J. Rubb. Res., **18(3)**, 127–138

Effects of Zeatin and Kinetin on in vitro *Regeneration of* Hevea brasiliensis *RRIM 2025*

NOR MAYATI C.H.*#

Efforts were undertaken to improve regeneration of Hevea brasiliensis *clone RRIM 2025 plants* via in vitro *culture technology. A total of 12,938 tapetum (anther walls) explants were cultured. Somatic* embryogenesis *(SEm) were induced on nine different embryogenesis induction media supplemented with zeatin or kinetin, in three separate experiments performed for three consecutive flowering seasons. Callogenesis was significant with the highest mean value at circa 97%. Although SEm and plantlets regeneration were achieved, the results were not significant. SEm formations were slightly insignificantly higher with RD1 supplemented with zeatin at 0.5 mg/L (9.99%) and RD1-E1 enriched with 0.3 mg/L kinetin (11.1%). A high variance of results discerned at embryogenesis and plantlets regeneration stages could be attributed to origin of explant and its susceptibility to variations in climatic conditions. In total, 42 plantlets were obtained and these results would prove invaluable for further improvement of* in vitro *propagation of RRIM 2025.*

Keywords: Anther; cytokinins; propagation; plant growth regulators; rubber; tissue culture

Somatic embryogenesis of *Hevea brasiliensis* (Mull. Arg.) serves as a platform for transgenic research[1–3] besides its potential use for mass rapid clonal propagation of selected economical genotypes[2,4]. The dwindling interest in cultivating rubber (*Hevea*) in recent years[5,6] could be partly attributed to drawbacks in the supply of conventional cuttings and bud grafted planting materials[2]. The volatility of commodity prices also forces rubber to compete with oil palm and other plantation commodities for available land[5,7]. Meanwhile, the supply of quality planting materials is critical to ensure appropriate returns on investment[8,9]. Latex timber clones (LTC) developed through conventional breeding at the Rubber Research Institute of Malaysia (RRIM) since 1995 has been envisioned for dual purposes, *i.e.* for high latex yield and for desired wood properties in anticipation of increasing demand from natural rubber (NR) and rubberwood based industry[10,11].

Rubber growers prefer to plant bud grafted LTCs to ensure genetic uniformity of scion while seedlings on the other hand are prone to irregular growth that often affects yield exploitation[11]. The rootstocks of LTCs derived from classic cross pollinated seeds are

* Genetic Transformation and Tissue Culture Programme, Production Development Division, Rubber Research Institute of Malaysia (RRIM), Malaysian Rubber Board, 47000 Sg. Buloh, Selangor, Malaysia.
# Corresponding author (e-mail: normayati@lgm.gov.my)

heterozygous and this may lead to intra-clonal variations due to stock-scion interactions. Discrepancy in individual tree productivity may undermine the overall land productivity in plantations. Thus, *in vitro* micro propagation of LTCs that stand on their own root systems could reduce variability that arises from root-scion interaction. *In vitro* culture technique has been developed by RRIM in an attempt to propagate elite with feasible genetically conserved *Hevea* clones. However, *in vitro* regeneration of modern *Hevea* clones proves challenging, thus far, only a few clones have shown to be successful.

Currently, efforts to improve tissue culture of local *Hevea* clones involve manipulation of organic additives, plant hormones, biocides and amino acids, including coconut water, sucrose, benzylaminopurine (BA), indolebutyric acid (IBA), α-naphthaleneacetic acid (NAA), and cytokinins (zeatin and kinetin). Both cytokinins involved in promoting cell division and shoot growth of *in vitro* plants by opposing apical dominance regulated by plant growth regulator, abscisic acid (ABA)[12]. Improved somatic embryogenesis with various concentration of kinetin has been reported for several *Hevea* clones *viz*. PB 260, PB 235 and RRIM 600[13–17]. The antagonistic effects of kinetin and BA in combination with NAA and gibberelic acid (GA_3) in the regeneration of plantlets from protoplast of *Hevea* clone RRII 105 were also evaluated[18]. Compared to kinetin, zeatin showed higher efficiency in promoting shoot proliferation in Gardenia[12]. In other studies, kinetin added in MS medium generated multiple shoot tips growth of apple cultivar, *Ziziphus spina-christi* wild[19]. However, nutrient media enriched with either kinetin or zeatin in combination with other hormones such as IBA, NAA, BA or N-phenyl-N-1,2,3-thidiazol-5yl urea (TDZ) showed different kinetics of shoot multiplication in various plant species[20,21]. In this research, the influence of exogenous zeatin and kinetin on somatic embryogenesis of RRIM 2025 (LTC) callus culture that originates from anther explant was investigated.

