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INDUCTION OF SHOOTS AND ROOTS FROM VEGETATIVE TISSUE CULTURE OF *HEVEA BRASILIENSIS* RRIM 2020

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ABSTRACT

Adventitious roots and shoot formation were induced from lateral meristem and shoot apical meristem of in vitro grown seedling of *Hevea brasiliensis* RRIM 2020 clone. Concurrently, shoots and roots were also induced for axillary buds (nodules) from matured bud stumps of this clone. The highest callogenesis at 93% was achieved from lateral meristem culture of RRIM 2020 initiated on Woody Plant media (WPM)-A which was WPM supplemented with 0.1 mg L^{-1} benzylaminopurine (BA), 0.5 mg L^{-1} 2,4-dinitrophenylhydrazine (2,4-D) and 3% sucrose. The second highest callogenesis at 61% was obtained from lateral meristem culture in WPM-C which was WPM supplemented with 0.5 mg L^{-1} BA, 0.5 mg L^{-1} 2,4-D, 0.5 mg/L zeatin, 10% coconut water (cw) and 7% sucrose, and this was followed by 49% callogenesis obtained from lateral meristem culture in Murashige and Skoog-Indian modification containing zeatin (MS(ID)Z). Embryogenesis induced in differentiation media, RD1 supplemented with kinetin or zeatin with a combination of plant growth hormones such as benzylaminopurine (BA), indolebutyric acid (IBA), 2,4-D and naphthaleneacetic acid (*NAA*) has successfully generated rooted embryoids. Rooting of lateral meristem culture was induced from calli initiated on WPM(C) containing 0.5 mg L^{-1} zeatin without casein hydrolysate. Meanwhile, the shoot apical meristem culture of RRIM 2020 initiated on developmental media, DM04 and a modified WPM (WPM(B)-M) has successfully enhanced shoot growth to 79% and 36% respectively. Apparently, adventitious shoots regeneration of shoot apical meristem was induced with two different meristem culture media containing thidiazuron (TDZ) (MC(A)-M and modified MC(B)-M). In both cases, while elongation and expansion of roots appeared to be retarded, further shoot development was triggered when the apical meristem was cultured on MS(ID)Z.

Keywords: Cytokinin, kinetin, micro-propagation, regeneration, tissue culture, thidiazuron, zeatin

INTRODUCTION

Tissue culture research in Malaysian Rubber Board (MRB) formerly known as Rubber Research Institute of Malaysia (RRIM) was initiated in 1960s. Until recently however, tissue culture of modern clones, particularly the RRIM 2000 series have been confined to explants such as anther walls (tapetum) and integument, reports on micropropagation of Hevea using vegetative organs such as meristem, shoot tips, nodal and leaves, however, were few and far between. Elsewhere, meristem culture techniques has been widely implemented for germplasm conservation, exchange of germplasm and virus elimination (Robbins 1972). Tissue culture systems are also capable of creating genetic variability and producing plants with novel characters (Roca 1979), which could be more favourable than existing crop varieties. Meristem culture has been successful in the removal of viruses from many plants such as potato, sugarcane and strawberry (Quak 1977), grapevine (Fayek et al. 2009), and carnation (Ashnayi et al. 2012). Meanwhile, shoot tips and nodal culture were shown able to produce pathogen-free plants (Morel 1960; Senula et al. 2000; Quak 1977), and has led to a large scale propagation and improvement of tree species (Bajaj 1986; Boulay 1987).

Moreover, micro-propagation techniques via adventitious organ culture have many advantages as the technique is able to produce plant under greenhouse condition and without seasonal interruption. Therefore, the technique enables rapid and mass production of selected superior planting materials, as well as provides platform for genetic engineering research. RRIM 2020 is among the RRIM 2000 series recommended for commercial field planting because of its higher yield at 1645 kg ha^{-1} yr^{-1}.

