Natural Rubber Research, **25**(1): 86-90, 2012

HISTOCHEMICAL CHANGES IN EMBRYOGENIC AND NON-EMBRYOGENIC CALLI OF *HEVEA BRASILIENSIS*

P. Kumari Jayasree, C.P. Reghu, R.G. Kala and A. Thulaseedharan

Rubber Research Institute of India, Kottayam-686 009, Kerala, India

Received : 22 February 2012 Accepted : 14 May 2012

Jayasree, P.K., Reghu, C.P., Kala, R.G. and Thulaseedharan. A. (2012) Histochemical changes in embryogenic and non-embryogenic calli of *Hevea brasiliensis*. *Natural Rubber Research*, **25** (1): 86-90.

It is well established that the accumulation of storage products is a reliable marker for the classification of embryogenic cells. The present study characterizes embryogenic and non-embryogenic calli of *Hevea brasiliensis* through histochemical localization of storage reserves. Inoculation of immature anthers on callus induction medium induced type I (soft and watery) and type II (semifriable / compact) callus. Observations showed that embryogenic callus consists of small cells with prominent nuclei, while non embryogenic calli were characterized by large cells having prominent nuclei. Histochemical examination revealed the accumulation of significant amount of storage starch, lipids and proteins which were dispersed throughout the cells of embryogenic calli, particularly, at later stage than in early phase, whereas low level accumulation of major storage reserves was detected in non-embryogenic calli.

Keywords: Embryogenic callus, *Hevea brasiliensis*, Histochemical characterization, Non- embryogenic callus, Storage reserves

INTRODUCTION

Rubber tree (*Hevea brasiliensis*) is the widely cultivated species as a commercial source of natural rubber. Over the past years, tremendous advancement has been made in latex yield through conventional breeding and by introducing several modern clones with high yield potential. However, many desirable secondary traits are limited due to the narrow genetic base. Genetic transformation provides a viable alternative approach for genetic improvement in this perennial tree species. However, an essential pre-requisite to the success of this approach is the availability of a suitable target tissue for genetic transformation whereby plants can be regenerated from single transformed cells through somatic embryogenesis. Embryogenic callus is generally considered to be a highly desirable target tissue for genetic transformation because of high population of totipotent cells for single cell origin of somatic embryos (Merkle *et al*., 1995).

Induction of embryogenic callus is the first and crucial step during somatic embryogenesis, which largely determines the success of embryogenesis. In most of the hard wood species, embryogenic callus formation is very difficult and *Hevea* is not an exception (Blanc *et al*., 1999). Screening

Correspondence: P. Kumari Jayasree (Email: kumarijaya@rubberboard.org.in)

of embryogenic cultures thus plays an important role, especially when the cultures are derived from numerous cell lines. An early identification of embryogenic potency would therefore be of great importance. Some of the early attempts to find indicators for embryogenic callus cultures relied on biochemical markers. Proteins (Sung and Okimoto, 1981), isozymes (Alves *et al.*, 1994; Asokan *et al*., 2001) and ethylene production (Wann *et al*., 1987) are some of biochemical variables which have been shown to discriminate between embryogenic and non embryogenic tissues. However, studying these biochemical systems are time consuming. Alternatively, histochemical marker is a simple and rapid approach. In several crops, histochemical localization of storage reserves were examined and reported that storage product accumulation may be a reliable marker for the classification of embryogenic cells (Brisibe *et al*., 1993; Sane *et al*., 2006). However, in *Hevea,* particularly Indian rubber clones, so far no information is available on the histochemical characterization of embryogenic potency. The present study therefore, reports the histochemical changes of storage reserves accumulation associated with embryogenic and non -embryogenic callus during somatic embryogenesis in *Hevea*.

