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SOMATIC EMBRYOGENESIS FOR *HEVEA*: THE TECHNIQUE OF ANTHER CULTURE

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ABSTRACT

Production of pollen plants via somatic embryogenesis might be cultivar dependent as successful production of pollen plants is reported only by Chinese Scientists. None of the locally available clones of Hevea produced even somatic embryos.The slow progress made on adoption of this technique suggests difficulties in reproducing, may be due to various critical factors involved in the the process. The rate of callus formation was influenced by the amount of auxins and cytokinins in the medium and the presence of both types was important for initiation of callus.

Key words: anther, *Hevea,* rubber, somatic embryogenesis

INTRODUCTION

The progress that has been made over the past two decades on *in vitro* techniques for multiplication and improvement of *Hevea* is vast. However, no large scale commercial application of tissue culture is available for *Hevea* though the progress at the research level suggests that the tissue culture technique of *Hevea* can and should be further developed.

Plantlets via somatic embryoids from anther wall-derived callus have been successfully produced as early as in 1977 (Chen *et al.*, 1979). Chinese Scientists reported the production of the first batch of anther derived plants. The first anther derived callus which could be subcultured was produced at the Rubber Research Institute of Sri Lanka in 1972 (Satchuthananthavale and Irugalbandara, 1972)

The potential uses of somatic embryogenesis either through anther culture or through seed inner integument culture has been discussed by many workers. One anticipated use is producing new cultivars or clones. It is likely that the production of variants will be required on a large scale as most of them may be undesirable. Also, success will depend usually on the type of change required. For instance, characteristics such as disease resistance, drought tolerance, wind resistance etc. will require screening & selection.

Production of haploids from pollen is another use in genetic improvement of *Hevea*. This is very useful in particular as *Hevea* is not only a perennial but also an out breeder. Haploids are rare in out-breeding species, except in amphidiploids (Kimber & Rilay, 1963). Chen (1984) points out that obtaining pure lines by successive inbreeding in *Hevea* is virtually

impossible and suggests that anther culture now provide a means of obtaining homozygous diploid lines. Some plantlets originated from pollen of good yielding genotypes, after doubling the chromosomes, may produce individuals with more promising economic characters that can be used as starting materials for selection. As haploid plants are deprived of dominance and suppression, recessive characters are also expressed in the first generation and need not to wait until the second generation. The possible uses of anther culture has been reported in detail (Chen 1984; Paranjothi, 1987). This approach has the advantage that the plantlets produce tap roots that resemble those of the normal seedlings. The procedure to produce plantlets from anthers consists of three main steps namely callus initiation, embryoid formation and plant development (Chen, 1984). More fundamental aspects of research including environmental parameters, histological studies, analysis of media etc. have already been made for better understanding of the process of somatic embryogenesis with anthers and inner integument tissue of seeds (Aubiron *et al.*, 1990, Chen *et al.*, 1981; Hadrami *et al.*, 1989; Michaux-Ferriere & Carron 1988).

Cytological observations did on anther derived calli embryoids and root tips of the plantlets have revealed that after 20-30 days of culture, 80% of the metaphase of mitotic division observed were diploids (2n=36), while after 50 days of culture only 10% were diploids and 69% were haploids where as the rest contained varying number of chromosomes. Therefore, it has been recommended to transfer the callusing anthers to the medium of differentiation after about 50 days of culture, for induction of calli and embryoids originating from pollen grains (Cen et al, 1981).

However, when the metaphase was observed in slides made with embryoids, a mixture of chromosome numbers, i.e., 9, 18, 27,32,36, & 45 have been observed but 80% of them with 18 chromosomes. When the embryoids were developed into plantlets, the number of cells with 18 chromosomes has decreased while the number with 27 has increased, suggesting that the origin of plantlets obtained by anther culture can be judged by examining the cells of embryoids (Cen *et al.*, 1981; Chen *et al.*, 1982).

The RRIM is also conducting studies on anther culture of *Hevea* but it was the Chinese who has done the major contribution in this area of study and developed the technique. As reported by Shijie *et al* (1990) pollen plants from about 13 clones have successfully been transplanted and established in the field. It has been reported by them that the variation between the clones in the frequency of embryoids and plantlet induction is very high. Only a few clones have shown a high rate of plantlet induction (about 22%) while many clones have given a very low embryoid and plantlet induction rates. Some clones have not shown any embryoid formation at all. Among the clones that have been successful in plantlet production are Haiken 1, Haiken 2, Haiken 6, RRIM 600, PR 107, Zhenxuan 1, Zhenxuan 5, Huapei 1, PB 5/51, IAN 717, Boating 155, Boating 3418 and SCATC 88-13. Statistics of their work on embryoid formation and plantlet development shows the frequency of 1.7 embryoids per culture tube and 0.054 plantlets per culture tube. The stage of flower buds used to harvest anthers, seems to be important in producing haploids (Shijie et al,1990).