MATERIALS AND METHODS

Explant Sources and General Procedures

Anther (tapetum) explant from greenish-yellow flowers[22] of *Hevea* clone RRIM 2025 were collected from adult trees planted in Field 68, RRIM Experimental Station, Sg. Buloh, Selangor. A total of 5880, 3878 and 3180 of anther explants from three consecutive flowering seasons were cultured *in vitro* in Experiment 1 (from trees no. 1 and 8 in March 2007), Experiment 2 (from trees no. 1 and 7 in September 2007), and Experiment 3 (from trees no. 2 and 3 in March 2008), respectively. The harvested flowers were kept in a Coleman storage box with bags of ice and transported to the laboratory within 4 to 6 hours. In the laboratory, flowers were washed twice with distilled water, soaked in 70% ethanol for 1 to 2 min, rinsed with distilled water and a final rinse with sodium hypoclorite (5% Clorox) solution for five minutes. Finally, the flowers were ozone sterilised for one minute. The sterile samples were then placed in an enclosed 500 mL beaker containing distilled water prior to inoculation onto selected basal media. Callogenesis and embryogenesis for explant culture were induced in 50 mL glass tubes containing 20 mL specific media and were kept in the dark at 27ºC.

Anther Culture

In Experiment 1, callogenesis was initiated onto three different media, (i) RRIM Initiation Media (RI) (Jaafar, H. *pers.comm*), (ii) Murashige and Skoog[23], with an addition of zeatin [MS(ID)]Z and (iii) Modified RI with hormones for MS(ID) namely RIMS(ID). RI

media contains 0.4 mg/L IBA + 1 mg/L 2, 4-D + 1 mg/L BA+ 0.5 mg/L zeatin + 7% sucrose. MS(ID)Z was MS media supplemented with 0.5 mg/L zeatin. Meanwhile, RIMS(ID) media is a combination of RI and MS(ID) containing 0.4 mg/L IBA + 1 mg/L 2,4-D + 1 mg/L BA + 0.2 mg/L NAA + 1 mg/L zeatin. Somatic embryo (SEm) formation was induced in the differentiation stage, using RRIM Differentiation Media 1 (RD1) as a control. The SEm induction with RD1-control was compared against eight modified RD1 comprising a combination of plant growth regulators and hormones with or without zeatin or kinetin at different concentrations (*Table 1*). The pH of the medium was adjusted to 5.8, solidified with Phytagel (Sigma®, Sigma-Aldrich Inc, USA) and autoclaved for 20 min at 121ºC.

In Experiment 2, callogenesis was induced onto Gamborg[24] (B5), MS(ID)Z, Schenk and Hildebrandt[25] (SH), and Heller[26] (H). Embryogenesis was induced onto (i) RD1-control, (ii) RD1-C1 and (iii) RD1-E2 (*Table 1*). Meanwhile in Experiment 3, callogenesis was promoted using six initiation media *i.e*. R1, B5, MS(ID)Z, Chinese media [CI(b)], Bourgin and Nitsch[27] (BN) and SH. Subsequently, embryogenesis was performed using differentiation media RD1- control and selected modified RD1 including RD1-A1, RD1-B1, RD1-C1, RD1-E1 and RD1-B2 (*Table 1*). Differentiation media evaluated in Experiments 2 and 3 was a narrowed-down screening process based on their performance in Experiment 1. Therefore, only the differentiation media that produced the highest embryogenesis and successfully regenerated plantlets in Experiment 1 were employed in Experiments 2 and 3.

After completion of the initiation process, the propagated calluses were subcultured for 1 to 3 months onto the listed differentiation media (*Table 1*) to induce embryogenesis. This was followed by desiccation of the calluses for ten days before transferring onto RD2 for one month and RD3 for another month. In the final stage, the recovered plantlets were transferred onto developmental media DM94 containing 5 mg/L GA_3 + 0.4 mg/L IBA + 7% sucrose to induce growth and multiplication of roots and shoots. Within a month, the complete tissue culture mother plants were transferred onto DM04 containing similar nutrient as DM94 but with reduced sucrose concentration at 3.5%, for further adaptation. During acclimatisation, the plantlets were placed in the growth chamber under TLD

TABLE 1. LIST OF DIFFERENTIAL MEDIA SCREENED FOR ABILITY TO INDUCE EMBRYOGENESIS OF *HEVEA* CLONE RRIM 2025

	Differential Media
1.	RD1-control: IBA (4 mg/L) + zeatin (0.5 mg/L) + $AgNO_3$ (0.5 mg/L) + sucrose (7%)
2.	RD1-A1: RD1 + NAA (0.2 mg/L) + zeatin (0.5 mg/L) + BA (1 mg/L) + sucrose (7%)
3.	RD1-B1: RD1 + NAA (0.2 mg/L) + zeatin (0.3 mg/L) + BA (1 mg/L) + sucrose (7%)
4.	RD1-C1: RD1 + NAA (0.2 mg/L) + BA (1 mg/L) + sucrose (7%)
5.	RD1-D1: RD1 + IBA (0.4 mg/L) + zeatin (0.5 mg/L) + BA (1 mg/L) + sucrose (7%)
6.	RD1-E1: RD1 + IBA (0.4 mg/L) + kinetin (0.3 mg/L) + BA (1 mg/L) + sucrose (7%)
7.	RD1-F1: RD1 + IBA (0.4 mg/L) + BA (1 mg/L) + sucrose (7%)
8.	RD1-B2: RD1 + NAA (0.7 mg/L) + kinetin (0.8 mg/L) + BA (1.5 mg/L) + sucrose (7%)
9.	RD1-E2: RD1 + IBA (1 mg/L) + kinetin (0.8 mg/L) + BA (1.5 mg/L) + sucrose (7%)

36W/54-765 cool daylight fluorescent tubes (Philips, Lifemax) for 12 h photoperiod at *ca*. 25-27ºC and the developments were monitored. After three months, the plantlets were transferred into glass jar containing 5 mg/L IBA solution for *ca*. two weeks before being transplanted into soil in polybags and were kept under controlled environment in the glass house.