MATERIALS AND METHODS

Immature anthers were inoculated on callus induction medium containing 2,4-D and kinetin as described by Jayasree *et al*. (1999). Different types of calli were induced and upon further subculture to embryo induction medium, embryogenic and non-embryogenic calli were obtained and were selected by visual observations. On microscopic observations, the embryogenic callus at early phase was found to be slightly mucilaginous in appearance which increased at the late phase. Two weeks before the initiation of histochemical investigations, both embryogenic and non-embryogenic callus cultures were subcultured onto a fresh medium and actively dividing calli were selected. Embryogenic callus at early and late phase and non embryogenic callus at the later phase were subjected to histochemical investigations. Samples were stained overnight in iodine potassium iodide (Johansen, 1940) for the localization of starch; sudan black B (Ruzin, 1999) and oil red O (Omman and Reghu, 2003) for lipids and mercuric bromophenol blue for storage proteins (Mazia *et al*., 1953). Samples were also stained with 0.1% toludine blue O for nuclei characterization. For all samples, three replications were made. The stained samples were washed with distilled water and pressed for uniform smearing on micro slides and mounted in glycerol. Observations were taken in Leica Q Win V.2.1 image analysis software attached to Aristoplan research microscope.

RESULTS AND DISCUSSION

It has been reported earlier that the accumulation of storage products may be noted as a reliable marker for the identification of embryogenic cells in many crops (Brisibe *et al*., 1993; Sane *et al*., 2006). In the present study, morphologically distinct callus types such as type I (soft, watery and friable callus) and type II (pale yellow and compact or semifriable callus) were formed after 40-50 days of culture of immature anthers on callus induction medium. After two months of subculture to embryo induction medium, yellowish friable or compact and mucilaginous callus was formed from type II, while creamy,

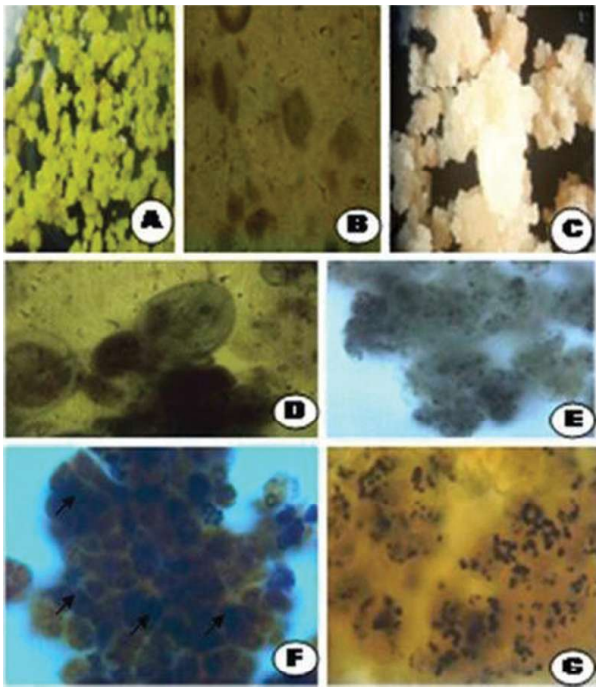

Fig. 1. Callus types and histochemical observations (X 200)
- A - Morphology of embryogenic callus
- B - Embryogenic callus stained with toludine blue showing small cells with prominent nuclei
- C - Morphology of nonembryogenic callus
- D - Non-embryogenic callus with large cells containing prominent nuclei
- E - Early stage embryogenic callus showing starch distribution
- F - Late stage embryogenic callus showing starch distribution (arrow indicates starch)
- G - Late stage non-embryogenic callus showing starch distribution

white friable callus was induced from type I. Upon two more subculture with 30 days interval, yellow highly friable compact embryogenic callus from type II and yellow friable, fast growing non-embryogenic callus from type I was produced. Salient histo-morphological variations were observed between embryogenic and non embryogenic calli. Embryogenic calli (Fig. 1A) consisted of small cells with prominent nuclei (Fig. 1B), in contrast to the non- embryogenic callus (Fig. 1C) with large cells possessing prominent nuclei (Fig. 1D). Electron microscopic and light microscopic investigations in various plant species revealed that the embryogenic cells that form somatic embryos are characterized generally as small, isodiametric in shape, having large and densely staining nuclei with dense cytoplasm as in pearl millet and cork oak (Namasivayam, 2007). Histochemical observations revealed large number of starch grains throughout the cells of embryogenic calli in *Hevea*. In early stage embryogenic cells, few starch grains with small size were randomly distributed (Fig. 1E). However, in later phase, almost all cells were densely accumulated with starch granules (Fig. 1F). In contrast, non embryogenic calli were poorly filled with starch grains (Fig. 1G). In *Aspidosperma polyneuron,* Verdeil *et al*. (2001) and Sane *et al*. (2006) reported starch as an indicator of somatic embryo development. According to Barciela and Vieitez (1993), starch may have provided energy for the onset of division and formation of embryogenic cells and embryos. However, starch accumulation cannot be considered as a histochemical marker for embryogenic process as large deposit of starch was also seen in non embryogenic callus (Barciela and Vieitez, 1993).

