Study on the Somatic Embryogenesis from Anthers of Immature Inflorescence of Some Selected *Hevea* Clones

U. K. D. K. DEEPIKA[1], P. SENEVIRATNE[2], C. NAYANAKANTHA[2] AND W. M. E. P. WICKRAMASINGHE[1]

[1] *Department of Horticulture and Landscape Gardening, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.*

[2] *Department of Plant Science, Rubber Research Institute of Sri Lanka, Dartonfield, Agalawatte, Sri Lanka.*

ABSTRACT

An experiment was conducted to find out the potential for somatic embryogenesis from anthers of immature inflorescence of *Hevea* clones: RRIC 100 and RRIC 121. The effect of hormone combination on callus initiation from the anthers depends on the type of Hevea clone. The highest callus induction was recorded when anthers of RRIC 121 were cultured on Murashige and Skoog (MS) medium supplemented with a hormone combination of 1.0mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5mg/l α-naphthalene acetic acid (NAA), 0.5mg/l kinetin (KIN) and 0.01mg/l thidiazuron (TDZ). Callus proliferation was favoured by thidiazuron in combination with 2, 4-D, NAA and KIN levels. However, embryo induction was not achieved in MS medium with 2mg/l gibberellic acid (GA_3) or with a combination of 2mg/l GA_3 and 0.01mg/l TDZ levels.

KEYWORDS: Anthers, Callus, Gibberellic Acid, *Hevea brasiliensis*, Micropropagation, Somatic Embryogenesis, Thidiazuron

INTRODUCTION

Hevea brasiliensis being the main source of natural rubber is an economically important perennial tree, belonging to the family Euphorbiaceae. It is cultivated mostly in high rainfall tropical areas within 20^0 North and 20^0 South of the equator (Chen, 1984). *Hevea* is an out breeding tree which has 2n=36 chromosomes and allotetraploid with x=9 (Wycherly, 1976).

Hevea is currently propagated by grafting buds from selected clones on to unselected seedling rootstocks (Combe, 1975). The application of conventional breeding techniques for crop improvement programmes in *Hevea* is limited due to its high heterozygosity and long generation cycle (Jayasree *et al.*, 2001). Therefore, propagation of *Hevea* via *in vitro* techniques has been attempted since 1970's (Seneviratne and Flegmann, 1996).

Plant regeneration via somatic embryogenesis has recently become an attractive tool for the production of transgenic plants, because of its single cell origin (Sushamakumari *et al.*, 2000). The first transgenic rubber trees were reported by Arokiaraj *et al.*, (1994), who used the particle bombardment method and then the *Agrobacterium tumefaciens* system (Arokiaraj *et al.*, 1998) on anther derived calli (Montoro *et al.*, 2003). Anther culture is important to produce haploid plants and thereby pure lines which will express all the dominants and recessives of *Hevea* genome. Since a characteristic of the pure line will be to produce homogenous progeny, these materials can be used in seed gardens to produce identical clonal seedlings which can be used directly in plantations (Withanage, 1992).

Plant regeneration by somatic embryogenesis of *Hevea brasiliensis* has been achieved in several experiments using different explant sources (Asokan *et al.*, 1992a, b; Carron *et al.*, 1995; Chen, 1984; Jayasree *et al.*, 1999). However, success rate in the conversion of somatic embryo into full plantlets still remains low (Sushamakumari *et al.*, 1999). Production of pollen plants via somatic embryogenesis might be cultivar dependent. Although successful production of pollen plants was reported by Chinese scientists, none of the locally available clones of *Hevea* produced even somatic embryos (Seneviratne *et al.*, 1996). The first anther derived callus, which could be sub cultured, was produced at the Rubber Research Institute of Sri Lanka in 1972 (Satchuthananthavale and Irugalbandara, 1972).

The main reason to work more with anther and nucellus tissue is that the regeneration capacity of these tissues is believed to be very high and use of juvenile tissue is an important requirement in the process of somatic embryogenesis (Seneviratne *et al.*, 1996). The procedure to produce plantlets from anther consists of three main steps namely callus initiation, embryoid formation and plant development (Chen, 1984).