The present study was carried out with the clones available in Sri Lanka; none of the clones used by the Chinese were included. The media composition, the culture conditions and

culture procedures were similar to those developed by Chen (1984) and Chen *et al.*(1991), and attempts were made to see whether somatic embryos can be formed from any of the clones.

MATERIALS AND METHODS

Clones, PB 86, RRIC 100, RRIC 102, RRIC 110, RRIC 121 and RRIC 130 were used. Flowers were collected in February March, *i.e.*, the main flowering season. Female flower buds about 3-3.5 mm long and light greenish yellow in colour were harvested as they contain majority of anthers at uninucleate state.

The compositions of the culture media were similar to those described by Chen *et al* (1990). Media were prepared in Macartny bottles.

Flower buds were wrapped loosely in pieces of gauze, about 15-20 buds per each pack, and they were dipped in 70% ethanol for 3 min followed by 10 min immerse in 10% NaOCl. After sterilization they were washed 3-4 times in sterilized water.

Media, water and instruments were sterilized in an autoclave under 300 k pas m^{-2} s^{-1} at 121°C for 20 min. Sterilized flower buds were dissected under a dissecting microscope. About 10-12 undamaged anthers were inoculated into each vial, well distributed on the medium. Cultures were incubated at 26°C under dark.

Anthers from different clones were cultured into the same callusing medium where kinetin, 2,4-D & NAA were supplied at 1 mg/l each. Sucrose was used at 70 g/l and coconut water at 0.5%. Agar was used at 6 g/l and pH was adjusted to 5-8 before autoclaving. After 50 days, calli were transferred to a medium containing kinetin, NAA & GA_3 at 0.5, 0.2 & 0.2 mg/l respectively.

For anthers of clone RRIC 130 the following combinations of kinetin and 2,4-D were tested while keeping the rest of the medium unchanged(Media were numbered as shown below). Medium containing kinetin, 2, 4-D and NAA each at 1 mg/l was used as the control. There were 25 replicates (vials) for each medium.

		Kinetin mg/l			
		0	.5	1	2
2,4-D mg/l	0	1	2	3	4
	.5	5	6	7	8
	1	9	10	11	12
	2	13	14	15	16

RESULTS

Culture contamination was not a serious problem but, fungal contaminations were observed rarely.

Calli were produced by most of the anther in each vial but some of them turned dark and did not show any growth.

Callus initiation was observed after the first week of culture and continued until they were transferred to the second medium after 50 days of culture. The calli were honey brown colour, loose, fragile and looked embryogenic (Plate 1).

Nevertheless, none of them produced any embryoids following their transfer to the second medium. Some cultures were left without transferring onto the second medium but the growth of those cultures ceased after about 3-4 months. By transferring the proliferating calli pieces into fresh callusing medium at 4-6 week intervals, the callus proliferation was maintained up to one year.

The results of the experiment with different combinations of kinetin and 2,4 -D are shown in Fig. 1 and Plate 1.

As evident from Fig.1 and Plate 1 no callus formation was observed in absence of kinetin. This shows the requirement of both auxin and cytokinin in the medium for the initiation of calli. Callusing was observed in rest of the media but on the medium containing kinetin at 1 mg/l and 2,4-D at 0.5 mg/l very good callus growth was observed.

When the calli were grouped according to their sizes, the calli masses on medium containing kinetin and 2,4-D at 1 and 0.5 mg/l respectively had a higher number of calli in the group of highest diameters of 6-7 mm. Nevertheless, the mean number of calli on that medium was less compared to media of 2,3 and 4. The reason for this was fusion of calli of 1-2 anthers on medium number 7 due to their fast growth.

After 50 days, calli were transferred onto the medium containing kinetin 1 mg/l, NAA 0.2 mg/l and GA_3 0.2 mg/l. However, no somatic embryos were formed on any of the calli. They turned brown and died after 5-6 weeks from transferring.

