing of anther of rubber tree. Zhang and Te-chato (2013) reported that a high numberof SEs from callus culture ofindica ricewas induced on phytagels up plemented medium. Phytagel solidifying culture medium was reported to increase plantlet formation in *Cleome rosea* (Simoes et al., 2010). Choudhury et al. (2008) reported that phytagel containing culture medium reduced water availability.When explants or callus could not absorb water it caused achange in osmotic potentialpromoting the development of plantlets. In case of oil palm, the increment of osmotic potentialwas reported to promote germination of somatic embryo (Hilae and Te-chato, 2005). However, increment of osmotic potential in this case was performed by adding sugar alcohol, sorbitol.

Conclusion

In rubber tree, seasonal collection of inflorescence influencedthe successful somatic embryo induction from anther culture. Callus was induced 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. SEs were developed on 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. Complete plantlets were regenerated on MS medium supplemented with 5% sucrose, 0.5 $mg.L^{-1}$ KN, 0.2 $mg.L^{-1}$ IAA, 1 $mg.L^{-1}$ BA, 0.3 $mg.L^{-1}$ GA_3 and 0.2% phytagel.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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