J. Rubb. Res., **8(2)**, 120–129

Histological Studies on in vitro *Somatic Embryogenesis from Anther Culture of* Hevea brasiliensis

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Somatic embryogenesis from in vitro *anther culture of rubber tree* (Hevea brasiliensis *Muell. Arg*.) *was analysed by using light microscopy. Two pathways of somatic embryogenesis were observed: internal origin, the embryogenesis initiated from internal embryogenic cells of callus mass, and surface origin from surface embryogenic cells. The somatic embryos with internal origin developed very similarly to the pattern formation of zygotic counterparts. They usually originated from single embryogenic cell and had a suspensor and seemed to be more competent for conversion into plantlets. The somatic embryos with surface origin usually initiated from a group of embryogenic cells. Their development had departed far from that of the zygotic embryos and rarely gave rise to plantlets. A high frequency of abnormal somatic embryogenesis occurred and this might account for the very low production of plantlets. An overwhelming majority of globular embryos failed to develop into torpedo and cotyledon embryos. The abnormalities in shoot apical meristem might be the main cause for the low conversion rate of the torpedo and cotyledon embryos into plantlets.*

Key words: somatic embryogenesis; abnormal somatic embryo; embryogenic cell; suspensor; tissue culture; *Hevea brasiliensis*; *in vitro*; anther culture; microscopy

Somatic embryogenesis of rubber tree (*Hevea brasiliensis*) has been successful through *in vitro* culture in the 1970s[1,2]. Since then, consistent efforts have been made to test various explants and to improve the culture conditions[3–8].

The technique of somatic embryogenesis is developed for two practical purposes: genetic engineering and mass vegetative propagation of rubber tree. By using the technique, transgenic rubber plants are obtained; they express in their latex two valuable recombinant proteins, human serum albumin and antibody against the coat protein of the bacterium *Streptococcus gordonii*, in early dental plaque[9,10]. On the other hand, maintained embryogenic callus lines are created for a few rubber clones, so that *in vitro* plantlets from somatic embryos of the clones can be obtained in large quantities in a reproducible way[11].

Although it has had some degree of success, the technique of *in vitro* somatic embryogenesis for rubber tree is far from perfect. In order to improve the technique,

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a deeper understanding of the histology of somatic embryo development is needed[2,12]. In this study, the somatic embryogenesis and plantlet regeneration from *in vitro* anther culture of rubber tree were analysed by using light microscopy. Two developmental pathways of the somatic embryos were observed and the various malformations of the *in vitro* cultures were described.

MATERIALS AND METHODS

Plant Material

The anthers of *H. brasiliensis* clone Haiken 2 were used as explants for inducing the somatic embryos. Anthers were collected as explants from green-coloured male flowers of 2.5 mm – 3 mm long.

Culture Media

Callus induction. The anthers were inoculated on de-differentiation medium based on MS medium[13] supplemented with 2 mg/L kinetin, 1 mg/L N-6-benzyl adenine (BA), 1 mg/L 2,4-dichlorophenoxy-acetic acid, 2 mg/L α-naphthalene acetic acid (NAA), 5% coconut water, and 10% sucrose.

Embryo induction. After 40 – 50 days of inoculation, the anthers with calli were transferred onto the differentiation medium, MS medium supplemented with 1.5 mg/L kinetin, 2 mg/L BA, 0.2 mg/L NAA, 0.5 mg/L gibberellin (GA3), and 7%–8% sucrose. On this medium the calli were differentiated into embryos at different development phases.

Embryo germination and plantlet regeneration. The embryos were transferred onto MS medium supplemented with 2 mg/L GA3, 0.5 mg/L indole acetic acid, 1 mg/L 5-bromo-uracil and 5% sucrose.

All media were solidified with 0.7%–0.8% agar and the pH was adjusted to 5.8.

Light Microscopy

Samples were collected at intervals of 7 days before the formation of torpedo embryo. Later the malformed cultures were sampled at irregular intervals. The samples were fixed in FAA (formalin 5 mL; glacial acetic acid 5 mL; 50% ethanol 90 mL). They were directionally embedded first in 0.8% agar and then post-fixed in FAA. Paraffin sections (8 μm – 10 μm thick) were prepared after ethanol/n-butanol dehydration and stained with haematoxylin method.