Several factors such as the developmental stage of the explant, quantity of growth regulators, basal media components, light intensity etc. appear to play crucial roles in somatic embryogenesis in many plant species including *Hevea* (Sushamakumari *et al.*, 2000). The role of auxins and cytokinins in different stages of somatic embryo development has also been reported as critical in many species (Komamine *et al.*, 1992).

Hence present work describes the potential for somatic embryogenesis from anther culture of some selected clones of *Hevea* under different auxin and cytokinin levels in initial stages.

MATERIALS AND METHODS

Plant material and culture media

This experiment was carried out at the tissue culture laboratory of the Department of Plant Science, Rubber Research Institute of Sri Lanka, Dartonfield, Agalawatte for a period of 5 months. Immature inflorescence from mature trees of *Hevea brasiliensis*, clone RRIC 100 (C1) and RRIC 121 (C2) were

collected during main flowering season in March. Male flower buds containing anthers at diploid stage were harvested from the inflorescence.

Explants were wrapped loosely in pieces of gauze, 40 buds per each pack, and were dipped in 70% ethanol for 3 min followed by 10 min immerse of 10% sodium hypochlorite solution containing 4% active chlorine. They were then washed 4 times in sterilized distilled water.

The basal medium used throughout the experiment was MS (Murashige and Skoog, 1962) supplemented with 2.5% coconut water, 70g/l sucrose and solidified with 6g/l agar. The pH of the medium was adjusted to 5.7. Media were prepared in Macartny bottles. Culture media, water and instruments were sterilized in an autoclave at 121°C (under 15 lb/inch^2) for 20 min. Sterilized flower buds were dissected under a dissecting microscope and the anthers were taken out in the laminar air flow cabinet.

Callus induction

Callus inducing ability of the two clones was tested in two hormone combinations of 1mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5mg/l α-naphthalene acetic acid (NAA) and 0.5mg/l kinetin (KIN) (T1) and 1mg/l 2,4-D, 0.5mg/l NAA,0.01mg/l thidiazuron (TDZ) and 0.5mg/l KIN (T2).

Twenty anthers were inoculated into each vial and were incubated at 26°C under dark condition. Twenty replicates were prepared for each hormone combination and the experiment was repeated twice. Callus was transferred on to new media once a month.

Callus induction, contaminated cultures and fresh weight of callus were recorded after 50 days.

Data were analyzed statistically using the chisquare procedure (SAS, 1991).

Embryo induction

In order to identify a suitable hormone combination for effective embryo induction, 50 day old calli were transferred on to the media containing 2mg/l gibberellic acid (GA_3) and 2mg/l GA_3 + 0.01 mg/l thidiazuron (TDZ) respectively. Observations on embryogenesis were made after one month from culture establishment.

RESULTS AND DISCUSSION

Callus initiation was started in 25-30 days after the inoculation of anthers on to the culture media followed by swelling of anthers. Initially produced friable embryogenic callus was pale yellow in colour and was highly proliferated until they transferred into embryo induction medium. However, most of the anthers did not respond to the culture media and turned dark even after 50 days from culture establishment. Contamination of cultures was 12% and was totally due to fungal contaminants.

Callus initiation was observed in both treatments in both clones. The present study was designed to include combinations of auxins and cytokinins as they were found to be essential for callus initiation from anther (Withanage, 1992).

Results of this experiment clearly showed that the effect of hormone combination on callus initiation depends on the type of *Hevea* clone. T1 showed a significantly higher callus formation than T2 in RRIC 100 (C1).