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RRIM 2020 is predominantly introduced for timber with special characteristics of high wood volume. In this study, *in vitro* propagation of lateral meristem, shoot apical meristem and axillary buds of Hevea RRIM 2020 clone was tested. The objectives of this study were to induce regeneration of roots and shoots, and to improve growth of aforementioned adventitious organ cultures of Hevea clone RRIM 2020. The callogenesis induction media in this study were enriched with various combinations of potential plant growth regulators and phytohormones including gibberelic acid (GA_3), indolebutyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dinitrophenylhydrazine (2,4-D). For further improvement, the media was also supplemented with cytokinins such as zeatin, kinetin, benzylaminopurine (BA) and thidiazuron (TDZ). The effects of these supplements on the regeneration of the adventitious organ cultures were also investigated.

MATERIALS AND METHODS

Lateral Meristem Culture

The lateral meristem explant was excised from *in vitro* grown seedling of Hevea clone RRIM 2020. In this experiment, the seedlings were germinated by embryo rescue technique using the pre-mature seeds freshly harvested from the trees planted in MRB Sungai Buloh Experimental Station. The fruits were sterilised with 70% ethanol, 5% bleach and two drops of Teepol. The epicarp, mesocarp, endocarp of the fruits and the shell of the seed were peeled and discarded. The embryo containing the epicotyl and hypocotyl were then cultured on MS media in 50 mL glass test tube and kept in the dark at 25 to 27 °C for two weeks. The *in vitro* grown seedlings were then transferred into growth chamber pre-set at 12 h daylight photoperiod at 27 °C, relative humidity (RH) at 82% and CO_2 level at 598 $\mu mol\ mol^{-1}$.

After 47 days, the *in vitro* grown seedlings were ready for sampling. The leaves were defoliated and lateral meristem was vertically sliced (0.5 mm thick) in a sterile condition with sterile dissection knife. The slicing meristem containing xylem and bark were rinsed with sterile water to discard latex debris that might obstruct nutrient uptake during culturing. All procedures involved in this experiment were carried out inside the laminar flow sterilised with UV lights for an hour prior to use.

Callogenesis was induced in 10 types of modified basal medium based on Woody Plant Media (WPM) (Minh & Thu 2001), Meristem Culture Media (MC) (Lineberger & Wanstreet 1983) and Murashige and Skoog media (1962) Indian modification enriched with zeatin (MS(ID)Z) (Table 1). MS medium used in this study was supplemented with a combination of growth regulators similar to that reported by Asseara-Batista et al. (1998). Embryogenesis was promoted using modified RRIM Differentiation Media 1 (RD1) where RD1-C1 was RD1 supplemented with 0.2 mg/L NAA, 1 mg/L BA, 4 mg/L IBA, 0.5 mg/L zeatin and 7% sucrose, and RD1-E2 was RD1 supplemented with 1.5 mg/L BA, 1 mg/L IBA, 0.8 mg/L kinetin, and 7% sucrose. The media were solidified with 2% commercial agar (Phytagel™, BoiReagent, Sigma-Aldrich Co. LLC) and autoclaved for 20 min at 121 °C. Callogenesis and embryogenesis induction were carried out in the dark at 25 to 27 °C for one to two months.

The developed plants were then transferred into the growth chamber as described above for induction of shoot greening and hardening. Throughout the regeneration stages, the rates of callogenesis, embryogenesis and contamination in percentage was recorded. The percentage of callogenesis refers to the number of successful callus generated from the total number of explants, while the percentage of embryogenesis refers to the number of embryos developed from the total number of successful calli. Percentage of contamination refers to the number of contaminated tubes from the total number of tubes cultured.

Table 1. Phytohormones and growth regulators in basal media used for lateral meristem, shoot apical meristem and axillary buds cultures of *Hevea brasiliensis* RRIM 2020 clone.