RESULTS

Callus Formation

One week after inoculation, the anthers became swollen and they were bright in their periphery. Callus was observed that formed from the endothecium and the residual filament of anthers (*Figure 1*). The initial callus composed of meristematic cells that were small and isodiametric with dense cytoplasm and big nucleus (*Figure 1*). Three to four weeks after inoculation, the anthers appeared yellowish and they increased 5-10 times in size due to considerable multiplication of the callus cells. The callus cells with the capacity to divide differentiated into parenchyma cells while they could continued to be meristematic at the surface area of the callus mass (*Figure 2*).

Embryo Development from Internal Embryogenic Cell

Embryogenic cells could be defined as the cells from which the somatic embryos initiated. The embryogenic cells occurred about 5 weeks after anthers were inoculated. They initiated from two origins: the internal cells and the surface cells of the differentiated callus masses (*Figure 3*). The internal embryogenic cells were present as separated single cell in the outer part of the differentiated callus masses. The single embryogenic cell had a thin wall, dense cytoplasm and a larger nucleus and was surrounded by parenchyma cells that often contained tannin-like substance (*Figures 3* and *4*). The embryogenic cell first divided into two cells (*Figure 4*): one cell located near the surface of the callus mass formed embryo and the other formed the suspensor. By several cell divisions, the former cell gave rise to multi-cellular proembryo (*Figures 5–7*) and further developed into globular embryo (*Figures 8* and *9*). The globular embryo had a surface cell layer with anticlinal cell division and internal cells with division in all directions. A large number of globular embryos could be observed about 10 days after the cultures were transferred onto the culture medium 2.

The globular embryo proceeded through the later development stages successively to form heart embryo, torpedo embryo and cotyledon embryo (*Figures 10–12*). At the stage of torpedo embryo, histo-differentiation became evident and a U-shaped procambium system was observed that consisted of densely stained, narrow, elongated cells (*Figure 11*).

The suspensor was a single line of cells at first (*Figures 6* and *7*) and became many lines of cells at the stage of globular embryo (*Figures 8* and *9*). The suspensor remained in the stage of torpedo and heart embryo (*Figures 10* and *11*) and even in the cotyledon embryo.

Embryo Development from Surface Embryogenic Cells

The surface cells of the differentiated callus mass could produce embryogenic cells (*Figure 13*). The embryos often developed from many embryogenic cells (*Figure 14*) but initiated from a single embryogenic cell occasionally. The embryos with multi-cellular origin usually could not give rise to normal morphogenesis and usually had no suspensor (*Figures 14–17*).

In the cultures, the embryo development was highly non-synchronic. Embryos of different developmental stages could be found in a culture.

Abnormal Somatic Embryogenesis

The efficacy of *in vitro* somatic plantlet formation was poor. This might be due to the developmental obstruction of the embryos at different stages. As shown in *Table 1*, a large number of globular embryos were produced but very few of them developed into torpedo embryos. Among the developed cotyledon embryos a few were normal (*Table 2*) and even fewer could finally give rise to plantlets.

Types of Abnormal Embryos Observed

Abnormal globular and heart embryos. The globular embryos had a part or all of their cells turned into mature parenchyma cells (*Figure 16* and *17*). The heart embryos had only one cotyledon primodium (*Figure 17*). The abnormal globular and heart embryos might mostly be the embryos with surface origin.

