J Tree Sci 20 (1&2) : 78-85 (2001)

Isozyme Markers for Distinguishing Embryogenic and Non-embryogenic Callus during Somatic Embryogenesis in *Hevea brasiliensis* (Muell). Arg.

M.P. Asokan, K. Narasimhan, P. Kumari Jayasree, A. Thulaseedharan, S. Sushama Kumari, R.G. Kala and R. Jayasree*

ABSTRACT

Isozyme pattern of six enzymes - aspartate amino transferase (GOT), superoxide dismutase (SOD), peroxidase (PR), aryl esterase (EST), shikhimate dehydrogenase (SDH) and glutamate dehydrogenase (GDH) were studied in the *in vitro* cultures derived from integumental tissues of the natural rubber producing tree, *Hevea brasiliensis*. Among early callus, embryogenic callus, non-embryogenic callus, embryo and mature plant leaves, the embryogenic and the non embryogenic calli showed marked difference in their zymogram profiles and thus useful in early identification of embryogenic calli.

Key words : *Hevea brasiliensis*, isozyme analysis, somatic embryogenesis.

INTRODUCTION

Although somatic embryogenesis has been extensively reported in a large number of angiosperms, generally the percentage of embryogenic tissue generated in a regeneration pathway is relatively less in woody species. The timely identification of embryogenic potency of such tissue for further sub culturing could enhance efficiency of the regeneration system as well as reduce the time span required for the completion of regeneration pathway. One way to achieve this goal is the biochemical characterization of the morphogenetic events. Since morphological differentiation or developments are often associated with a continuous synthesis and degradation of enzymes and structural proteins (Scandalios 1994), correlating the changes in enzyme profile with specific morphogenetic development would help not only to understand the molecular pathway of

Biotechnology Division, Rubber Research Institute of India, Kottayam - 686 009, India
*Corresponding author

regeneration, but also would help to enhance its efficiency. Previously the biochemical difference between embryogenic and non-embryogenic calli have been identified with antigens (Khavkin et al. 1977). The quantification of trypsin inhibitor level has also been used for this purpose (Carlberg et al. 1987). There are some recent reviews on this topic in particular on differentiation. The use of isozyme as well as protein markers were reported previously also to determine the embryogenic potential of *in vitro* cultures (Blanco et al. 1997; Devi and Radha 1997). In a study on isozyme modifications and plant regeneration through somatic embryogenesis in sweet potato, the expression of esterase, peroxidase, glutamate oxaloacetate transferase and acid phosphatase were found useful to distinguish compact embryogenic callus from friable non-embryogenic callus (Alves et al. 1994). In our investigation on rubber somatic embryogenesis, we have extended our studies with biochemical parameters on the five steps of the somatic embryogenesis with zymogram patterns of 6 enzyme systems. Our primary objective was to differentiate the embryogenic callus from non-embryogenic and to determine the point at which a tissue has undergone induction and become embryogenic by biochemical characterization. This information may help for early identification of embryogenically potent callus before other visible characteristics appear.

MATERIALS AND METHODS

Somatic embryogenesis: Five week old immature fruits of *Hevea brasiliensis* (Clone RRII 105) were collected from trees and thoroughly washed in tap water. They were surface sterilised with 0.25% mercuric chloride for 5 min. and washed thoroughly with sterile distilled water. Integumental tissues were excised and cultured for callus induction on B5 medium (Gamborg et al. 1968) supplemented with 2.0 mg l^{-1} 2, 4-D, 3.0% (w/v) sucrose and 0.2% gelrite dispensed in test tubes and covered with cotton plugs wrapped in cheese cloth. The cultures were incubated in dark at 25±1°C for six weeks for callus induction. Thereafter, the callus was subcultured onto B5 medium supplemented with 0.5 mg l^{-1} NAA, 2.0 mgl kinetin and 0.5 mg l^{-1} IAA and incubated under 16 h photoperiod (6.9 W m^{-2}) for embryo induction.

Selection of material for enzyme study: Following five stages of the somatic embryogenesis pathway were used in the study. (1) Expanded explant (EX) was the inner integument cultured for 3 weeks in

callus induction medium and kept in the dark at 25±1°C. (2) Early callus (CE) was the first mass of callus appearing on the explant after 4 weeks on the callus induction medium. (3) Embryogenic callus (EC) was selected (Fig. 1) from a population of embryogenically competent callus tissue and (4) The non-embryogenic callus (NC) was selected from a population of non-embryogenic calli (Fig. 2) which was identified from their morphology. (5) Embryos (EM) were identified (Fig. 3) when their bipolarity was evident. Cotyledonary stage embryos were used.