DISCUSSION

Micropropagation of *Hevea* by means of somatic embryogenesis has gained a considerable interest of research workers over the past one or two decades, due to the number of advantages it contained over the other tissue culture techniques. Among the various explants tested, only anthers and nucellus tissues have given encouraging results so far on somatic embryogenesis. The progress made on anther culture is mostly limited to Chinese Laboratories while that on nucellus tissue is limited to the Rubber Research Laboratories in Montpelier, France. Moreover, Chinese scientists have used their own clones for most successful reports on anther culture. None of the cultivars available in Sri Lanka showed any development beyond very good callus formation. Nevertheless, it is very unlikely that the embryogenic capacity of all the cultivars available in Sri Lanka is zero. Other factors may be governing embryogenesis, such as medium composition and subculture period, which are as much as or even more important than the clone or the cultivar.

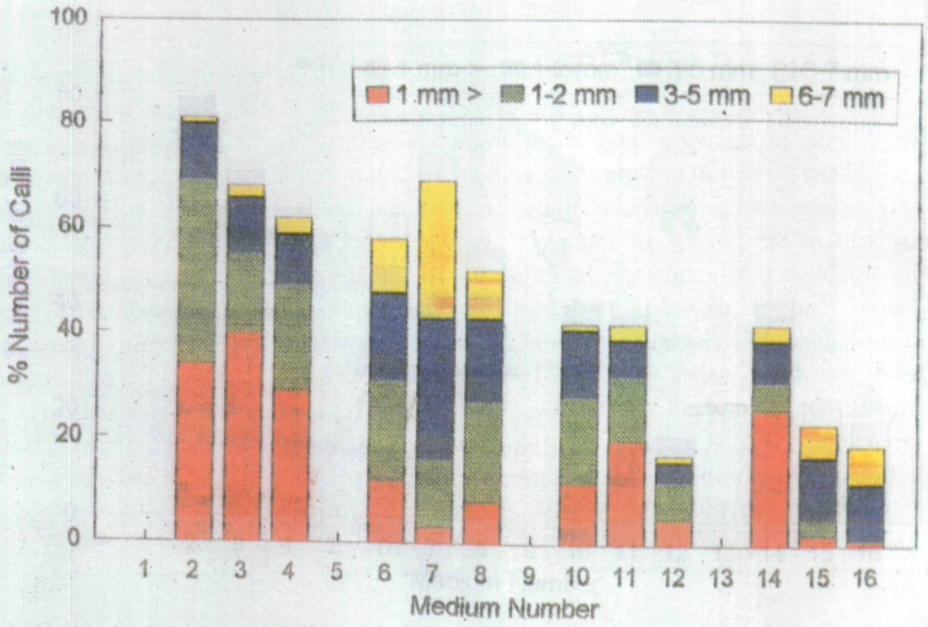

Fig.1. Callus formation on media 1-16 (calli masses are grouped according to the diameter).

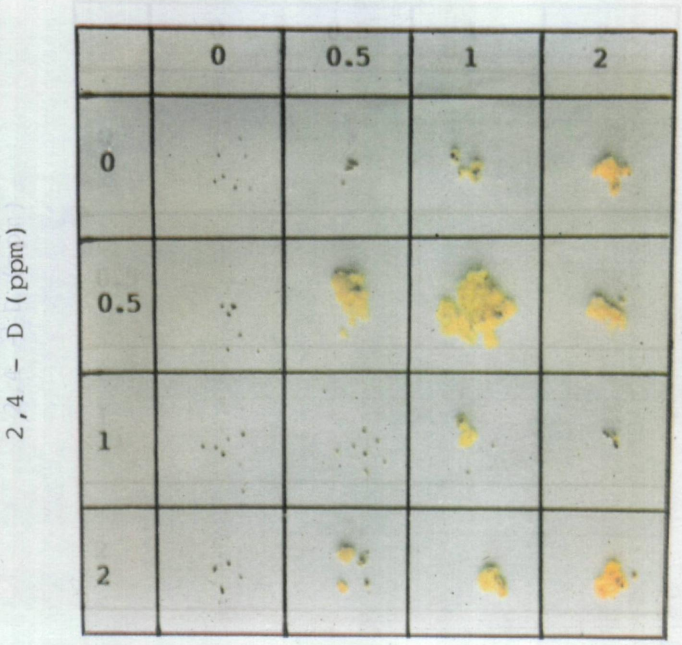

Plate 1. Callus formation on different combinations of kinetin & 2,4-D (Results at 50 days of culture)

Ever since the report of Satchuthananthavale (1972) on successfully transferable anther culture, thousands of anther cultures were done at the Rubber Research Institute of Sri Lanka, without producing a single plant or even an embryoid.