Basal media	Phytohormones and growth regulators									
	BA (mg L^{-1})	NAA (mg L^{-1})	2,4-D (mg L^{-1})	TDZ (mg L^{-1})	IBA (mg L^{-1})	GA_3 (mg L^{-1})	Zeatin (mg L^{-1})	Casein hydrolysate (mg L^{-1})	Sucrose (%)	Coconut water (%)
WPM(A)	0.1	-	0.5	-	-	-	-	-	7	10
WPM(B)	0.1	0.1	-	-	-	-	-	-	7	10
WPM(C)	0.5	-	0.5	-	-	-	0.5	-	7	10
MC(A)	1.0	-	-	-	-	-	-	100	7	10
MC(B)	2.5	0.1	-	-	-	-	-	100	7	10
MS(ID)Z	0.5	0.1	0.5	-	0.2	-	0.5	-	7	5
WPM(A)-M	0.1	-	0.5	-	-	-	-	-	3	-
WPM(B)-M	0.1	0.1	-	-	-	-	-	-	3	-
MC(A)-M	1.0	-	-	1.0	0.4	1.0	-	100	3	10
MC(B)-M	2.5	0.1	-	-	1.0	5.0	-	100	3	10
DM04	-	-	-	-	0.4	5.0	-	-	3	-

WPM = Woody Plant Media
MC = Meristem Culture Media
MS(ID)Z = Murashige and Skoog Media (Indian modification) with zeatin
DM = Developmental media

Shoot Apical Meristem and Axillary Bud Cultures

The shoot apical meristem (SAM) and axillary buds generated from the above experiment were used in the following investigation. Axillary buds were also obtained from vegetative branches (young bud sticks) harvested from well-maintained Hevea budding stumps in the field. For the budding stumps, the vegetative branches with three to four newly protruded buds were washed under running tap water and surface sterilized with 70% alcohol with two drops of Teepol for 1 min. They were then rinsed with distilled water followed by 100% commercial bleach (Clorox) with two drops of Teepol for 20 min. They were rinsed again three times with distilled water before washing with sterile water using ozone sterilizer at ozone output M 0.065 for 15 min. The leaf and leaf primordial of the remaining *in vitro* grown seedlings from the above experiment and vegetative branches of budding stumps were defoliated and small segments of the explants were excised with sterile dissecting knife. All procedures were performed in the laminar flow sterilised with UV light as indicated above.

Shoot regeneration and multiplication were induced on four types of media namely DM04, modified WPM(B) (WPM(B)-M), MC(A)-M and MC(B)-M (Table 1). The shoots evolved from shoot apical meristem and axillary bud explants were developed further in the growth chamber at similar condition as mentioned above.

RESULTS AND DISCUSSIONS

Lateral Meristem Culture

In this early stage of the experiments, promising results for the development of lateral meristem culture for *Hevea brasiliensis* clone RRIM 2020 were apparent. The calli were successfully initiated when the lateral meristem explant was cultured on WPM(A), WPM(B), WPM(C), MC(A), MC(B) and MS(ID)Z (Table 2, Fig. 1). The callogenesis achieved was from 49% to 93% (Table 2). The highest callogenesis rate was induced by WPM(A) at 93%. It was followed by WPM(C) at 61 % and MS(ID)Z at 49% (Table 2). Two and three axillary bud growth was observed for the meristem cultured on

WPM(A) and MC(A), respectively (Table 2). WPM(B) and MC(B) showed limited capability, with regeneration of seven and one callus sections, respectively (Table 2). Furthermore, the initiation media MC(A) also successfully induced formation of three callus sections (Table 2). At this stage, high contamination was also observed from 23% to 95% (Table 2). The use of explants originated from *in vitro* grown seedlings is suggested to eliminate and/or reduce contamination in the culture system. Thus, the sterilization techniques used in this study need to be further optimised in future research.

Table 2. Successful regeneration of lateral meristem culture from *in vitro* grown seedlings of Hevea clones RRIM 2020.

