

Isozyme Analysis of Embryogenic and Non-Embryogenic Callus of Diploid Banana Cultivars

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ABSTRACT

Isozyme analyses were carried out on protein extracts of embryogenic and non-embryogenic callus derived from bract meristem of four diploid *Musa* cultivars using polyacrylamide gel electrophoresis. We examined the isozyme pattern of peroxidase, catalase, malate dehydrogenase and alcohol dehydrogenase for their utility as biochemical markers of banana embryogenic callus cultures. Peroxidase, catalase and alcohol dehydrogenase exhibited high intensity bands in the embryogenic samples, while the corresponding bands were either absent or faint bands were observed in the non-embryogenic callus. Malate dehydrogenase enzyme pattern exhibited homogenous bands in both the embryogenic and non-embryogenic samples.

Highlights

- Isozyme such as peroxidase, catalase and alcohol dehydrogenase exhibited high intensity bands in the embryogenic callus.
- For embryogenic calli identification, isozyme analysis can be used as biochemical markers.

Keywords: Peroxidase, Catalase, SOD, MDH, ADH

During the process of somatic embryogenesis and plant regeneration the screening of an embryogenic culture plays an important role, especially when cultures are derived from numerous cell lines. In many cases, embryogenic potential is identified by morphological characteristics. However such a visual screening is subjective and often applicable only after prolonged culture periods. An early biochemical identification of embryogenic potency would therefore, be of great help for an efficient plant regeneration. Biochemical characterization of cultured tissues and cells may be utilized in differentiating regenerable and non-regenerable cultures, which is a pre requisite for understanding the morphogenesis *in vitro*. Changes that occur in different enzyme system during cell or callus cultures have been studied in order to distinguish the organogenic from non-organogenic tissue. Few reports were published on biochemical differences between embryogenic and non-embryogenic callus

cultures with respect to polypeptide pattern (Chen and Luthe 1987). Many investigations have been made into the physiological changes taking place during organogenesis in callus cultures (Kavi Kishore and Mehta 1988). Several biochemical variables have been shown to discriminate embryogenic and non-embryogenic tissues. Distinct proteins (Sung and Okimoto 1983), isozyme forms and ethylene are among them. Everet *et al.* (1985) characterized maize callus types and demonstrated differences in the secreted polysaccharides and isozymes.

MATERIALS AND METHODS

Embryogenic and non-embryogenic calli derived from bract meristem of four diploid banana cultivars viz. *Musa acuminata* cv. Matti, cv. Sannachenkadali, cv. Chingan and cv. Njalipoovan were selected for isozyme profiling. Embryogenic callus was initiated from bract meristem inoculated on MS medium supplemented with TDZ (0.45 μ M) and



non-embryogenic callus was obtained from MS medium supplemented with 2, 4-D (0.45 μ M). After electrophoresis the gels were stained for Peroxidase, Malate dehydrogenase, Catalase and Alcohol dehydrogenase activity (Vallejos, 1983). Samples of 20 μ l were directly loaded in the gel along with bromophenol blue. The gels were stained in freshly prepared solution according to the standard procedures.

Peroxidase (E.C.1.11.1.7)

For peroxidase activity, the gel was incubated in the staining solution containing 0.1 M phosphate buffer (pH- 6.1), 480 μ l guaiacol and 280 μ l hydrogen peroxide. The gel was incubated in the staining solution for 10 -15 minutes and was transferred to 70% acetic acid for fixation.

Catalase (E.C.1.11.1.6)

For catalase activity, the gel was first incubated in 0.01% hydrogen peroxide for 5 - 10 min and then incubated in substrate solution containing equal proportion of 1% potassium ferric cyanide and ferric chloride. The solution was incubated in dark until the development of achromatic bands (~ 30 min).

Malate dehydrogenase (E.C.1.1.1.37)

The gel was incubated in 0.1M Tris buffer (pH - 7.5) containing 30 mg NAD⁺, 20 mg MTT (Thiazoyl blue) and 4mg PMS (Phenazonium metho sulfate) in 200 ml buffer. The gel was incubated in dark at 30°C for 15 - 60 min.