Statistical Analysis

In this study, the treatments for each experiment were repeated two to three times. Mean of data collected was analysed by Analysis of Variance (ANOVA) using a statistical software, Statistical Analysis System (SAS®, SAS Institute Inc. NC, USA) release 9.2. To evaluate the effects of every media tested, the means of treatment variables were compared by least significant difference (LSD) test at P = 0.05.

RESULTS

The ability of all the eight initiation media tested in this study to induce callogenesis was apparent. Callogenesis of RRIM 2025 was significantly (P = 0.0123) induced in Experiment 1, induction of callogenesis with R1 (97.2%), and RIMS(ID) (96.3%) media were significantly higher compared to that induced on MS(ID)Z (89.3%) (*Table 2*). In Experiment 2, a high level of callogenesis was induced on B5, MS(ID)Z, SH and H media with mean value ranging from 88.8% to 94.2%. However, the difference was not significant (P > 0.05) (*Table 2*). Similarly, the six initiation media tested in Experiment 3, *i.e*. R1, B5, MS(ID)Z, CI(b), BN and SH, also induced high level of callogenesis with mean value ranging from 88.8% to 95% with no significant (P > 0.05) difference among them (*Table 2*). Highest level of callogenesis induction was achieved with RI media, followed by B5 and SH media *i.e*. ranging

TABLE 2. CALLOGENESIS OF RRIM 2025 ANTHER CULTURE IN EXPERIMENTS 1 (5880 ANTHER EXPLANTS), 2 (3878 ANTHER EXPLANTS) AND 3 (3180 ANTHER EXPLANTS)

Initiation media	Mean callogenesis (%) ± se		
	Experiment 1	Experiment 2	Experiment 3
RI	97.2±0.53[a]		95.0±0.45[a]
RIMS(ID)	96.3±1.76[a]		
MS(ID)Z	89.3±1.52[b]	88.8±3.14[a]	88.8±2.37[b]
B5		94.2±0.99[a]	94.2±1.14[ab]
SH		91.5±0.16[a]	93.4±0.78[ab]
H		90.0±2.53[a]	
CI(b)			91.9±1.06[ab]
BN			91.8±0.08[ab]
F test probability	0.0123	0.5365	0.1847
LSD	4.7665		
CV	2.53	3.95	2.27

Mean values followed by the same superscript letter(s) within a column are not significantly different at P = 0.05. Se is Significant Errors, LSD (Least significant difference) is written for significant value within column and CV is coefficients of variation.

from 91% - 97% (*Table 2*). Overall, MS(ID)Z gave rise to the lowest level of callogenesis in all experiments and the differences recorded were significant when compared to success of callogenesis with RI media in Experiments 1 and 3 (*Table 2*). However, there was little variation in the percentage of callogenesis achieved with initiation media tested in each experiments, as indicated by the small values of coefficient of variation (CV) and standard errors (SE) (*Table 2*).

The promotion of embryogenic calluses were recorded significant in Experiment 1 (P = 0.0059), Experiment 2 (P = 0.001) and Experiment 3 (P = 0.0114) (*Table 3*). In Experiment 1, RI and MS(ID)Z enriched with 0.5 mg/L zeatin produced significantly higher embryogenic calluses, of about 4.4 folds (12.2%) and 4.1 folds (11.4%) respectively, as compared to RIMS(ID) that induced significantly the lowest embryogenic calluses at mean value of 2.78% (*Table 3*). Induction of embryogenic calluses by MS(ID)Z, B5 and SH in Experiment 2 was also significant (P = 0.001), with the highest score given by B5 media at 7.24%. Meanwhile, SH media did not give a good response, with the lowest score at 0.77% (*Table 3*). In Experiment 3, the higher embryogenic calluses were obtained from BN and B5 media with mean embryogenesis ranging from 7.54% to 13.5% (*Table 3*). Meanwhile, the other media tested did not result in significant induction of embryogenic calluses with low mean values ranging from 1.26% to 2.82% (*Table 3*).

SEm formation from callus induced in all initiation media tested was not a guarantee the subsequent formation of plantlets with the exception of B5 media, that significantly (P = 0.0034) produced an average of 2 plantlets (*Table 3*) in Experiment 2. The proportion of plantlets was only significant (P = 0.0006) for Experiment 1, while RI media gave rise to the highest score at 3.76% *i.e*. 2.5 folds higher than RIMS(ID) at 1.52% . Meanwhile, the highest percentage recorded for B5 at 31.5% in Experiment 2, was not significant (P = 0.0582). Together, large variations in the percentage of embryogenic calluses originating from callogenic explant induced by the various initiation media were discerned, as illustrated by the large CV and SE value (*Table 3*). The results also revealed that no SEm were induced from callogenic calluses obtained from MS(ID)Z, CI(b) and H media (*Table 3*).