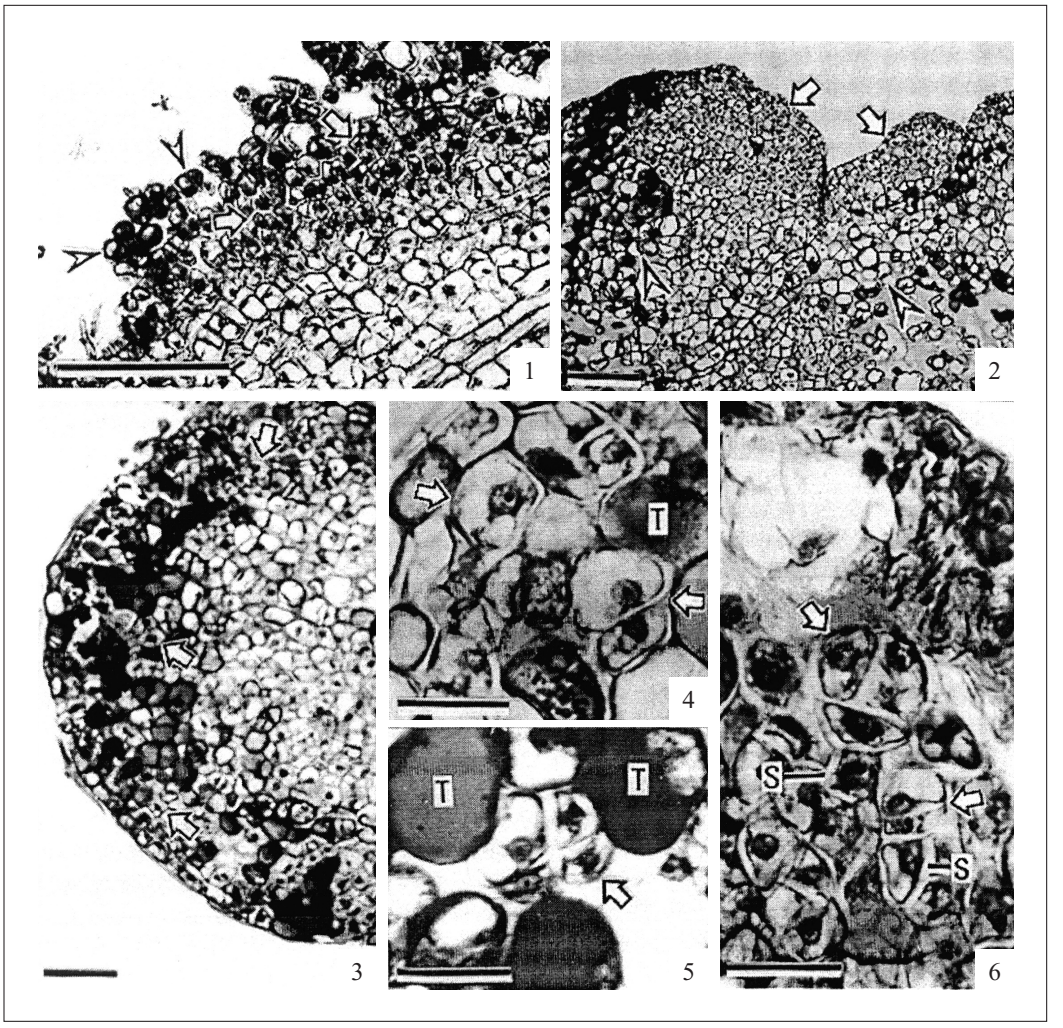


Figure 1. Callus formed from endothecium. Note meristematic callus cells (arrows) and pollen (arrow-heads).

Figure 2. Callus cells with considerable multiplication. Arrows: Meristematic callus cells; arrow-heads: Differentiated callus cells.

Figures 3–6. Somatic embryogenesis from internal embryogenic cell.