Alcohol dehydrogenase (E.C.1.1.1.1)

The gel was incubated in 0.1M Tris buffer (pH - 7.5) containing 30 mg NAD⁺, 20 mg MTT, 4 mg PMS and 6 ml ethanol and kept in dark at 30 °C for 15 to 60 minutes.

RESULTS AND DISCUSSION

Embryogenic calli initiated from bract explants inoculated on MS medium supplemented with 0.45 μ M TDZ (Fig. 1) and non - embryogenic calli initiated from bract explant inoculated on MS medium supplemented with 0.45 μ M 2, 4-D (Fig. 2) of four cultivars were selected for isozyme analysis. The embryogenic calli showed higher activity of enzymes such as peroxidase, catalase, malate

dehydrogenase and alcohol dehydrogenase. Peroxidase enzyme pattern showed homogenous banding pattern in all the embryogenic samples. A single major band with high intensity (Rm value 1.77) was observed in the embryogenic calli, while the band was completely absent in the non-embryogenic calli (Fig. 3). Catalase zymogram revealed homogenous banding pattern consisting of a single band (Rm value 3.2) in the embryogenic calli, which was totally absent in the non-embryogenic callus (Fig. 4). Malate dehydrogenase showed homogenous banding pattern in embryogenic and non-embryogenic calli and the bands were anodic in nature. The total number of bands present in the zymogram consisted of two intensively stained major bands (1 and 2) in both the embryogenic and non-embryogenic calli (Fig. 5). Alcohol dehydrogenase zymogram revealed banding pattern consisting of two bands (Rm value 0.75 and 1.25) in the embryogenic calli of cvs. Matti and Sannachenkadali respectively. In cvs. Chingan and Njalipooovan two bands (Rm value 2.8 and 1.75) were observed. The bands were absent in the non-embryogenic samples (Fig. 6).

The developmental programme of a cell involves the appearance or disappearance of specific proteins at a particular stage, which reflects that the differentiation is regulated by differential gene expression. The isozymes analysis at different stages of culture may unravel the physiological and or biochemical changes underlying the process of differentiation. It was also important in tissue specific biochemical characteristics that were expressed and maintained in culture (Scandalios 1974). The application of isozymes as markers in embryogenic cultures was reported in several studies (Everett *et al.* 1985; Chawla 1988). Isozymes catalyzed identical reactions during growth and differentiation and it was regulated by the activity of plant growth regulators. It followed the co-dominant mode of inheritance. In the present study, embryogenic calli exhibited higher enzymatic activity of peroxidase, catalase and alcohol dehydrogenase when compared to non-embryogenic calli, suggesting the low metabolic rate in the later.

The present study observed that peroxidase enzyme pattern showed homogenous banding pattern in all the embryogenic samples. Peroxidases as constitutive

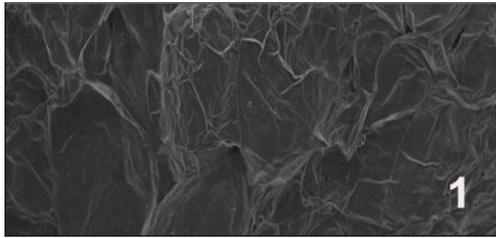


Fig. 1: Scanning electron micrograph of non - embryogenic callus

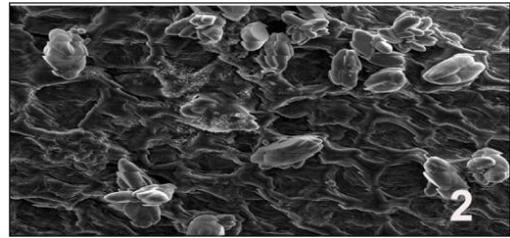


Fig. 2: Scanning electron micrograph of embryogenic callus

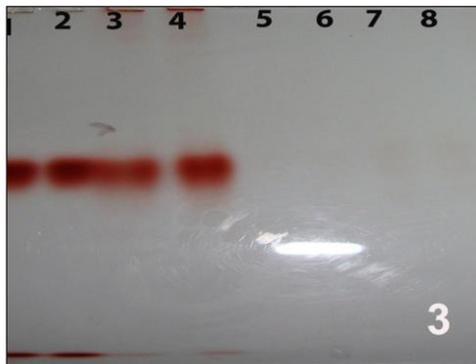


Fig. 3: Peroxidase enzyme pattern showing homogenous banding pattern in embryogenic calli (Lane 1 - 4) and bands that are absent in non - embryogenic calli (Lane 5 - 8)