Experiment	Media	Callus formation	% Contamination	Embryo formation
RRIM 2020	WPM(A)	93% with 8 callus section and 2 axillaries buds growth	90	RD1-E2: 2 rooted, the rest were dormant
	WPM(B)	7 callus section	95	Dormant
	WPM(C)	61% callus formation	23	RD1-C1: 2 rooted
	MC(A)	3 axillaries buds growth and 3 callus section	70	Dormant
	MC(B)	1 callus section	80	Dormant
	MS(ID)Z	49% callus formation	45	RD1-E2: 1 rooted RD1-C1: 2 rooted

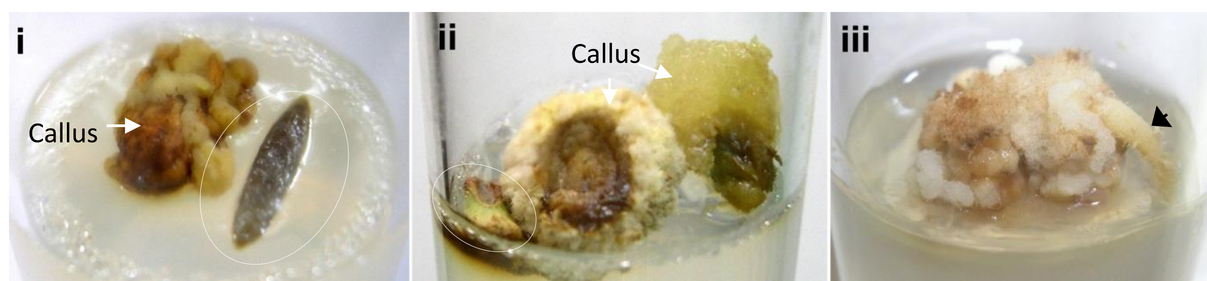

Figure 1. Lateral meristem culture originating from *in vitro* grown seedlings of Hevea RRIM 2020. Calluses were induced on basal media MS(ID)Z. Panel (i) and (ii) show excised meristem (in the white circle) and callus formation (white arrow). (iii) Embryogenic calli with root regeneration (black arrow head).

In the differentiation stage, the calli initiated on WPM(A) successfully generated two rooted embryoids after transfer into RD1-E2 (Table 2, Figs. 2A-C) that was supplemented with BA, IBA, kinetin and sucrose. Meanwhile, two rooted embryoids were obtained from the calli initially induced on WPM(C) and differentiated on RD1-C1 (Table 2, Figs. 2D-E) supplemented with NAA, BA, IBA, zeatin and sucrose. For the calli that were initiated on MS(ID)Z, at least one and two rooted embryoids were successfully produced after transfer into RD1-E2 and RD1-C1, respectively [Table 2, Fig. 1(iii)]. This result suggests that kinetin and zeatin in the media successfully induced rooting of the calli, similar to the report on root formation in wheat culture (Dudits et al. 1975).

Furthermore the medium with a combination of auxin (BA and 2,4-D) and zeatin at different concentrations with or without NAA and IBA were also able to induce rooting for lateral meristem culture of Hevea clone RRIM 2020. Zeatin (a type of cytokinin) added in the culture media has been reported to induce cell division and growth of shoots *in vitro* of other plant species such as Gardenia (Duhoky & Rasheed 2010; Al-Juboory et al. 1998), *Citrus reticulata* Blanco and *Citrus limon* (Singh et al. 1994), and in apple cultivar Zizyphus spina-christi wild (Arya & Shekhawat 1986). An addition of casein hydrolysate in MC(A) and MC(B) media did not succeed in inducing root for lateral meristem culture of Hevea RRIM 2020. This finding contradicts with the report on root development

with casein hydrolysate in *Dactylis glomerata* (Gramineae) cell suspension culture (Gray & Conger 1985).