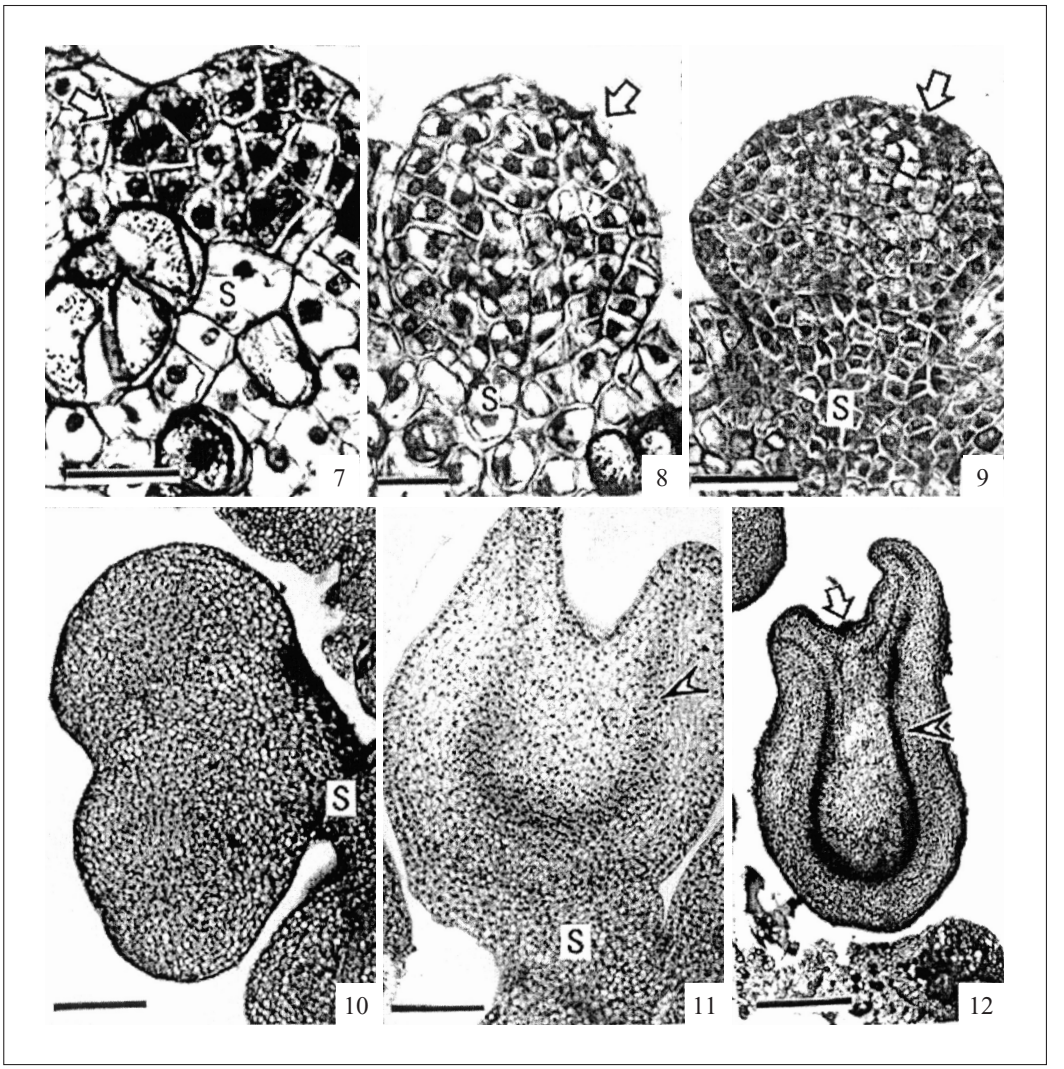
Figure 3. Differentiated callus cell mass. Embryogenic cells (arrows) occur among tannin-containing cells (black).

Figure 4. Embryogenic cells (arrows). One of them divided into two cells (right). T: Tannin-containing cell.

Figure 5. Multicellular proembryo (arrow). T: Tannin-containing cell.

Figure 6. Multicellular (left) and two-cellular (right) proembryos (arrow). S: Suspensor.

Bars in Figures 1–3 = 50 μm; Figures 4–6 = 20 μm.



Figures 7–12. Somatic embryogenesis from internal embryogenic cell; S= suspensor:

Figure 7. Multicellular proembryo (arrow).