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 tissues is an important requirement in the process of somatic embryogenesis. Good proliferating calli could be readily established with a wide range of tissues of *Hevea* such as stems, roots, leaves, cotyledons, nucellus etc. Unlike the calli of other explants, the calli produced by mature cotyledons always developed roots if the calli were left without transferring to a fresh media for more than eight weeks (Plate 2). This was not observed with any other calli including anther calli. Obtaining calli from fragments of epicotyls, petioles, internodes and cotyledons has been reported by Carron et al (1984). These calli have produced roots after several subcultures. Induction of roots may be easy with most of the explants of *Hevea*.

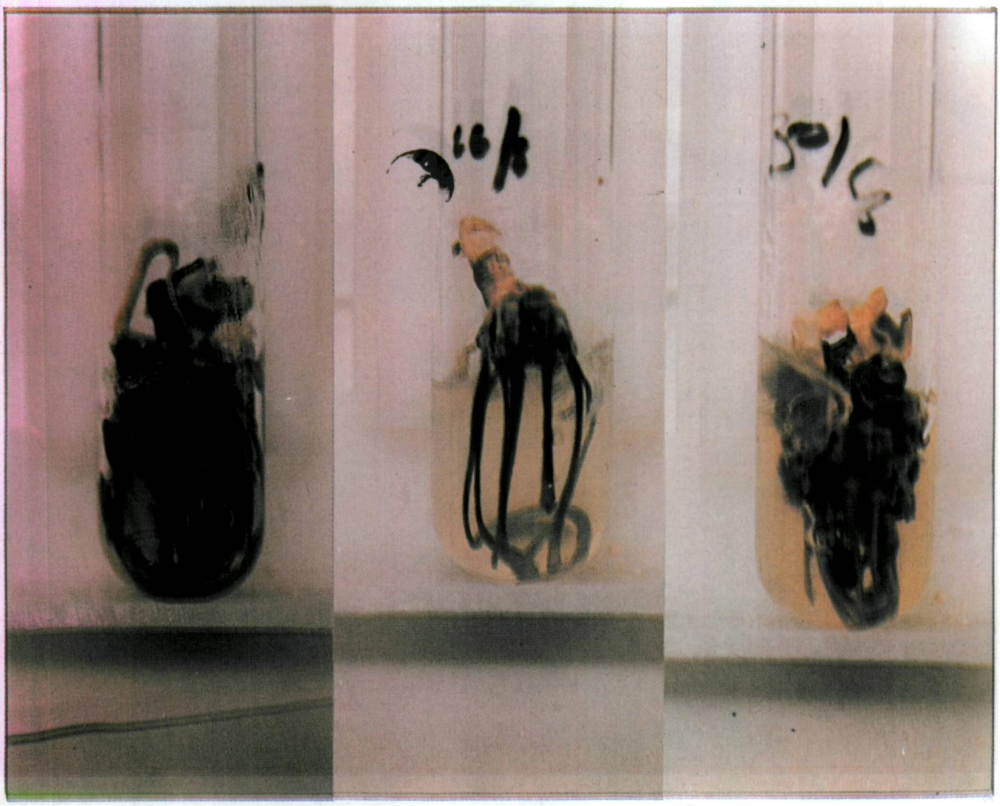

Plate 2. Root formation on calli derived from cotyledons (about three months old callus)

Plantlets were produced from anther derived calli much earlier than the production of plants from nucellus tissue. Nevertheless, since the production of the first few anther derived plants, in 1976, i.e., about 20 years ago, the progress toward implementing the technique to produce anther derived plants, seems slow. Had the technique developed was an easily adopted one, commercial scale plant production by anther culture should now be available at least in some rubber growing countries.

The advantages that can be achieved by adopting anther culture technique are discussed earlier. Therefore, every effort must be taken to adopt this technique of anther culture. At the Rubber Research Institute of Sri Lanka, experiments on anther culture were conducted, every year during the flowering season, since 1973, under the supervision of five or six Research Officers, without obtaining anything beyond very good embryogenic looking calli.

A report published in 1990 by the Scientists from the same laboratories in China where the first anther derived plantlets were produced, suggests that even after 13 years, the success rates at every stage are below commercial level application.