After a maximum of 45 days initiation, the embryonic calluses or zygotic embryos obtained (*Figures 1A* and *B*) were subcultured onto the listed differential media (*Table 1*). Overall, no significant effects of differentiation media on SEm formation were observed (*Table 3*). A high variance of SEm and subsequent plantlets formation was indicated by the large CV values recorded (*Table 3*). The highest SEm induction were obtained from RD1-CI (11.5%) and RD1-E1 (11.1%) in Experiment 1 and RD1-control (9.99%) in Experiment 3. However, these were not significant when compared to SEm induced by the other differentiation media tested (*Table 3*). Further, in the developmental stage of the experiments, plantlet (*Figure 1C*) regeneration was also very low and was not significant (P > 0.05) (*Table 3*). However, in Experiment 1, a slightly higher and significant number (P = 0.0026) of plantlet regeneration (1.33), with mean value of 5.7% was achieved from SEm differentiated on RD1-E1 as compared to the results of the other differentiation media (*Table 3*).

From the above described SEm lines, a total of 42 tissue culture plants were obtained (*Table 4*). Plantlet regeneration was highest in Experiment 1 with a total of 18 plantlets, while Experiments 2 and 3 resulted in a total of 16 and 8 plantlets, respectively (*Table 4*). They were derived mostly from SEm differentiated

TABLE 3. PERFORMANCE OF MEDIA ON REGENERATION SUCCESS: EMBRYOGENESIS AND PLANTLETS RECOVERY OF RRIM 2025 ANTHER CULTURE IN EXPERIMENTS 1, 2 AND 3

MEDIA	Mean Embryogenesis (%) ± se			Mean Plantlet (no) ± se			Mean plantlets (%) ± se		
Initiation media	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
RI	12.2±1.96^{a}		1.26±0.91^{b}	0.71±0.24^{a}		0^{a}	3.76±1.25^{a}		0^{a}
RIMS(ID)	2.78±0.6^{b}			0.57±0.57^{ab}			1.52±0.84^{b}		
MS(ID)	11.4±2.5^{a}	6.18±1.71^{a}	2.82±0.87^{b}	0^{b}	0^{b}	0^{a}	0^{b}	0^{b}	0^{a}
B5		7.24±2.03^{a}	13.5±4.13^{a}		2.00±0.37^{a}	0.25±1.13^{a}		31.5±14.3^{a}	1.57±0.83^{a}
SH		0.77±0.32^{b}	1.81±0.92^{b}		0.67±0.49^{b}	0.17±0.17^{a}		13.3±8.82^{ab}	4.17±4.17^{a}
H		0^{b}			0^{b}			0^{b}	
CI(b)			2.71±1.31^{b}			0^{a}			0^{a}
BN			7.54±2.94^{ab}			0.25±0.18^{a}			7.23±5.66^{a}
F probability	0.0059	0.001	0.0114	0.0714	0.0034	0.3606	0.0006	0.0582	0.4212
n	14	6	12	14	6	12	14	6	12
LSD	5.97	3.47	7.29		1.03		1.69		
Differential media									
RD1-control	8.40±5.32^{a}	4.97±1.94^{a}	9.99±3.54^{a}	0.67±0.67^{ab}	0.50±0.38^{a}	0.08±0.08^{a}	0.79±0.79^{b}	1.98±1.42^{a}	0.46±0.46^{a}
A1	8.02±2.73^{a}		5.36±2.47^{ab}	0.33±0.21^{b}		0.08±0.08^{a}	2.08±1.37^{b}		0.56±0.56^{a}
B1	5.70±1.66^{a}		4.58±2.64^{ab}	0.33±0.21^{b}		0.08±0.08^{a}	1.52±0.98^{b}		0.56±0.56^{a}
C1	11.5±3.86^{a}	2.24±1.31^{a}	1.98±0.97^{b}	0.33±0.21^{b}	0.75±0.41^{a}	0.17±0.17^{a}	2.23±1.42^{b}	19.0±12.3^{a}	5.56±5.56^{a}
D1	9.67±3.78^{a}			0^{b}			0^{b}		
E1	11.1±2.90^{a}		4.39±2.92^{ab}	1.33±0.61^{a}		0^{a}	5.70±2.65^{a}		0^{a}
F1	7.17±2.71^{a}			0^{b}			0^{b}		
B2			3.32±1.26^{ab}			0.25±0.18^{a}			5.83±4.34^{a}
E2		3.43±1.47^{a}			0.75±0.41^{a}			12.6±6.87^{a}	
F probability	0.8332	0.1816	0.3467	0.1273	0.7828	0.7496	0.0026	0.2705	0.5088
n	6	8	12	6	8	12	6	8	12
LSD							2.59		
CV	86.39	77.7	178.27	190.52	122.47	367	122.49	179.76	471.31

Mean values followed by the same superscript letter(s) within a column are not significantly different at $P \leq 0.05$. Se is significant errors, LSD (Least significant difference) is written for significant value within column and CV is coefficients of variation.