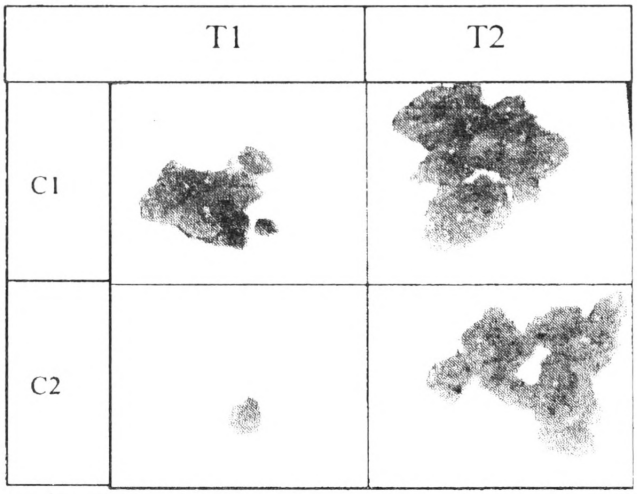

Plate 1.Callus formation of RRIC 100 (C1) and RRIC 121 (C2) under different hormone combinations (T1 and T2) at the 50th day of culture

Table 1. Effect of hormone combination and clone on callus induction

Treatment	Callus induction (%)	
	RRIC 100 (C1)	RRIC 121 (C2)
T1	13.89[a]	3.57[a]
T2	10.34[b]	45.83[b]

Values denoted by different letters in a column are significantly different (P<0.05).

The highest percentage of callus induction was observed in RRIC 121 (C2) when anthers were cultured on MS medium supplemented with T2. The lowest callus induction percentage was recorded in the same clone under T1 hormone level. This reveals that the induction of callus from anthers of a given *Hevea* clone varies significantly according to the hormone combinations included in the culture medium.

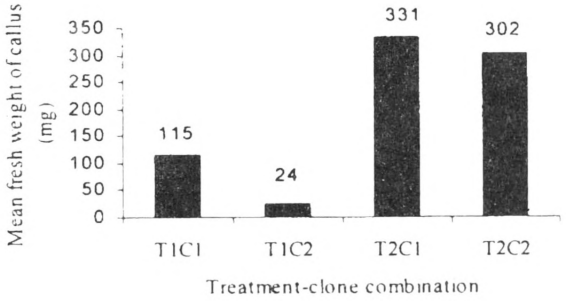

Figure 1. Mean fresh weight of callus at 50th day of culture

Even though the callus induction was low, the highest mean fresh weight of calli masses was observed at T2 in C1 clone. The reason could be due to the fast callusing and thereby fusion of 2 to 3 calli masses to form a single mass. RRIC 121 (C2) also showed a better callus proliferation at T2 hormone combination. According to the figure 1, the lowest mean fresh weight of callus was recorded at T1 hormone level in RRIC 121 (C2) similar to the poor performance resulted in callus induction.

The experiment showed that T2 has considerable potential in increasing weight of callus than T1. The thidiazuron present in T2 increased the rate of callus proliferation compared to that of T1. Thidiazuron was found to be effective on callus induction and organogenesis in several crops (Seneviratne and Flegmann, 1996). Furthermore, Capelle *et al.* (1983) reported that thidiazuron was extremely active in stimulating callus growth of *Phaseolus lunatus*.

Among two clones tested, early callus initiation occurred in RRIC 121 compared to that in RRIC 100 (data not shown). According to Montoro *et al.* (1993), the structure of calli and their morphogenetic capacities are not strictly specific to the genotype but rather to a genotype and medium interaction. According to Chen *et al.* (1990), the development stage of the flower bud is critical in callus induction. Since it is related to the external morphological characteristics, for any of a clone, the proper stage has to be identified by the size and the colour unique to the clone (Withanage, 1992).

Even though calli were transferred on to the embryo induction medium, no somatic embryo formation was observed in any of the cultures even after one month. Some of the calli were turned brown and the proliferation was ceased. Others remained without showing any morphological change in the medium containing only GA_3. But the media containing both thidiazuron and GA_3 showed further callus proliferation. According to Seneviratne *et al.* (1996), it is very unlikely that the embryogenic capacity of all the cultivars available in Sri Lanka is zero. Other factors such as medium composition and subculture period, may govern embryogenesis, which are as much as or even more important than the clone or the cultivars.