Fig. 4: Catalase zymogram showing a single band in embryogenic calli (Lane 1 - 4), which is absent in non - embryogenic callus (Lane 5 - 8)

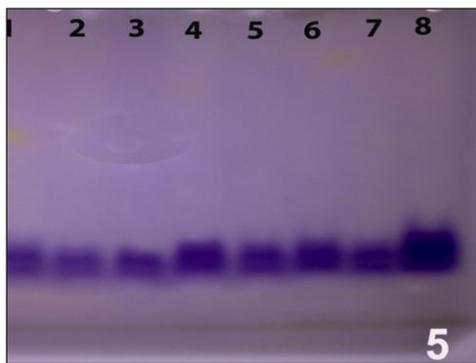


Fig. 5: Malate dehydrogenase showing two distinct bands in embryogenic (Lane 1 - 4) and non - embryogenic calli (Lane 5 - 8)



Fig. 6: Alcohol dehydrogenase showing two distinct bands in embryogenic calli (Lane 1 - 4) and absent in non - embryogenic callus (Lane 5 - 8) (Lane 1 - 4, Embryogenic calli of cv. Matti, cv. Sannachenkadali, cv. Chingan, cv. Njalipoovan and Lane 5 - 8, Non - embryogenic calli of cv. Matti, cv. Sannachenkadali, cv. Chingan, cv. Njalipoovan)

enzymes of the cell wall, of the free intercellular spaces and vacuoles have a role in modifying the cell wall properties (Brett and Waldron 1990). An isoperoxidase with pH 7.0 was revealed in carrot cell suspension cultures grown under conditions stimulating embryogenesis (Joersbo *et al.* 1989). A rise in specific peroxidase activity was noticed a day before the globular embryoids were seen. They also revealed an isoperoxidase that correlated with the early stages of somatic embryogenesis. Low peroxidase activity was a distinct characteristic of

habituated tissue reported in pumpkin callus lines, carrot cell lines (Joersbo *et al.* 1989) and sugar beet calli (Hagge *et al.* 1990). Coppens and Dewitte (1990) proposed an enzyme - based marker system including esterase and peroxidase for embryogenic cultures of barley. In maize, Rao *et al.* (1990) detected two cathodical isoperoxide specific for embryogenic callus. A similar study conducted in wheat revealed high amount of isoperoxide in embryogenic calli (Cialacu *et al.* 1996). Peroxidase involved in several developmental processes in the plant cell including



cell division and cell differentiation is related with auxin /cytokinin ratio in the medium (Booij *et al.* 1993).