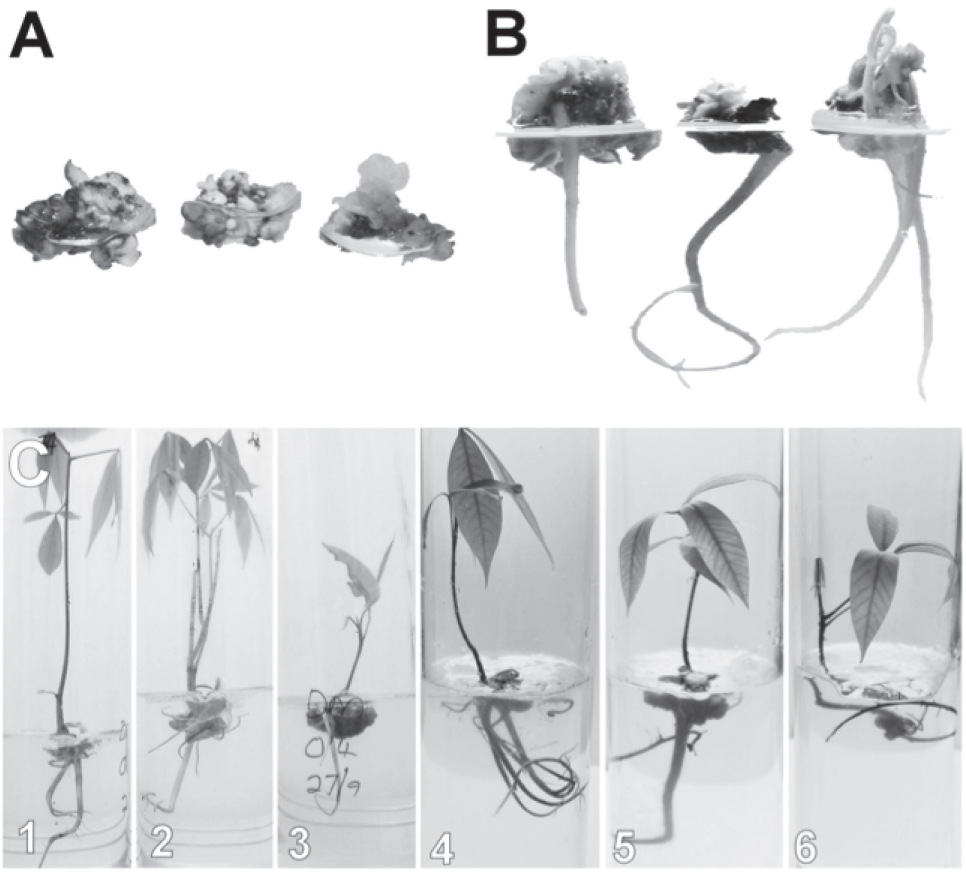

Figure 1. In vitro *regeneration of anther culture* Hevea brasiliensis *clone RRIM 2025; (A) Embryogenic calli and zygotic embryos, (B) Rooted embryoids and (C) Plantlets.*

on RD1-control (9 plantlets), RD1-A1 (3 plantlets), RD1-B1 (3 plantlets), RD1-B2 (3 plantlets), RD1-C1 (10 plantlets), RD1-E1 (8 plantlets), and RD1-E2 (6 plantlets). At the end of the study period, all plantlets have undergone acclimatisation with 11 of them successfully transplanted into the soil. However, only two plantlets that were obtained from SH/RD1-B2, and one plantlet each from SH/RD1-C1, SH/RD1-E2, B5/RD1-B1 and B5/RD1-E2 survived in the glasshouse before wilting and died a few months later.

DISCUSSION

Anther explants (tapetum) culture from flowers of *Hevea* RRIM 2025 used in this study was initiated to ensure a high level of SEm. The technique also ensures ontogenic rejuvenation of clonal progeny[14,28] and this success could be partly attributed to their proximity to rejuvenating sexual cells[29–31]. The proficiency of anther explant to progress into SEm stage was scrutinised using various types of embryogenesis expression media

TABLE 4. NUMBER OF PLANTLETS RECOVERY FROM ANTHER CULTURE OF RRIM 2025 IN EXPERIMENTS 1, 2 AND 3

Differentiation media	Total plantlets (No.)		
	Exp. 1	Exp. 2	Exp.3
RD1-control	4	4	1
A1	2		1
B1	2		1
C1	2	6	2
E1	8		
B2			3
E2		6	
Total	18	16	8

based on successful earlier experiments in related research.

The various modifications made to previously established differentiation media (control treatment) did not improve embryogenesis of *Hevea* RRIM 2025 anther culture. The higher SEm lines produced also did not show correlation to plantlet regeneration in the following developmental stage. Results suggest that embryogenesis was mainly independent of medium composition, similar to an earlier finding for PB 260 clone[14]. Thus, it is believed to be largely dependent on the origin of the sample and climatic or seasonal influences during explant harvesting.