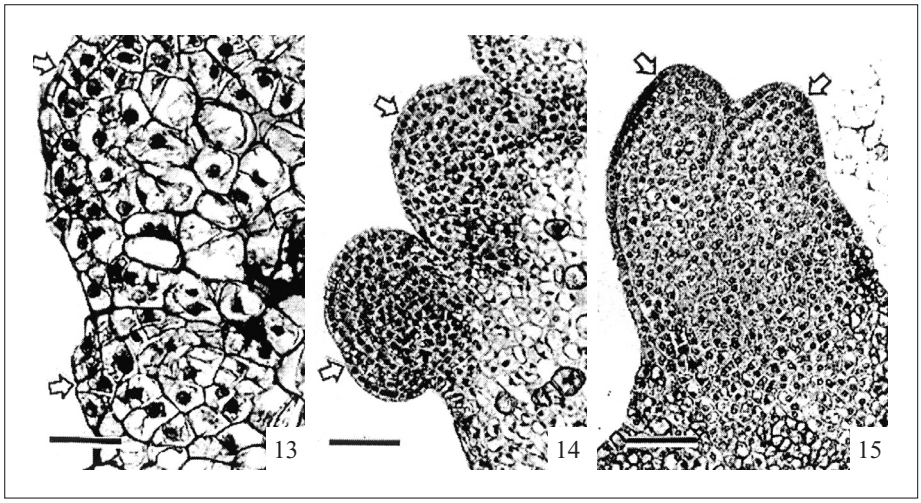
Figures 8 and 9. Globular embryo (arrow).

Figure 10. Heart embryo.

Figure 11. Torpedo embryo with U-shaped procambium (arrow-head).

Figure 12. Cotyledon embryo with shoot apical meristem (arrow) and procambium (arrow-head).

Bars in Figure 7 = 25 μm; Figures 8 and 9 = 50 μm; Figures 10–11 = 100 μm; Figure 12 = 120 μm.



Figures 13–15. Somatic embryogenesis from surface embryogenic cells.

Figure 13. Embryogenic cells (arrows) at the surface of the callus cell mass.

Figure 14. Globular embryos (arrows) initiated from many embryogenic cells.

Figure 15. Heart embryo; Arrows: Cotyledon primodia.

Bars in Figure 13 = 25 μm; Figures 14 and 15 = 50 μm.

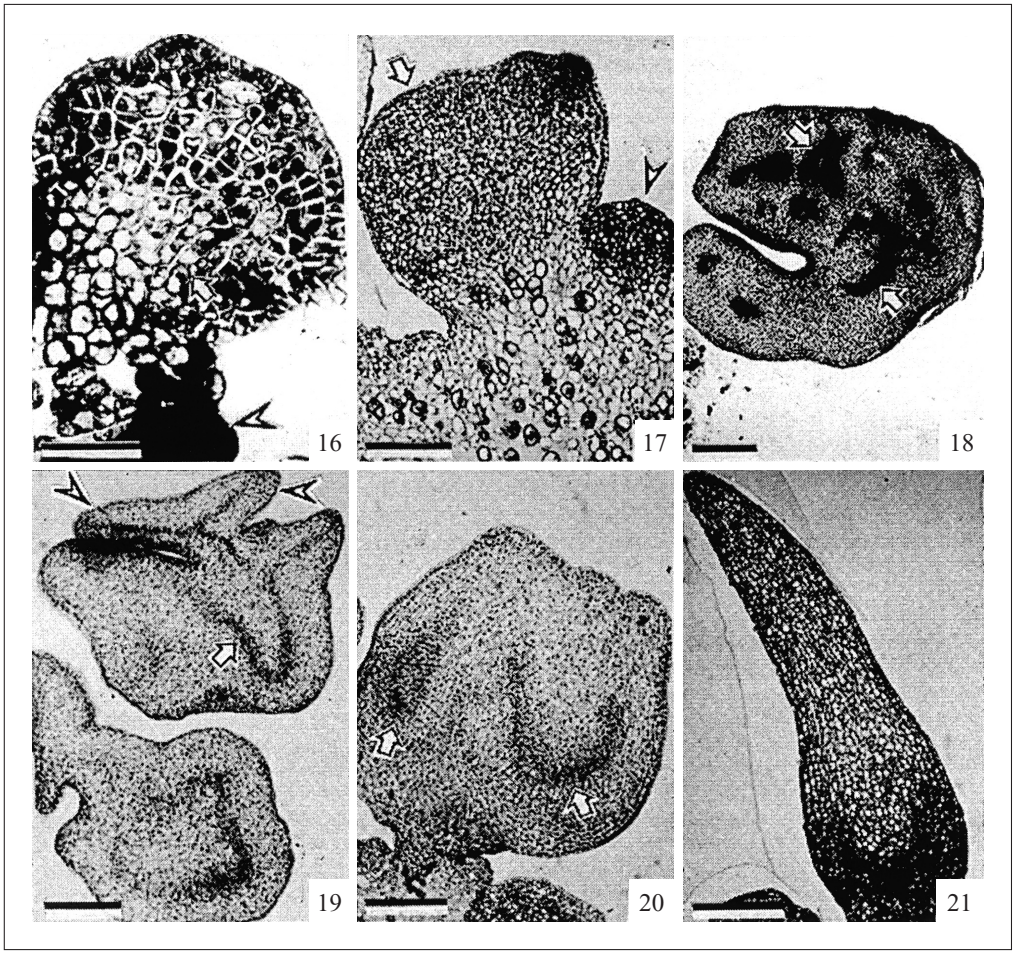
Abnormal torpedo and cotyledon embryos. The abnormal cotyledon embryos have been shown in *Table 2*. The embryos could have cup-like, trumpet-like, leaf-like or gobbet-like shoot pole. The embryos with gobbet-like shoot pole had disrupted procambium system (*Figures 18* and *19*). Two or more embryos could occur as a fused mass. *Figure 20* shows a cup-like embryo where two cotyledons were fused. Some embryos had a finger-like shoot pole without apical meristem and procambium (*Figure 21*).