In the present investigation, it was observed that catalase zymogram revealed homogenous banding pattern consisting of a single band in the embryogenic calli, which was totally absent in non - embryogenic callus. Catalase, as a tetrameric heme - containing enzyme catalyzes hydrogen peroxide to oxygen and water. It prevents harmful hydrogen peroxide accumulation. This enzyme played a central role in protecting cells from the toxic effect of activated oxygen species (Halliwell 1974). Callus initiation and subsequent plant regeneration involved the promotion of meristematic activity, its maintenance and its suppression where differentiation takes place (Racchi and Terragna 1993). Malate dehydrogenase also showed homogenous banding pattern consisting of two distinct bands. Monomorphic banding pattern was observed in embryogenic and non-embryogenic calli and the bands were anodic in nature. Over 100 different members of malate dehydrogenases belonging to the NAD - dependent dehydrogenases, largest and best - studied families of nucleotide - binding proteins have already been identified. In the present study, alcohol dehydrogenase exhibited banding pattern consisting of two bands in the embryogenic calli, whereas, these bands were absent in non - embryogenic callus. Previous studies revealed that ADH activity increased during the anaerobic stress in many plant species including Maize (Drew *et al.* 1997; Crawford 1977). It was also reported that the activity of ADH was high throughout callus tissue development in *Cactaceae* (Torquato *et al.* 1995). Isozymes were found appropriate in detecting various types of callus because of the difference in the isozyme pattern that were observed between two types of calli. More over absence or reduced number of bands were observed in non - embryogenic callus. Jarret and Litz (1986) and Rani and Raina (2000), reported the presence or absence of isozymes in genotype identification. Isoenzyme systems were reported as biochemical tools for identifying interspecific hybrids and genetic variants as well as for detecting genetic alterations in plants as a consequence of *in vitro* culturing, as reported for *Lycopersicon esculentum* (Evans *et al.* 1984), *Solanum phureja* (Pehu *et al.* 1986), *Rubus idacus* (Cousineau and Dounelly

1989), *Ipomoea batatas* (Alves *et al.* 1994), *Saccharum officinalis* (Taylor *et al.* 1995), *Musa sp.* (Ulisses *et al.* 2002) and *Ananas comosus* (Feuser *et al.* 2003).

Even though the embryogenic and non-embryogenic callus were derived from the same genotype explant, specific differences in isozyme patterns of peroxidase, esterase and catalase were observed, which may be correlated to their difference in the degree of dedifferentiation. Sellés-Marchart *et al.* (2006) reported that differences in optimum parameters for enzyme activity is dependent on the plant sources, extraction methods, and purity of the enzyme, buffers, and substrates. Variable number of isozymes of alcohol dehydrogenase callus suggests the differential gene expression. In the present study, embryogenic and non- embryogenic cultures clearly exhibited difference in enzyme activities and in isozyme patterns. These changes or patterns may be used as biochemical attributes in identifying embryogenic cultures from non- embryogenic cultures.

REFERENCES

- Alves, C.M.J., Sihachakr, D., Allot, M., Tizroutine, S., Mussio, I., Servaes, A. and Ducreux, G. 1994. Isozyme modification and plant regeneration through somatic embryogenesis in sweet potato (*Ipomoea batatas* (L.) Lam. *Plant Cell Rep.* **13**: 437 - 441.
- Booij, I., Monfort, S. and Macheix, S. 1993. Reaction between peroxidases and budding in datepalm tissues cultured *in vitro*. *Plant Cell Tiss. Org. Cult.*, **35**: 166 - 171.
- Brett, C. and Waldron, K. 1990. Cell wall structure and the skeletal functions of cell walls. In: Black M, Chapman (eds) *Physiology and Biochemisrly of Plant Cell Walls. Topics in Plant Physiology: Series 2*, Unwin Hyman Ltd, London, pp. 4 - 47.
- Chawla, H.S. 1988. Isozyme modification of callus from barley and wheat. *Plant Cell Tiss. Org. Cult.*, **12**: 299 - 304.
- Chen, L.J. and Luthe, D.S. 1987. Analysis of proteins from embryogenic and non-nembryogenic rice (*Oryza sativa* L.) calli. *Plant Sci.*, **48**: 181 - 188.
- Cialăcu, M., Hagima, I. and Verzea, M. 1996. Caracterizarea embriogenezei somatice la grâu - aspecte morfohistologice, biochimice și parametri cantitativi. *Analele ICCPT*, **LXIII**: 239 - 240.
- Coppens, L. and Dewitte, D. 1990. Esterase and peroxidase zymograms of barley (*Hordeum vulgare* L.) callus as a biochemical marker system of embryogenesis and organogenesis. *Plant Sci.*, **67**: 97 - 105.
- Cousineau, J.C. and Dounelly, D.J. 1989. Identification of raspberry cultivars *in vivo* and *in vitro* using isozymes analysis. *Hort Sci.*, **24**: 490 - 492.