Figure 2 (A-G) shows the distribution of lipids and proteins in different callus types and stages. When stained with sudan black, lipid bodies appeared as brown droplets and as orange droplets with oil red dye. In the early stage, the embryogenic cells showed small granules of lipids with feeble stainability (Fig. 2A). However, at late stage, the lipid bodies increased both in number and size (Fig. 2B & C). In non- embryogenic calli, at late phase, the lipid content was very less in number or quantity (Fig. 2D). This is

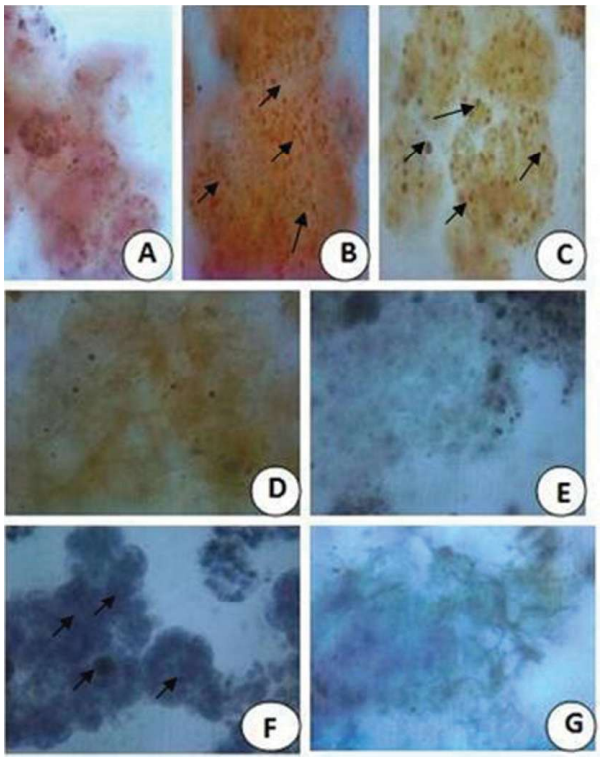

Fig. 2. Distribution of lipids and proteins in different callus types and stages

A - Early stage embryogenic callus showing lipid distribution (stained with oil red)

B - Late stage embryogenic callus showing lipid distribution (stained with oil red)

C - Late stage embryogenic callus showing lipid distribution stained with sudan black (arrow indicates lipids)

D - Late stage non- embryogenic callus showing lipid distribution stained with sudan black

E - Early stage embryogenic callus showing protein distribution

F - Late stage embryogenic callus showing protein distribution (arrow indicates proteins)

G - Late stage non-embryogenic callus showing protein distribution

in agreement with the observations reported earlier in oil palm by Schwendiman *et al*. (1988), where the accumulation of lipid reserves could be a good indicator of the acquisition of the embryogenic potential.

Storage protein was stained bluish colouration with mercuric bromophenol blue during formation of embryogenic callus. During early phase, the cells were faintly stained (Fig. 2E) whereas in later stage, protein bodies were localized abundantly (Fig. 2F). This might be a reflection of their high synthetic metabolism prior to embryo development. However, compared to embryogenic calli, less accumulation of protein was observed in non- embryogenic calli (Fig. 2G). In sugarcane, high level of protein accumulation has been reported earlier (Brisibe *et al*., 1993) which can be considered a reliable marker for classification of embryogenic cells. Similarly, Sane *et al*. (2006) reported high content of protein accumulation associated with embryogenesis in date palm. The low level accumulation of storage reserves in non-embryogenic calli may be related to the less metabolic activity of cells that hinders embryo development.

CONCLUSION

The present histochemical study revealed the accumulation of considerable amount of storage starch, lipids and proteins in embryogenic calli at late stage than in early phase in contrast to non-embryogenic calli. Hence the accumulation of storage reserves may be used as a histochemical marker for identification of embryogenic cells from non-embryogenic cells in the calli of *Hevea*. Early selection of embryogenic callus could also avoid the frequent subculture of non-embryogenic callus at each passage, thereby, enhancing the regeneration potential of elite clones through somatic embryogenesis and genetic improvement *via* transgenic approaches.