According to Jayasree *et al.* (2001), *Hevea* needed continuous dark till the acquisition of embryo induction. However, light favoured embryo maturation and further plant regeneration. In the present study both anthers and callus were incubated under dark.

According to Sushamakumari *et al.* (2000), addition of GA_3 strongly stimulated the embryogenesis. They have obtained better results with the combination of Zeatin and GA_3 in embryo induction. Generally somatic embryogenesis occurs under stress conditions in the culture medium. Linossier *et al.* (1997) observed enhancement of somatic embryo development in *Hevea* at the presence of abscisic acid and high concentration of Poly Ethylene Glycol (PEG). It was reported that there were some beneficial effects of glutamine and casein hydrolysate on somatic embryogenesis of *Hevea* (Jayasree *et al*, 2001). However, none of the above chemicals could be tested in the present study.

CONCLUSIONS

The present study demonstrated that the callus initiation from anthers of immature inflorescence of *Hevea brasiliensis* depends on the type and concentration of growth regulators and the nature of genotype. Thidiazuron had a great influence in improving callus proliferation. Somatic embryo induction was not possible with MS medium treated with hormone combinations of 2mg/l GA_3 and 2mg/l GA_3 + 0.01mg/l TDZ in *Hevea* clones RRIC 100 and RRIC 121. The potential for somatic embryogenesis from other clones yet to be studied.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. C.K. Jayasinghe, Deputy Director (Research), Rubber Research Institute of Sri Lanka (RRISL), Dartonfield, Agalawatta, for giving permission to conduct this research. The assistance given by the staff of the tissue culture laboratory is acknowledged. Sincere thanks are also due to Mr. Keminda Herath at RRISL for assisting statistical analysis.

Authors gratefully acknowledge the assistance given by Professor S.J.B.A. Jayasekara, Dean, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka and Dr. B. Ranaweera, Head, Department of Horticulture and Landscape Gardening, throughout the study. A special thanks goes to staff of the computer services unit at Makandura for providing necessary computer facilities.

REFERENCES

Arokiaraj, P., H. Jones, K.F. Cheong, S. Coomber, B.V. Charlwood (1994). Gene Insertion into *Hevea brasiliensis*. Plant Cell Rep.**13**:425-431.

Arokiaraj, P., H.Y. Yeang, K.F. Cheong, S. Hamzab, H. Jones, S. Coomber and B.V. Charlwood (1998). CaMV 35 s promoter directs β-glucuronidase expression in the laticiferous system of transgenic *Hevea brasiliensis* (rubber tree). Plant Cell Rep.**17**:621-625.

Asokan, M.P., P. Jayasree and S. Sushamakumari (1992a). Plant regeneration by somatic embryogenesis of rubber tree (*Hevea brasiliensis*). *International Natural Rubber Conference*, 1992, Bangalore, India, p.49.

Asokan, M.P., P. Jayasree, S. Sushamakumari and P. Sobhana (1992b). Plant regeneration from anther culture of rubber tree (*Hevea brasiliensis*). *International Natural Rubber Conference*, 1992, Bangalore, India, p.51.

Capelle, S.C., D.W.S. Mok, S.C. Kirchner and M.C. Mok (1983). Effects of thidiazuron cytokinin autonomy and the metabolism of N^6- (2-isopentinyle) (8^{14} C) adenosine in callus tissues of *Phaseolus lunatus* L. *Plant Physiology*, **73**, 796-802.

Carron, M.P., H. Etienne, L. Lardet, S. Campagna, Y. Perrin, A. Leconte and C. Chaine (1995). Somatic embryogenesis in rubber (*Hevea brasiliensis* Muell.Arg.). In: *Somatic embryogenesis in woody plants, Angiosperms* (Eds. S.Jain, P.Gupta and R.Newton). Kluwer Academic Publisher, The Netherlands, pp **2**:117-136.