- Crawford, R.M.M. 1977. Tolerance of anoxia and ethanol metabolism in germinating seeds. *New Phytol.*, **79**: 1224 - 1246.
- Drew, M.C. 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**: 223 - 250.
- Evans, D.A., Sharp, W.R. and Medina - Filho, H.P. 1984. Somaclonal and gametoclonal variation. *Am. J. Bot.*, **71**: 759 - 774.
- Everret, N.P., Wach, M.J. and Ashworth, D.J. 1985. Biochemical markers of embryogenesis in tissue cultures of the maize inbred B73. *Plant Sci.*, **41**: 133 -140.
- Feuser, S., Meler, K., Daquinta, M. and Guerra, M.P. 2003. Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by isoenzyme and RAPD markers. *Plant Cell Tiss. Org. Cult.*, **72**: 221 - 227.
- Hagge, D., Kevers, C., Ledlly, F., Gaspar, T. and Boucaud, J. 1990. NaCl dependent growth rate of normal and habituated sugar beet calli, ethylene production and peroxidase activity. *CR Acad. Sci.*, **310**(3): 259 - 264.
- Halliwell, B. 1974. Superoxide dismutase, catalase and glutathione peroxidase: solutions to the problems of living with oxygen. *New Phytol.*, **73**: 1075 - 1086.
- Jarret, R.L. and Litz, R.E. 1986. Isoenzymes as genetic markers in bananas and plantains. *Euphytica.*, **35**: 539 - 549.
- Joersbo, M., Anderson, J.M., Okkles, F.T. and Rajagopal, R. 1989. Isoperoxidases as markers of embryogenesis in carrot cell suspension cultures. *Physiol Plant.*, **76**: 10 - 14.
- Kavi Kishor, P.B. and Mehta, A.R. 1988. Changes in some enzyme activities during growth and organogenesis in dark-grown tobacco callus cultures, *Plant Cell Physiol.*, **29**: 255-259.
- Racchi, L.M. and Terragna, C. 1993. Catalase isozyme as useful markers of differentiation in Maize tissue cultures. *Plant Sci.*, **93**: 105 - 202.
- Rani, V. and Raina, S.N. 2000. Genetic fidelity of organized meristem - derived micropropagated plants: A critical reappraisal. *In Vitro Cell Dev Biol- Plant*, **36**: 319 - 330.
- Rao, K.V., Suprasanna, P. and Reddy, G.M. 1990. Biochemical changes in embryogenic and non-embryogenic calli of *Zea mays*. *Plant Sci.*, **66**: 127 - 130.
- Scandalios, J.W. 1974. Isozymes in development and differentiation. *Ann. Rev. Plant Physiol.*, **25**: 225.
- Sellés-Marchart, S., Casado-Vela, J. and Bru-martinez, R. 2006. Isolation of a latent polyphenol oxidase from loquat fruit (*Eriobotrya japonica* Lindl.): Kinetic characterization and comparison with the active form. *Archives of Biochemistry and Biophysics*, **446**: 175-185.
- Taylor, P.W.J., Fraser, T.A., Ko, H.L. and Henry, J. 1995. RAPD analysis of sugarcane during tissue culture. In: Terzi M, Cella R, Falavigna A (eds) *Current issues in plant molecular and cellular biology*. Kluwer Academic Publisher, pp. 241.
- Torquato, E.F.B., Prioli, A.J. and Machado, M.F. 1995. Differential alcohol dehydrogenase and malate dehydrogenase isozyme expression in long term callus tissue culture of *Cereus peruvianus* (cactaceae). *Biochem. Genet.*, **33**: 11 - 12.
- Ulisses, C., Camara, T.R., Willadino, L., Albuquerque, C.C., Martins, L.S.S. and Freitas, N.S.A. 2002. Caracterizaco isoenzimatica de clones de bananeira nanicao submetidos a salinidade. *Revista Brasileira de Engenharia Agrícola e Ambiental*, **6**: 358 - 361.
- Vallejos, E. 1983. Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) *Isozymes in plant genetics and breeding, part A*. Amsterdam: Elsevier Science Publishers BV, pp. 469 - 516.