Leaf samples (LE) were selected from plants of the same cultivar grown under green house conditions.

Isozyme analysis - The isozyme expression of GOT, PR, EST, SOD and GDH was analysed by activity staining (Vallejos 1983) after polyacrylamide gel electrophoresis (Hames 1990). The source of each sample extract came from random selection of material of the respective tissue category in order to ensure true representation from each category. Not less than seven extracts were run for each enzyme to ascertain the repeatability of isozyme profile.

RESULTS AND DISCUSSION

The expression of aspartate amino transferase (GOT) in the extracts of embryogenic callus, embryos and leaves displayed distinct and unique banding pattern (Fig. 4). The non embryogenic callus displayed no bands. Embryogenic callus and the embryos showed two similar bands just above the mid migration zone, but the leaf indicated only some limited enzyme activity representing 4 minor bands with in the same region. A third band was visible at the fast migration zone for embryogenic callus and the leaves but not for the embryos. No other stage indicated any clear expression of this enzyme

The embryogenic callus showed a remarkably distinct zymogram profile of superoxide dismutase (SOD) with four distinct bands, one in the slower, another broad band in the mid and two closely linked bands in the faster migration zones, respectively (Fig. 5). Non-embryogenic callus showed only limited enzyme activity in the mid zone. The embryos showed three bands comparable to that of the embryogenic callus. SOD is identified as a very common and essential component of defense mechanism against environmental stress in biological systems and superoxide radicals, hydrogen peroxide and singlet oxygen are formed in numerous cellular reactions (Asada and Takahashi 1987). The SOD gene expression is generally easier to detect in biological systems.

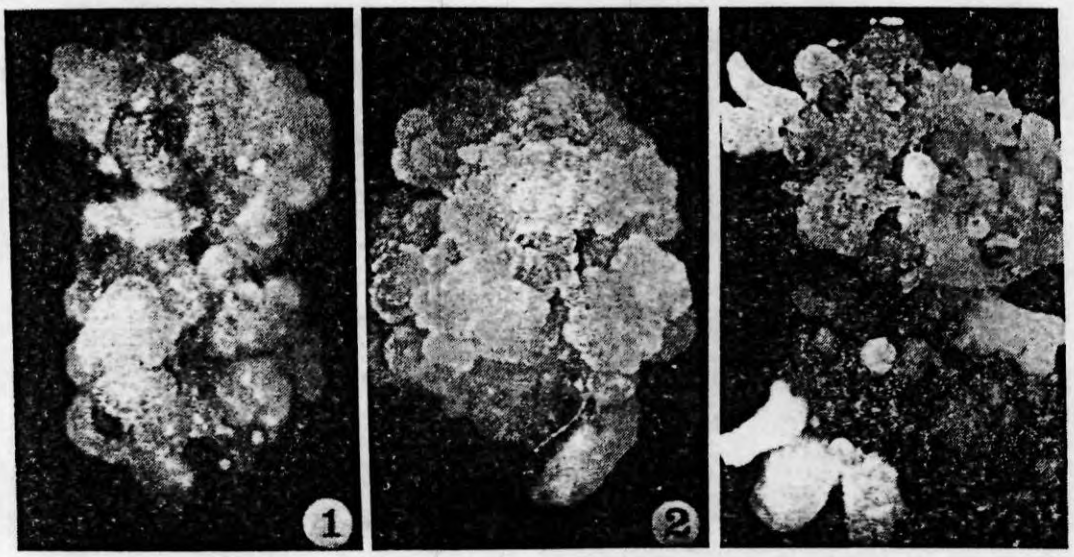

Fig 1-3. Embryogenic callus (fig. 1), nonembryogenic callus (fig. 2) and emerging somatic embryos (fig. 3) of *Hevea brasiliensis*.