The juvenility of explants[32], the origin and physiological ages of donor plants[28] also appear to play a crucial determinant in somatic embryogenesis. Anther explant used in this study was harvested from different trees in three consecutive flowering seasons. Variation in embryogenesis capacity among experiments could also be associated with the origin of flowers, as they were collected from the different donor plants. Due to the nature of the trees and seasonal difference in each year, sampling could not be fixed to the same trees. Sometimes the same tree did not flower in every season/year and flowering season is normally within a window of three weeks. Elsewhere, the difference in physiologic age of flowers within trees as well as the zonation in relation to their development and proximity of their roots system have been shown[30,33]. It is also evident that variations in microclimatic conditions including changes in lights intensity, temperature, humidity and rainfall[15] could affect the photosynthesis rate that leads to the growth rates of meristematic tissues. These have been identified as among the determining factors of the quality of inflorescences, and thereby influence frequency of plant regeneration[15]. Similarly, these could be among the factors that have contributed to the reduced percentage of callus and embryogenesis induced from Experiments 1 to 3 (*Tables 2* and *3*).

Induction of significant callogenesis of above 90% for all basal media tested except MS(ID)Z with a potential to form SEm and regenerate complete plantlets is noteworthy. However, some of the vigorously differentiating embryoids succumbed to necrosis before a complete differentiation was achieved. It was discerned that the presence of zeatin at a low concentration of 0.5 mg/L

in RD1-control, and kinetin at 0.3 mg/L in RD1-E1 were apparently sufficient to maintain somatic embryogenesis and plantlet regeneration of RRIM 2025. On the contrary, maximum plantlets number obtained with RD1-C1 containing 0.2 mg/L NAA + 1 mg/L BA without an addition of both cytokinins was also discerned. Thus, it appears that induction of SEm and RRIM 2025 plantlet formation can be zeatin or kinetin-independent similar to as shown for tobacco in an earlier report[34]. Another interesting observation is that the low embryogenesis in Experiment 1 and reduced frequency in Experiments 2 and 3 achieved by the differentiation media RD1-C1 (without kinetin or zeatin), had little bearing on plantlet formation. As the media without zeatin and/or kinetin might be suboptimal, an addition of kinetin at a minimum concentration of 0.3 mg/L was supposed to improve embryogenesis of RRIM 2025 anther culture. However, the insignificant improvement discerned in this study, perhaps, was due to insufficient cytokinin concentration applied. This finding calls for further investigation.

Generally, the negative effects of kinetin in inducing regeneration of somatic embryos of RRIM 2025 contradicted those reported in other *Hevea* clones[13,14]. The authors reported that a specific concentration of kinetin at 0.004 mg/L promoted high percentage of somatic embryos for RRIM 600, PB 260, and PB 235 that were able to regenerate into complete plantlets. Meanwhile, other groups have indicated that kinetin at 0.5 to 1 mg/L induced callusing, somatic embryos and complete plantlets formation (15.63%) in *Hevea* (clone was not mentioned)[15,16]. Furthermore, kinetin at 0.7 mg/L to 2 mg/L had been shown to induce 12.83% polyembryony while zeatin at 0.3 mg/L has been successful in promoting more than 60% multiple embryos from leave culture of RRII 105[35–36]. The antagonistic effects of kinetin when applied with BA, NAA and GA_3 on *Hevea* clone RRII 105 has also been reported[18]. In our study, application of 0.5 mg/L zeatin with IBA and $AgNO_3$ or NAA and BA appeared to have a synergistic effect on embryogenesis and consecutive plantlet regeneration of RRIM 2025. Likewise, the synergistic effects were also evident from the use of 0.3 mg/L kinetin with IBA and BA.

Hence, the complete regeneration of plants in this study after a period of six to seven months of anther inoculation is promising, particularly for mass propagation and genetic transformation of RRIM 2025. However, as in the earlier reports, the plantlet developmental stage is still a major limiting factor in *Hevea* tissue culture research[13]. There are a number of factors that could limit the ability of plantlets to overcome environmental attributes during transition from *in vitro* phase to the soil. Acclimatisation conditions, in particular, has to be optimised by improving (i) the balance between relative humidity and temperature[2,37] (ii) the optimum quantum light spectrum and radiation[38] for proliferation of new leaves and greening of shoots and (iii) the optimum nutrient concentration for meristem hardening to ensure ability to perform photosynthesis for survival.

CONCLUSION

An addition of 0.5 and 1 mg/L zeatin in basal media successfully induced callogenesis of RRIM 2025 anther culture. On the other hand, differentiation media with zeatin at a concentration of 0.3 and 0.5 mg/L and kinetin at concentrations of 0.3 and 0.8 mg/L did not significantly induce SEm and plantlet regeneration. The high variance in the results also suggests that RRIM 2025 tissue culture regeneration in this study was seasonal and origin of explant-dependent.

ACKNOWLEDGMENTS

The author sincerely thanked Jamnah Abd Rahman, Mohd Annuar Malek and Hj Jafri Shariff for their excellent technical assistance and Hashima Idris for the help in statistical analysis. The author would also like to acknowledge Dr E Sunderasan for his critical review of the manuscript.