Abnormal plantlets. The plantlets had abnormal shoot poles, which developed from the abnormal cotyledon embryos described above (*Table 2*). Some plantlets lacked shoot apical meristem or had abnormal shoot apical meristem (*Figures 22* and *23*). The shoot apex of the embryos could turn into leaf-like structure (*Figure 24*). If the apical meristem was normal, the plantlets could develop into normal plants even among the plantlets with abnormal external morphology. Root was absent in some embryos.

DISCUSSION

Two Pathways of Somatic Embryogenesis from Rubber Tree Anther Culture

Heterogeneity in somatic embryogenesis was reported in many species[14,15]. In this study it was found that at least there were two developmental pathways that the somatic



Figures 16–21. Abnormal embryos.

Figures 16 and 17: Abnormal embryos with surface origin.

Figure 16. Globular embryo with a part of its cells turned into parenchyma cells (arrow).

Figure 17. A heart embryo (arrow) with single cotyledon primodium and a globular embryo turned into parenchyma cell mass (arrow-head).

Figure 18–19. Gobbet embryos without apical meristem. Arrows: abnormal procambium. Arrow heads: Abnormal cotyledon primodia.

Figure 20. Cup-like embryo with fused cotyledons. Arrows: Procambium.

Figure 21. Embryo having a finger-like shoot pole without apical meristem and procambium.

Bars in Figure 16 = 50 μm; Figures 17–21 = 100 μm.

TABLE 1. SOMATIC EMBRYO DEVELOPMENT: FROM GLOBULAR EMBRYO INTO TORPEDO EMBRYO[a]

Number of globular embryo per culture 10 d after transfer on culture medium 2	Number of torpedo embryo per culture 20 d after transfer on culture medium 2
20.6±10.3	1.3±1.0

[a]Fifteen cultures of *Hevea* clone Haiken 2 were observed.

TABLE 2. NUMBER OF NORMAL AND ABNORMAL COTYLEDON EMBRYOS ONE MONTH AFTER TRANSFER ON CULTURE MEDIUM 3[a]

Embryos observed	Normal embryos	Embryos with abnormal shoot pole			
		Cup-like	Trumpet-like	Leaf-like	Gobbet-like
30(100%)	9(30%)	3(10%)	2(6.7%)	9(30%)	7(23.3%)

[a]*Hevea* clone Haiken 2

embryogenesis initiated from rubber tree anther culture: internal origin, namely the embryogenesis initiated from internal embryogenic cells of callus mass, and surface origin, which embryos developed from surface embryogenic cells.