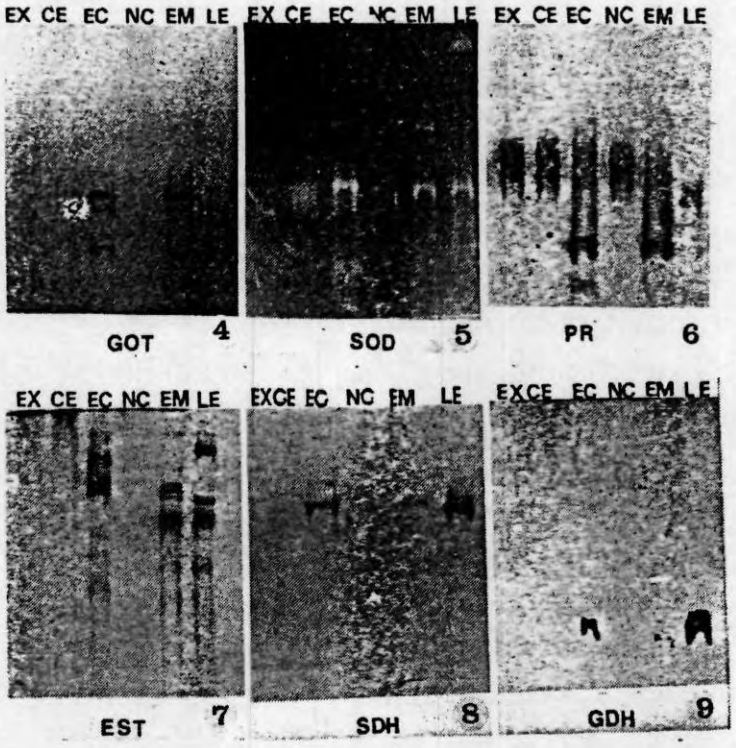

Fig 4-9. Zymograms of aspartate amino transferase (fig. 4), superoxide dismutase (fig. 5), peroxidase (fig. 6), aryl esterase (fig. 7), shikhimate dehydrogenase (fig. 8) and glutamate dehydrogenase (fig. 9) in *Hevea brasiliensis*. EX (expanded explants): CE (early callus): EC (embryogenic callus): NC (nonembryogenic callus): EM (embryos) and LE (leaf).

Peroxidase (PR) isozyme in the expanded explant, early callus and non embryogenic callus displayed a similar expression in a wider area in the mid migration zone (Fig. 6). Among all the enzymes studied, peroxidase was the only enzyme that displayed some activity in the expanded explant. Presumably the lack of activity of other enzymes in the expanded explants may be due to the unique physiological status of the expanded explant being in between differentiation and redifferentiation phase since this tissue was in callus induction medium for three weeks prior to enzyme analysis. Leaf extract expressed two bands in the mid migration zone which were absent in all other extracts perhaps indicating a different allelic or gene function. In the slow migration zone, embryogenic callus and embryo had a broad band but the same was very weak in leaf. Interestingly, embryogenic callus showed the most enzyme activity with four bands, three in the slower migration and one in the mid zone. Presumably the embryogenic callus after having undergone embryo induction may be an activity site for an array of regulatory genes functioning prior to the initiation of further morphogenic events. Devi and Radha (1997) observed the appearance of specific peroxidase isozyme bands in the embryogenic calli of *Vigna radiata* which are useful as biochemical markers for somatic embryogenesis. The presence of several peroxidases in plant systems have been implicated in a variety of secondary metabolic reactions such as lignification, polysaccharide cross linking, indole-3-acetic acid oxidation, wound healing, phenol oxidation, pathogen defense etc. and hence it may be considered as a convenient enzyme marker for biochemical studies.

Several electrophoretically detectable isozyme bands were visible for aryl esterase (EST). Embryogenic callus, embryo and leaf extract had maximum activity. Embryogenic callus had 4 minor bands in the mid migration zone, two densely stained major bands and two minor bands in the faster migration zone (Fig. 7). Embryo had a similar expression but differed markedly above the mid zone where one broad band and four minor bands appeared. Leaf had similarity with the embryo till the mid zone. Two major bands above this zone were distinctly dissimilar. A broad band in the fast migration zone was very different than any observed in other extracts. Non-embryogenic callus indicated no bands. Easterase has been successfully utilized to identify embryogenic and organogenic calli in barley (Coppens and Dewitte 1990). Among sample extracts, leaf and

embryogenic callus displayed one distinct band each of Shikimate dehydrogenase (SDH) on the same migration zone (Fig. 8). A smaller but fainter narrow band appeared for the embryo extracts. The early callus, non-embryogenic callus and expanded explant showed very faint activity. The appearance of a band in the extracts of leaf, embryo and embryogenic callus was on the same zone of activity, which may presumably be indicative of the same allelic action. The primary function of shikhimate pathway in plant metabolism is the production of aromatic amino acids for protein synthesis and such aromatic amino acids are precursors for the synthesis of many plant products such as coumarins, cyanogenic glycosides, glycoinolates, indole acetic acid etc. (Hrazidna and Jensen 1992).

Like SDH, Glutamate dehydrogenase (GDH) isozyme expression was confined to a distinct single band in the embryogenic callus and leaf. In the embryo a very low activity was observed (Fig. 9). The enzyme activity was in the slow migration zone. GDH is a key enzyme in somatic embryogenesis since glutamine and glutamic acids are products during biosynthesis of nitrogenous compounds from ammonia. Ammonia is a key exogenous substrate provided during *in vitro* culture. A majority of amino acids for protein synthesis originate from glutamate by transamination (Miflin and Lea 1982). These transamination reactions leading to protein synthesis are considered to account for ammonia assimilation in dividing cells in culture. Also GDH having a low affinity for ammonia may be responsible for ammonia assimilation at higher levels (>1 mM) (Durzan 1987). The role of GDH may be primarily indicative of the influence of ammonia in embryogenesis and this role needs to be better understood.