Date of receipt: August 2014
Date of acceptance: July 2015

REFERENCES

1. MONTORO, P., TEINSEREE, N., RATTANA, W., KONGSAWADWORAKUL, P. AND MICHAUX-FERRIERE, N. (2000) Effect of Exogenous Calcium on Agrobacterium Tumerfaciens-mediated Gene Transfer in *Hevea brasiliensis* (Rubber Tree) Friable Calluses. *Plant Cell Rep*., **19(9)**, 851−855.

2. PRIYADARSHAN, P.M. (2011) *Biology of Hevea Rubber*. Oxforshire, UK: CAB.

3. SUNDERASAN, E., BADRUL-EZAM, B., AZLINA, A. AND AROKIARAJ, P. (2012) *Hevea* Genetic Transformation with Human Atrial Natriuretic Factor. *J. Rubb. Res*., **15(4)**, 255−264.

4. PARANJOTHY, K. AND GANDIMATHI, H. (1976) Tissue and Organ Culture of *Hevea International Rubber Conference*. Kuala Lumpur, 20 October 1975, 59−84.

5. CHEN, Y. (2012) Planting Profit. *M'sian Bus*. Kuala Lumpur: Berita Publishing.

6. MATHEWS, A.S. (2012) Overseas NR Plantation Ventures: Yes, the Rush for "White Gold" is on. *Rubb. Asia*, **26(3)**, 66−72.

7. SMITH, H.P. (2012) NR Prices Will Take a Downturn After 2012. *Rubb. Asia*, **26(3)**, 51−54.

8. ANON (2012) Resurgence of Nurseries in NR Producing Countries. *Rubb. Asia*, **26(3)**, 158−160.

9. ANON (2012) Rubber Board Plans: Models Nurseries to Support Growers. *Rubb. Asia*, **26(3)**, 154−155.

10. KILLMANN, W. AND HONG, L.T. (2000) Rubberwood: The Success of an Agricultural By-product. *Unasylva*, **51(201)**, 66−72.

11. ONG, S.H., OTHMAN, R., BENONG, M., HASHIM, O., AZIZ, M.Z.A. AND GHANI, M.N. (1995) RRIM Planting Recommendations 1995-1997. *Proc. Rubber Grower's Conference*, Kuala Lumpur, Malaysia.

12. DUHOKY, M.M.S. AND RASHEED, K.A. (2010) Effect of Different Concentrations of Kinetin and NAA on Micropropagation of *Gardenia jasminoides. Journal of Zankoy Sulaimani-A*, **13(1)**, 103−120.

13. MONTORO, P., ETIENNE, H., CARRON, M.P. AND NOUGAREDE, E. (1992) Effects of Cytokinins on the Induction of Embryogenesis and the Quality of Somatic Embryos in *Hevea brasiliensis* Mull. Arg. *Comptes Rendus des Seances de l'Academie des Sciences: Serie 3(France)*.

14. LARDET, L., DESSAILLY, F., CARRON, M.P., MONTORO, P. AND MONTEUUIS, O. (2009) Influences of Aging and Cloning Methods on the Capacity for Somatic Embryogenesis of a Mature *Hevea brasiliensis* Genotype. *Tree Physiol*., **29(2)**, 291–298.

15. SRICHUAY, W., KALAWONG, S. AND TE-CHATO, S. (2014) Effects of Seasonal Collection on Callus Induction, Proliferation and Somatic Embryogenesis from Anther Cultures of *Hevea brasiliensis* Muell Arg. *Afr. J. Biotechnol*., **13(35)**, 3560−3566.

16. SRICHUAY, W., KALAWONG, S., SIRISOM, Y. AND TE-CHATO, S. (2014) Callus

Induction and Somatic Embryogenesis from Anther Cultures of *Hevea brasiliensis* Muell Arg. *Kasetsart J. (Nat. Sci.)*, **48(3)**, 364−375.

17. ZHUO, Q.N., JIANG, Z.H., HUANG, T.D., LI, W.G., SUN, A.H., DAI, X.M. AND LI, Z. (2010) Plant Regeneration *via* Somatic Embryogenesis from Root Explants of *Hevea brasiliensis*. *Afr. J. Biotechnol*., **9(48)**, 8168−8173.

18. SUSHAMAKUMARI, S., ASOKAN, M.P., ANTHONY, P., LOWE, K.C., POWER, J.B. AND DAVEY, M.R. (2000) Plant Regeneration from Embryogenic Cell Suspension-Derived Protoplast of Rubber. *Plant Cell, Tiss. Org*., **61(1)**, 81−85.

19. ARYA, H.C. AND SHEKHAWAT, N.S. (1986) Clonal Multiplication of Tree Species in the Thar Desert Through Tissue Culture. *Forest Ecol. Manag*., **16(1)**, 201−208.

20. AL-JUBOORY, K.H., SKIRVIN, R.M. AND WILLIAMS, D.J. (1998) Callus Induction and Adventitious Shoot Regeneration of Gardenia (*Gardenia jasminoides* Ellis) Leaf Explants. *Sci. Hortic-Amster*., **72(3−4)**, 171−178.