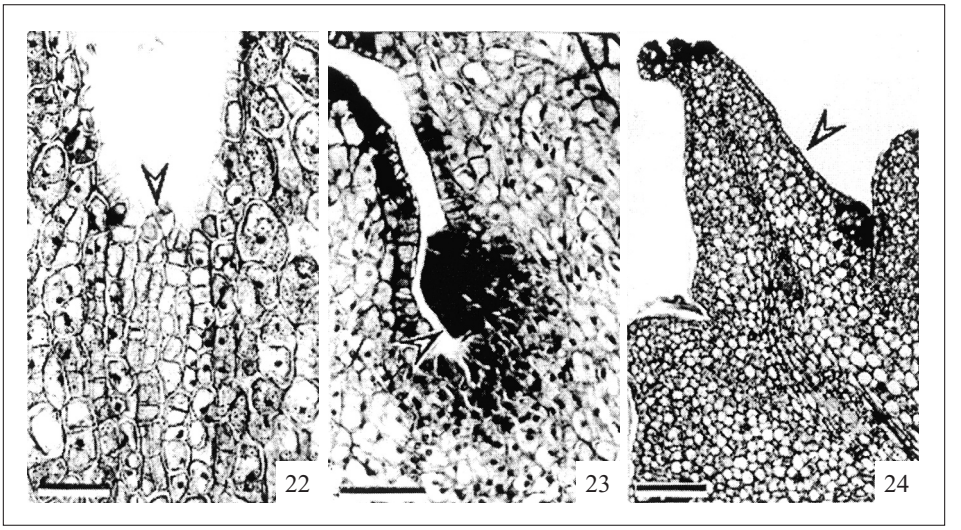
The pattern formation of somatic embryos with internal origin was very similar to that of their zygotic counterparts[16]. They were initiated usually from the single embryogenic cells. A suspensor was present in the somatic embryos as in the zygotic ones although the suspensor was absent in somatic embryos from most species cultures[14,15]. The somatic embryos with internal origin seemed to be more competent for conversion into plantlets.

The somatic embryogenesis with surface origin was usually initiated from a group of embryogenic cells. Their developmental pattern was departed far from that of the zygotic embryos and rarely gave rise to normal dicotyledon embryos and plantlets.

Same somatic embryos were occasionally found initiated from single cells within the surface embryogenic cells of callus mass. These embryos developed in the same manner as the embryos with internal origin and might have a higher conversion rate into normal plantlets. In this regard, the embryogenesis initiated from single cell might be more highly competent for conversion into plantlet regardless of internal or surface origin.

Variation in External Morphology and Internal Anatomy of Somatic Embryos was Related to Competence to Form Plantlets

We observed in this study that occurrence of abnormal somatic embryos was highly frequent in rubber tree anther culture. This may account for the very low production of plantlets from these cultures. An overwhelming majority of the globular embryos failed to develop into torpedo embryos (*Table 1*). The formation of torpedo embryos might be a critical stage of the somatic embryogenesis for rubber tree. At



Figures 22–24: Abnormal plantlets.

Figure 22. Shoot apex (arrow-head) without apical meristem.

Figure 23. Shoot apex with abnormal apical meristem (arrow-head).

Figure 24. Shoot apex turned into leaf-like structure (arrow-head).

Bars in Figures 22–23 = 50 μm.

this stage, the sites of the cotyledons, the root axis and other morphological and histological features could be identified. However, among the formed torpedo and cotyledon embryos, a large number were abnormal and failed to convert into plantlets (*Table 2*, *Figures 18–21*). In this connection, the abnormalities in the shoot apical meristem might be the main cause for low conversion rate. The embryos with abnormal shoot apical meristem never gave rise to plantlets while normal plantlets could be produced from cotyledon embryos with abnormal cotyledon but normal shoot apical meristem.

In order to efficiently regulate the formation of plants *via* somatic embryogenesis, it is important to understand how the somatic embryos developed. By using histological approach in this paper, this study provided the primary knowledge of somatic embryogenesis from rubber tree anther culture which would give the basis for improved regeneration efficiency.

Date of receipt: January 2005
Date of acceptance: June 2005

REFERENCES

1. PARANJOTHY, K. AND GHANDIMATHI, H. (1975) Tissue and Organ Culture of *Hevea*. *Proc. Int. Rubber Conf., Kuala Lumpur*, 59–84.