In summary there was marked difference in the expression of enzymes between embryogenic and non-embryogenic calli and this difference may be utilized to identify, isolate and multiply calli of embryogenic potency. Interestingly, the activity of all the 6 enzymes studied was very negligible in the expanded explant (EX) and early callus (CE) and were not adeqate enough to be picked up in photography. A possible hypothetical answer to this phenomenon could be that expanded explant and early callus symbolise the late stage of dedifferentiation when structural gene expression might have been very minimal or absent. Regarding the non-embryogenic calli, despite growing in the optimal media formulations in several replications and repeated subculture did not alter

this non-embryogenic phenomenon. Callus not undergone the induction phase may remain biochemically inactive and will be unable to surpass the threshold of dedifferentiation. Generally in angiosperms, embryogenic tissue may be friable and a physical appearance may be indicative of the embryogenic nature of the tissue. However, prior to this physical appearance biochemical identification of embryogenic tissue from the non-embryogenic may be useful especially when the regeneration pathways are elaborate and complex, as in many woody species.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. NM Mathew, Director of Research and Dr. MR Sethuraj, former Director of Research, Rubber Research Institute of India for the encouragement.

REFERENCES

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

Asada K and Takahashi M 1987 Production and scavenging of active oxygen in photosynthesis. In : DJ Kyle, CB Osmond and CJ Arntzen (Eds) *Photoinhibition*. Amsterdam, Elsevier Science Publications : 227-87 pp.

Blanco MA, Nieves N, Sanches M, Borroto CG, Kartillo R, Gonzalez JL, Escalona M, Baez E and Hernandez Z 1997 Protein changes associated with plant regeneration in embryogenic calli of sugarcane (*Saccharum* sp). *Plant Cell Tiss Org Cult* **51:** 153-158.

Carlberg I, Jonsson L, Bergensrahle A and Soderhall K 1987 Purification of a trypsin inhibitor secreted by embryogenic carrot cells. *Plant Physio* **84:** 197-200.

Coppens L and Dewitte D 1990 esterase and peroxidase zymograms from barley (*Hordeum vulgare* L.) callus as a biochemical marker system of embryogenesis and organogenesis. *Plant Sci* **67:** 97-105.

Devi P and Radha P 1997 Isozyme analysis of embryogenic callus obtained from mung bean. *In Vitro* **33:** 47.

Durzan DJ 1987 Ammonia: Its analogues, metabolic products and site of action in somatic embryogenesis. In: JM Bonga and DJ Durzan (Eds) *Cell and Tissue Culture in Forestry Vol 2.* Bonston-Lancaster, Martinus Nijhoff Publications : 92-136 pp.

Gamborg OL, Miller RA and Ojima K 1968 Nutrient requirements of suspension cultures of soybean root cell. *Exp Cell Res* **50:** 151-58

Hames BD 1990 One - dimensional polyacrylamide gel electrophoresis. In:BD Hames and D Rickwood (Eds). *Gel Electrophoresis of Proteins. A Practical Approach.* Oxford University Press, New York: 174 p.

Hrazdina G and Jensen RA 1992 Spatial organization of enzymes in Plant metabolic pathways. In:WR Briggs, RL Jones and V Walbot (Eds), *Annu Rev Plant Physiol Plant Mol Biol* **43:** 241-267.

Khavkin EE, Misharin SI and Ivanov VN 1977 Embryonal antigens in maize caryopeses. The temporal order of antigen accumulation during embryogenesis. *Planta* **135:** 225-231.

Miflin DJ and PJ Lea 1982 Ammonia assimilation and amino acid metabolism. In: D Boulter and B Partheir (Eds). *Nucleic Acids and Proteins in Plants 1. Structure, Biochemistry and Physiology of Proteins.* Springer-Verlag, New York: 5-64 pp.

Scandalios JG 1974 Isozymes in development and differentiation. In: WR Briggs, PB Green and RL Jones (Eds), *Ann Rev Plant Physiol* **25:** 225-258 pp.

Vallejos CE 1983 Enzyme activity staining. In: SD Tanksley and TJ Orton (Eds) *Developments in Plant Genetics and Breeding 1. Isozymes in Genetics and Breeding Part A.* Elsevier Publications, Amsterdam: 469-516 pp.