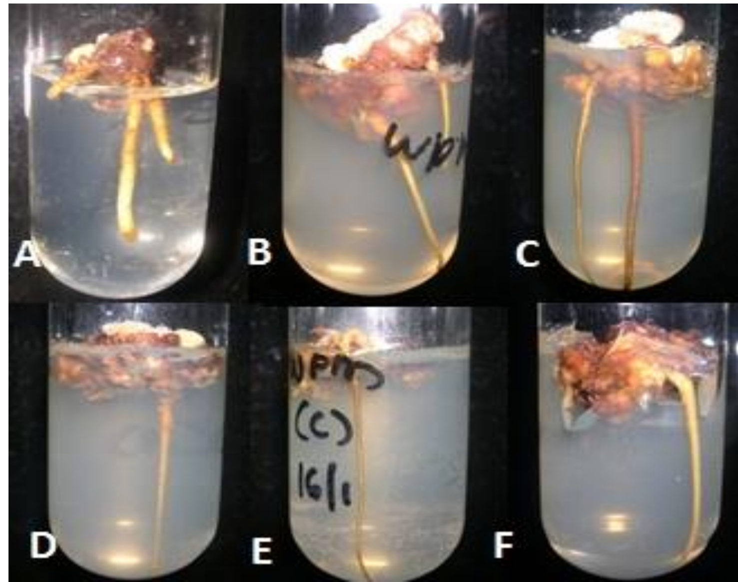

Figure 2. Rooted embryoid from lateral meristem culture of Hevea clone RRIM 2020 initiated on Woody Plant media (WPM)-A (panel A-C) and WPM-C (panel D-F). The rooted embryos were induced in RD1-E2 and RD1-C1, respectively.

Calli that developed at the injured surface of excised stem have been shown able to develop into healthy plants (Delmer & Amor 1995). However, in the present study, most of the calli and axillary bud growth became dormant (Table 2). This is probably due to insufficient nutrients prior to the extended differentiation stage. Thus, sub-culturing on freshly prepared differentiation media is permitted. Besides, callose deposit was observed in some of the stem segments (data not shown), possibly induced by wounding during stem excision (Delmer & Amor 1995). Callose restricts solutes movement in healed tissues (Canny 1995). It is a complex carbohydrate of β-1, 3-glucan, which is commonly deposited in wounded plants cell walls (Stone & Clark 1992) and thereby could be misidentified as callus.

Shoot Apical meristem and Axillary Buds Cultures

In this study, shoot regeneration and multiplication of shoot apical meristem (SAM) and axillary buds explants from Hevea RRIM 2020 were examined. Shoot induction of SAM on DM04, and modified WPM(B)-M media showed 79% and 36% [Table 3, Figs. 3 (i) and (ii)] growth, respectively. Meanwhile, for the explants cultured in modified MC(A)-M containing 1 mg L^{-1} TDZ and modified MC(B)-M containing 0.1 mg L^{-1} NAA, poor growth with tiny shoots was observed for shoot apical meristem [Figs. 3 (iii) and (iv)] and axillary bud explants (data not shown). Elongation and expansion of induced shoots were observed but generally appeared to be retarded [Figs. 3 (iii) and iv)]. Thus, further investigations are required before the findings could be recommended as a possibility of genome preservation (in the form of nodules) for Hevea RRIM 2000 series.

TDZ (urea-type cytokinin) have been used to efficiently induce adventitious shoots in many woody plant species such as *Acer x freemanii* (Kerns & Meyer 1986) and apple (van Nieuwkerk et al. 1986). TDZ used in leaf segment culture of Japanese persimmon (at 1 μM) (Yokoyama et al. 2011), and hydrangea (at 0.05 to 0.5 μM) (Ledbetter & Preece 2004) had successfully induced adventitious buds growth in these species. However, TDZ inhibited and slowly elongated their further growth, which was only perceived following the transfer to the medium containing zeatin (Yokoyama et. al. 2011). TDZ in these studies has prolonged the shelf life of the meristematic nodules for more than five years,

thus provided long-term conservation of *in vitro* plants of the species (Yokoyama et. al. 2011; Ledbetter & Preece 2004).