2. CHEN, Z., CHEN, F., CHIEN, C., WANG, C., HSU, H., OU, H., HO, Y. AND LU, T. (1978) Induction of Pollen Plant of *Hevea brasiliensis* Muell-Arg. *Acta Genet. Sin*., **5**, 99–107.

3. CARRON, M.-P., ENJALRIC, F., LARDET, L. and deschamps, a. (1989) Rubber (Hevea brasiliensis Müll. Arg.), *Biotechnology in Agriculture and Foretsry, Vol. 5, Trees.* (*Bajaj, Y.P.S. ed*.), pp. 222–245. Berlin : Springer-Verlag. Springer-Verlag.

4. MONTORO, P., ETIENNE, H., MICHAUX-FERRIEREY N. AND CARRON M.P. (1993) Callus Friability and Somatic Embryogenesis in *Hevea brasiliensis*. *Plant Cell Tissue Organ. Cult*., **33**, 331–338.

5. CAILLOUX, F., JULIEN-GUERRIER, J., LINOSSIER, L. AND COUDRET A. (1996) Long-term Somatic Embryogenesis and Maturation of Somatic Embryos in *Hevea brasiliensis*. *Plant Sci*., **120**, 185–196.

6. LINOSSIER, L., VEISSEIRE, P., CAILLOUX, F. AND COUDRET, A. (1997) Effects of Abscisic Acid and High Concentrations of PEG on *Hevea brasiliensis* Somatic Embryos Development. *Plant Sci*., **124**, 183–191.

7. SUSHAMAKUMARI, S., SOBHA, S., REKHA, K., R. JAYASREE, R. AND ASOKAN, M.P. (2000) Influence of Growth Regulators and Sucrose on Somatic Embryogenesis and Plant Regeneration from Immature Inflorescence of *Hevea brasiliensis*. *Indian J. nat. Rubb. Res*., **13**, 19–29.

8. 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 Protoplasts of Rubber. *Plant Cell Tissue and Organ Culture*, **61**, 81–85.

9. AROKIARAJ, P., RÜKER, F., OBERMAYR, E., SHAMSUL BAHRI, A.R., HAFSAH, J., CARTER, D.C. AND YEANG, H.Y. (2002) Expression of Human Serum Albumin in Transgenic *Hevea brasiliensis*. *J. Rubb. Res*., **5**, 157–166.

10. YEANG, H.Y., AROKIARAJ, P., HAFSAH JAAFAR, ARIJA, S.A.M., RAJAMANIKAM, S., CHAN, J.L., JAFRI SHARIB, LEELA-VATHY, R., SAMSIDAR HAMZAH AND VAN DER LOGT, C.P.E. (2002) Expression of a Functional Recombinant Antibody Fragment in the Latex of Transgenic *Hevea brasiliensis*. *J. Rubb. Res*., **5(4)**, 215–225.

11. CARRON, M.P., LARDET L. AND DEA, B.G., (1998) *Hevea* Micropropagation by Somatic Embryogenesis. *Plant. Rech. Dév*., **5**, 187–192.

12. WANG, Z., WU, H., ZENG, X., CHEN, C. AND LI, Q. (1984) Embryogeny and Origin of Anther Plantlet of *Hevea brasiliensis*. *Chinese J. Trop. Crops*, **5(1)**, 9–13.

13. MURASHIGE, T. SKOOG (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiol. Plant*., **15**, 473–497.

14. DODEMAN, V.L., DUCREUX, G. AND KREIS, M. (1997) Zygotic Embryogenesis *Versus* Somatic Embryogenesis. *J. Exp. Bot*., **48**, 1493–1509.

15. VON ARNOLD, S., SABALA, I., BOZHKOV, P., DYACHOK, J. AND FILONOVA, L. (2002) Developmental Pathways of Somatic Embryogenesis. *Plant Cell Tissue and Organ Culture*, **69**, 233–249.

16. XIE, S., QIU, D. AND ZHEG, J. (1981) Studies on the Development of Embryo and The Formation of Endosperm in *Hevea brasiliensis*. *Chinese J. Trop. Crops*, **2(2)**, 1–6.