REFERENCES

Alves, J.M.C., Sihachakr, D., Allot, M., Tizroutine, S., Mussio, I., Servaes, A. and Ducreux, G. (1994). Isozyme modifications and plant regeneration through somatic embryogenesis in sweet potato (*Ipomoea batatas* (L.) Lam.). *Plant Cell Reports*, **13**: 437-441.

Asokan, M.P., Narasimhan, K., Jayasree, P.K., Thulaseedharan, A., Sushamakumari, S., Kala, R.G. and Jayashree, R. (2001). Isozyme markers for distinguishing embryogenic and non embryogenic callus during somatic embryogenesis in *Hevea brasiliensis* (Muell.) Arg. *Journal of Tree Sciences*, 20(1&2): 78-85.

Barciela, J. and Vieitez, A.M. (1993). Anatomical sequence and morphometric analysis during somatic embryogenesis on cultured cotyledon explants of *Camellia japonica* L. *Annals of Botany*, **71**: 395-404.

Blanc, G., Michaux-Ferriere, N., Teisson, C., Lardet, L. and Carron, M.P. (1999). Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. *Plant Cell Tissue Organ Culture*, **59**: 103-112.

Brisibe, E. A., Nishioka, D., Miyake, H., Taniguchi,T. and Maeda, E. (1993). Developmental electron microscopy and histochemistry of somatic embryo differentiation in sugarcane. *Plant Science*, **89**: 85-92.

Jayasree, P.K., Asokan, M.P., Sobha, S., Sankariammal, 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.). *Current Science*, **76 (9)**: 1242-1245.

Johansen, D.A. (1940). *Plant Microtechnique*. Mc-Graw Hill, New York

Mazia, D., Brewer, P.A. and Alfert, M. (1953). The cytochemical staining and measurement of protein with mercuric bromophenol blue. *Biological Bulletin*, **104**: 57-67.

Merkle, K.A., Parrott, W.A. and Flinn, B.S. (1995). Morphogenic aspects of somatic embryogenesis. In: *In Vitro* Embryogenesis in Plants. (Ed. T.A. Thorpe). Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 155-203.

Namasivayam, P. (2007). Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell Tissue Organ Culture*, **90**: 1-8.

Oomman, P. and Reghu, C.P. (2003). Staining procedure for lacticiferous system of *Hevea brasiliensis* using oil red O. *Indian Journal of Natural Rubber Research*, **16 (1&2)**: 41-44.

Ribas, L.L.F., Guerra, M.P., Zanette, F. and Kulchetscki, L. (2000). Somatic embryogenesis in *Aspidosperma polyneuron* Mull. Arg. In: *Somatic Embryogenesis in Woody Plants*. (Eds. S.M. Jain, P.K. Gupta and R. J. Newton). (Vol 6), Kluwer Academic Publishers, Dordrecht, pp. 509-537.

Ruzin, S.E. (1999). *Plant Microtechnique and Microscopy*. Oxford University Press, New York

Sane, D., Aberlenc-Bertossi, F., Gassama-Dia, Y.K., Sagna, M., Trouslot, M.F., Duval, T. and Borgel, A. (2006). Histological analysis of callogenesis and somatic embryogenesis from cell suspension of Date Palm (*Phoenix dactylifera*). *Annals of Botany*, **98**: 301-308.

Schwendiman, J., Pannetier, C. and Michaux-Ferriere, N. (1988). Histology of somatic embryogenesis from leaf explants of the oil palm, *Elaeis guineensis*. *Annals of Botany*, **62**: 43-52.

Sung, Z.R. and Okimoto, R. (1981). Embryonic proteins in somatic embryos of carrot. *Proceedings of the National Academy of Sciences*, 3683-3687.

Verdeil, J.L., Hocher, V., Huet, C., Grosdemange, F., Escoute, J., Ferriere, N. and Nicole, M. (2001). Utrastructural changes in coconut calli associated with the acquisition of embryogenic competence. *Annals of Botany*, **88**: 9-18.

Wann, S.R., Johnson, M.A., Noland, T.L. and Carlson, J.A. (1987). Biochemical differences between embryogenic and non embryogenic callus of *Picea abies*. *Plant Cell Reports*, **6**: 39-42.