Chen, Z. (1984). Rubber (*Hevea*). In: *Handbook of plant*

cell culture vol. 2. Crop species (Eds. Sharp, W.R., D.A.Evans, P.V.Ammirato and Y.Yamada). Macmillan publishing Co. Newyork, pp **2**: 546-571.

Chen, Z., X. Xuen and P .Rensheng (1990). Rubber tree-Anther and ovule culture. In: *Handbook of plant cell culture* vol.6: *perennial crops* (Eds. Z. Chen, D.A. Evans, W.R. Sharp, P.V. Ammirato and M.R. Sondahl) McGraw-Hill Publishing company, pp 453-467.

Combe, J.C. (1975). Demonstration of intraclonal variability in young grafted trees. *Revue Generale des Caoutchouces et Plastiques* **52**, 91-94.

Jayasree, P.K., M.P. Asokan, S. Sobha, L. Sankariammal, K. Rekha, R.G. Kala, R. Jayasree and A. Thulaseedharan (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell.Arg.) *Current Science*, **76** (9): 1242-1245.

Jayasree, P.K., V. Thomas, C.K. Saraswathyamma and A. Thulaseedharan (2001). Optimization of parameters affecting somatic embryogenesis in *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **14** (1): 20-29.

Komamine, A., R .Kawara, M. Matsumoto, S. Sunabori, T. Toya and T .Fujimura (1992). Mechanisms of somatic embryogenesis in cell cultures: Physiology, biochemistry and molecular biology. *In vitro cellular and Developmental Biology*, **28**: 11-14.

Linossier, L., P. Veisseire, F. Cailoux and A. Coudret (1997). Effects of abscisic acid and high concentration of PEG on *Hevea brasiliensis* somatic embryos development. *Plant science*, **124**:183-191.

Montoro, P., H. Etienna and M.P. Carron (1993). Callus friability and somatic embryogenesis in .*Hevea brasiliensis*. *Plant Cell Tissue and Organ Culture*. **33**: 331-338

Montoro, P., W. Rattana, V. Pujade-Renaud, N. Michaux-Ferrier, Y. Monkolsook, R. Kanthapura and S. Adunsadthapong (2003). Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Plant Cell Rep, **21**: 1095-1102.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiologia Plantarum*, **15**: 473-497.

SAS(1991). SAS/ETS *User's Guide for Personal Computers, Version* 6, 4th Edition. Cary, NC USA, SAS Institute Inc.

Satchuthananthavale, R. and Z.E. Irugalbandara (1972). Propagation of callus from *Hevea* anther. *Quarterly Journal of Rubber Research Institute of Ceylon*. **49** (3&4), 65-68.

Seneviratne, P. and A. Flegmann (1996). The effect of thidiazuron on axillary shoot proliferation of *Hevea brasiliensis in vitro*. *Journal of the Rubber Research Institute of Sri Lanka*, 77, 1-14.

Seneviratne, P., S.P. Withanage, A. Nugawela, G.A.S. Wijesekara and G.M. de Zoysa (1996). Somatic embryogenesis for *Hevea* : The technique of anther culture. *Journal of the Rubber Research Institute of Sri Lanka* (1996) **78**, 79-88.

Sushamakumari, S., K. Rekha, V. Thomas, S. Sobha and R. Jayasree (1999). Multiple shoot formation from somatic embryos of *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **12** (1&2):23-28.

Sushamakumari, S., S. Sobha, K. Rekha, R. Jayasree and M.P. Asokan (2000). Influence of growth regulators and sucrose on somatic embryogenesis and plant regeneration from immature inflorescence of *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **13** (1&2): 19-29.

Withanage, S.P. (1992). Anther culture and establishment of field grown shoot materials of *Hevea brasiliensis* (Muell.Arg.). A research report submitted in partial fulfilment of the Advance course in Genetics and Plant breeding for the Degree of Bachelor of Science in Agriculture 1992 University of Ruhuna, Sri Lanka. 1-51.

Wycherly, P.R. (1976). Rubber (*Hevea brasiliensis*). In: Evaluation of crop plants (Ed.N.W.Simonds) 77-88.