After the production of first five plants in 1977, another three plants have been produced in 1979. During the following 10 year period, they have produced 1700 plants by inoculating 31584 culture tubes. By 1988, pollen plants belong to 13 clones had been successfully transplanted and established. However, it had been reported of great variation among the clones for their induction frequency. Some clones such as Haiken 2 have given a high induction rates for both embryoid and plantlets. Yet, many clones have given very low induction rates for embryoid and plantlet formation, while on the same media, some clones have not responded at all. Apart from the high clonal variation, many critical factors are included in the process of anther culture. The large living pollen in the anthers to be inoculated seems a prerequisite for success. Flower buds 3 mm in length with green corollas have been found to be in this stage, i.e., anthers at uninuclear stages. Obviously the correlation between the size, and external appearance and the development stage of the pollens inside may vary from clone to clone.

The composition of the medium, specially the composition and concentration of nitrogen, seems important. For differentiation of somatic tissues of anther require a lower level of NH_4^+ that is unfavourable for development of pollen embryoids. This requires a higher concentration of total nitrogen. When the total nitrogen decreased to 30.3 mM, the percentage of callusing anthers has increased to 80%, while that of the embryos decreased to 0.8%. Higher frequency of callus & embryo induction has occurred with a higher concentration of KH_2PO_4 i.e., 3.75 mM. Further, both presence and concentration of kinetin have been reported to be very important. Moreover, incorporation of coconut water at 5-10% has increased the frequency of induction of pollen.

Addition of sucrose at 7-8% has been reported to be important for callus induction and embryoid formation. However, for plantlet forming medium, a reduced level, i.e., 4-6% has been better. Also, the transferring time has been reported as very critical for embryoid induction.

The medium composition and subculture period can be reproduced in any tissue culture laboratory. Nevertheless, those related to selection of flowers are difficult to be reproduced specially with different clones. Furthermore, detection of the causes responsible for the failure, in order to rectify, seems more difficult as so many critical factors are involved in the process.

The very low success rate at every stage, i.e., callusing embryoid formation, plant regeneration and transplanting makes the situation worse. Thousands of anthers need to be inoculated to get a few embryoids formed, and this makes factorial experiments very difficult.

As stated by Chen (1984) the success rate of anther culture, though very low, is partly dependent on the incorporation of coconut water into the medium. Incorporating ingredients such as coconut water prevents proper reproduction of experiments. Plant growth regulators such as kinetin seems to present in coconut water. The type, concentration etc. may vary with the maturity and the type of coconuts used.

After extensive and time-consuming research work carried out by the French workers since 1979, plantlets were produced from nucellus tissue via somatic embryogenesis (Michaux-ferriere & Carron, 1988). It was found that the embryogenic cells on callusing medium continued to develop, only if subculturing was carried out when they were in a specific physiological stage. The optimum period for subculturing obviously depends on the experimental conditions chosen, the type of explants and the clone. True bipolar embryos were obtained by accurate timing of sub cultures and by proper balance of hormones, minerals and sugar in the culture medium. Excess of auxin and cytokinin in the medium and the prolonged exposure to them has been found to suppress the embryogenic capacity of the calli (Hadrami *et al.*, 1991). Further, the relative water content and water potential of callus has been found to be good markers of the embryogenic state of the calli (Etienne et al, 1991). The decrease in 3,4-D and BAP supply in the culture medium and the addition of ABA have both stimulated the embryogenesis (Etienne *et al.*, 1993). Furthermore, the development stage of the seed from which the nucellus tissue was taken, seemed critical. They recommended the seeds after 45 to 75 days of anthesis, to harvest nucellus tissues.

They used four step procedure that takes minimum of three weeks for the first callusing medium, three to five weeks for the second callusing medium, and five to six months for the differentiation of embryoids which takes another 4 months for the multiplication of embryoids. This stage was successful with only one clone (RRIM 600) and the differentiation frequency was only 1%. The time required for the final stage of maturation of embryoids and plantlet formation is not yet certain, because the plantlet formation was a very rare occurrence. Carron *et al.* (1989) reported of obtaining only a few tens of plants out of thousands of embryos produced.

However, the difficulty in the use of somatic embryogenesis technique to *Hevea* is supported by the little progress made with anthers & nucellus tissue over a period of 10-15 years. The progress made on the technique of anther culture at the Rubber Research Institute of Sri Lanka is little and very discouraging as mentioned before. The objectives of the anther culture, mainly to induce variation, seem very challenging and difficult to achieve.

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