Table 3. The performance of shoot apical meristem culture from *Hevea brasiliensis* RRIM 2020

Clone	Media	% contamination	% growth
RRIM 2020	DM04	21	79
	WPM(B)-M	64	36
	MC(A)-M	100	-
	MC(B)-M	100	-

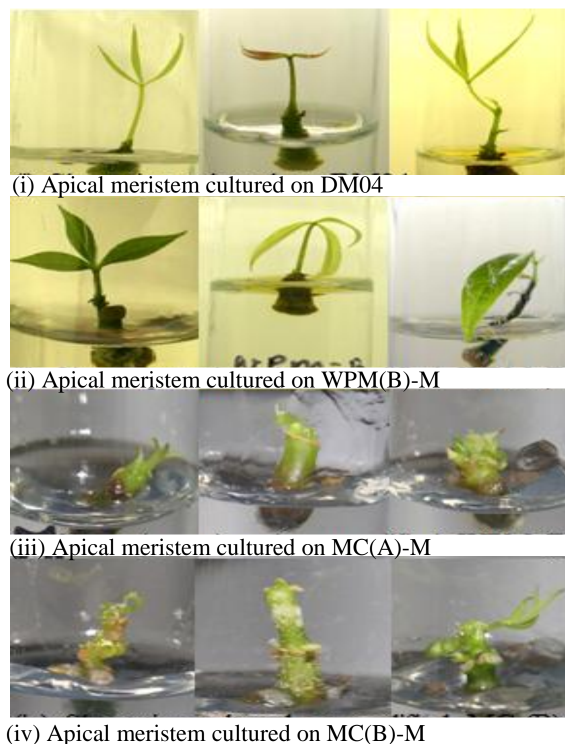

Figure 3. Adventitious shoot regeneration and multiplication from shoot apical meristem of Hevea RRIM 2020 tested on four different media i.e. (i) DM04, (ii) WPM(B), (iii) MC(A)-M and (iv) MC(B)-M.

Casein hydrolysate had successfully stimulated rooting and supported root development of the *Dactylis glomerata* culture (Gray & Conger 1985). On the contrary, an addition of casein hydolysate in MC(A), MC(B), MC(A)-M and MC(B)-M media used in this study did not induce rooting of shoot apical meristem and axillary bud cultures of RRIM 2020. The presence of coconut water in the medium also did not enhance regeneration of adventitious shoot and root growth, although a positive response has been observed in *Eucalyptus globulus* culture (Trindate & Pais 2003). Concurrently, a high percentage of contaminations were observed from 21 to 100% (Table 3), perhaps due to inadequate sterilization of the budding stumps. After the second multiplication, the cultures were all contaminated and therefore discarded.

Shoot and root tissue cultures were performed in the present research in an attempt to explore additional reliable and sustainable explants for plant regeneration. Shoot apical meristem (SAM) has been employed to regenerate well-developed shoot without an adventitious propagation (Grout 1999; Hu & Wang 1984). Generally, buds display high organogenesis (plant/organ regeneration) capability for woody plants (McCown et al. 1988) because they contain dense cell clusters with a consistent

internal cell differentiation. The developmental pathway of bud culture is similar to that of embryogenic stages, where the regeneration strategy has proven to sustain totipotency (McCown et al. 1988), that will ensure genetic stability and minimize somaclonal variation of woody plant culture.

CONCLUSION

Propagation of tissue culture plants for *H. brasiliensis* using adventitious organ in this study resulted in a relatively low regeneration of RRIM 2020. However, successful root generation for lateral meristem culture of Hevea clone RRIM 2020 with zeatin was obtained. TDZ in MC(A)-M has promoted adventitious shoots formation for apical meristem culture, but inhibited its elongation and expansion. Induced roots for lateral meristem culture with WPM(A), WPM(B) and WPM(C) and induced shoots of shoot apical meristem culture with DM04 and WPM(B)-M, were well developed. The results thereby, suggested the insignificant effects of TDZ and casein hydrolysate on Hevea RRIM 2020 regeneration. The results obtained so far have shown the additional possibilities of *in vitro* clonal propagation of Hevea RRIM 2000 series and have great potential to be further exploited.

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