21. SINGH, S., RAY, B.K., BAHTTACHARRYYA, S. AND DEKA, P.C. (1994) *In vitro* Propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm. *HortScience*, **29(3)**, 214−216.

22. SHIJE, Z., ZHENGUA, C. AND XUENG, X. (1990) A Summary Report on Ather Culture for Haploids Plants of *Hevea brasiliensis*. *Breeding of Hevea brasiliensis*: *Proc. IRRDB Symp*., Kunming, China, 5-6 October, 69−78.

23. MURASHIGE, T. AND SKOOG, F. (1962) A Revised Medium for Rapid Growth and Bio-Assays with Tobacco Tissue Cultures. *Physiol. Plantarum*, **15(3)**, 473−497.

24. GAMBORG, O.L., MILLER, R.A. AND OJIMA, K. (1968) Nutrient Requirement of Suspensions Cultures of Soybean Root Cells. *Exp. Cell Res*., **50(1)**, 151−158.

25. SCHENK, R.U. AND HILDEBRANDT, A.C. (1972) Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Culture. *Can. J. Bot*., **50(1)**, 199−204.

26. HELLER, R. (1953) *Recherche sur la nutrition minérale des tissus végétaux cultivés in vitro. Ann. Sci. Nat. Bot. Bio. Vég. (Paris)*, **14**, 1−123.

27. BOURGIN, J.P. AND NITSCH, J.P. (1967) *Obtention de Nicotiana haploïdes à partir d'étamines cultivées in vitro* (Production of haploid *Nicotiana* from Excised Stamen). *Ann. Physiol. Veg*., **9**, 377−382.

28. FORTANIER, E.J. AND JONKERS, H. (1976) Juvenility and Maturity of Plants as Influenced by their Ontogenetical and Physiological Aging. *Acta Hortic*., **56(1)**, 37−43.

29. BONGA, J.M. (1982) Vegetative Propagation in Relation to Juvenility, Maturity and Rejuvenation. In: Bonga, J.M. and Durzan, D.J. (Eds) *Tissue Culture in Forestry*. The Hague: Matinus Nijhoff, 387−412.

30. BONGA, J.M. AND VON ADERKAS, P. (1992) Rejuvenation of Tissues from Mature Conifers and its Implications for Propagation *In Vitro. Clonal Forestry I: Genetics and Biotechnology*. Berlin: Springer-Verlag, 182−199.

31. ZHOU, Q.N., SUN, A.H., LI, Z., HUA, Y.W. AND JIANG, Z.H. (2012) Cryopreservation and Plant Regeneration of Anther Callus in *Hevea* by Vitrification. *Afr. J. Biotechnol*., **11(28)**, 7212−7217.

32. CARRON, M.P., ETIENNE, H., LARDET, L., CAMPAGNA, L., PERRIN, Y., LECONTE, A. AND CHAINE, C. (1995) Somatic Embryogenesis in Rubber (*Hevea brasiliensis* Meull. Arg.) In: JAIN, S., GUPTA, P. and NEWTON, R. (Eds) *Somatic Embryogenesis in Woody Plants: Angiosperms*. Norwell, MA: Kluwer, 117−136.

33. HACKETT, W.P. (1985) Juvenility, Maturation and Rejuvenation in Woody Plants. In: *Hortic. Rev*., Vol. 7, Janick, J. (Ed). Hoboken, NJ: John Wiley & Sons Inc., 109−155.

34. ANGELOVA, Y., PETKOVA, S., ZOZIKOVA, E., KOTSEVA, E. AND ILIEV, L. (2001) Effects of Kinetin and 4PU-30 on the Growth and the Content of Polyphenols in Tobacco Callus Tissue. *Bulgar. J. Plant Physiol*., **27(1-2)**, 36−42.

35. SUSHAMAKUMARI, S., REKHA, K., DIVYA, U.K., JAYASHREE, R., KALA, R.G., KUMARI JAYASREE, P. AND THULASEEDHARAN, A. (2014) Biotechnology Interventions for Crop Improvement in *Hevea brasiliensis*: Present Status at Rubber Research Institute of India. *IRRDB Biotechnology Workshop on* Hevea *Tissue Culture and Micropropagation*. Bogor, Indonesia.

36. JAYASREE, K., ASOKAN, M.P., SOBHA, S., SANKARI-AMMAL, L., REKHA, K., KALA, R.G., JAYASHREE, R. AND THULASEEDHARAN, A. (1999) Somatic Embryogenesis and Plant Regeneration from Immature Anthers of *Hevea brasiliensis* (Muell. Arg.). *Curr. Sci. India*, **76(9)**, 1242−1245.

37. LECONTE, A. AND CARRON, M.P. (1988) Acclimatization of Micro-Cuttings from Rubber (*Hevea brasiliensis* Meull. Arg.): Problems and Perspectives. In: *Compte Rendu de Colloque Exploitation Physiologie et Amelioration de L'Hevea*, 499−503.

38. SDOODEE, S. AND RONGSAWAT, S. (2012) Impact of Climate Change on Smallholders' Rubber Production in Songkla Province, Southern Thailand. *The 2012 International and National Conference for the Sustainable Community Development of "Local Community (AEC)"*, 81−89.