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Biochemical marker studies in tissue cultured and naturally growing plants of *Asclepias curassavica (L).*

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ABSTRACT

In the present study, the genetic stability of in vitro propagated Asclepias curassavica plants were assessed by using biochemical enzyme markers - peroxidase, esterase, polyphenol oxidase and acid phosphatase enzymes and were compared to the naturally grown plant. The results indicate the clonal purity of the in vitro raised plants. However variations were observed in callus regenerated plants which may have been occurred due to somaclonal variations.

Keywords

Isozyme, peroxidase, esterase, somaclonal variation

Introduction

Cells in prolonged *in vitro* culture are prone to be genetically changes. They frequently tend to become polyploids, aneuploids and show chromosomal structural changes. Genetic variability in cultured plant cells was first reported by Murashige and Nakano (1967). The genetic variability of the plants obtained from undifferentiated cell cultures (callus, cell suspension and protoplast cultures) was agronomically more valuable. Isozymes are used for investigating the genetic structure of a natural population. They were first used in genetic analysis of forest trees by Feret and Bergmann (1976). It is very important to detect the variations quite early in the life of a plant, especially in long living forest trees. Plant regenerated from cultured cells may exhibit heritable variation called somaclonal variations (Hashmi *et al.*, 1997; Eapen, 1999) which may either lead to karyotypic changes or alteration at the DNA level. Mode of regeneration and the source of explant play a key role in determining the presence or absence of variation (Damasco*et al.*, 1996). Various approaches like karyological analysis (or) isozyme markers to assess the genetic stability of *in vitro* derived plants were revealed by Isabel *et al.*, (1993). Rout and Das (1995) established isozyme profiles during embryogenesis and *in vitro* flowering of *Bambusa*

vulgaris. The Isozymes, peroxidase and esterase patterns of *in vitro* regeneration in *Zizypusmauritiana* was reported by Gupta and Srivastava, (1996). In *Hemidesmusindicus*, comparative banding patterns of peroxidase in direct and indirect regenerated plants were first reported by Chandrasekhar (2001). Isozymes are the best source for monitoring genetic diversity in forest trees (Adams, 1981) in a stipulated time with limited resources. They can be used very effectively to identify parent trees, clones and for certifying commercial clones in reforestation (Feret and Bergmann, 1976).

Materials and methods

Clonal purity was determined by using biochemical markers. Peroxidase (EC 1.11.1.7), Esterase (EC 3.1.1.2), Polyphenol oxidase (EC 1.10.3.2) and acid phosphatase (EC 3.1.3.2) isozyme/allozyme analysis of the *in vitro* regenerated plants and mature parent plant were performed and compared to trace the clonal purity.

Preparation of Enzyme Extract of Peroxidase

Peroxidase enzyme extract was prepared by homogenizing fresh young leaves of tissue cultured and field grown plants of randomly selected plants in a pre-chilled mortar with 3 ml of 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 18,000 rpm at 5°C for 15 minutes. The supernatant was stored on ice and used as an enzyme extract (Reddy & Garber, 1971). Each slot of the gel was loaded with 10µl of enzyme extract, containing 20 µg of protein, estimated by Lowry's method (Lowry et al, 1951).

Preparation of Enzyme Extract of Esterase

Esterase enzyme extract was prepared by homogenizing fresh young leaves of tissue cultured and field grown plants of randomly selected plants in a pre-chilled mortar and pestle with 3 ml of 10 mM sodium phosphate buffer (pH 9.5). The homogenate was centrifuged at 5°C for 10 minutes at 10,000 rpm. The supernatant was stored on ice and used as an enzyme extract.

Preparation of Enzyme Extract of Polyphenol Oxidase

Polyphenol oxidase enzyme extract was prepared by homogenizing fresh young leaves of tissue cultured and field grown plants of randomly selected plants in a pre-chilled mortar and pestle with 3ml of 0.01 M K_2PO_4 buffer (pH 7.0) containing 1% nonionic detergent at 0^0C. The homogenate was centrifuged at 5^0C for 15minutes at 18,000 rpm. The supernatant was stored on ice and used as an enzyme extract.

Preparation of Enzyme Extract of Acid Phosphatase

Acid phosphatase enzyme extract was prepared by homogenizing fresh young leaves of tissue cultured and field grown plants of randomly selected plants in pre chilled mortar and pestle with 3ml of 50 mM citrate buffer (pH 5.3). The homogenate was centrifuged at 5^0C for 10 minutes at 10,000 rpm. The supernatant was stored on ice and used as an enzyme extract.

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Preparation of sample buffers

The sample buffer for peroxidase was prepared with 0.5 M Tris-HCl (pH 6.8), Glycerol, 0.05 % Bromophenol blue and made up to 10 ml with distilled water and stored at room temperature. Sample buffers for esterase, polyphenol oxidase and acid phosphatase were prepared with10% sucrose and made up with distilled water.

Native Polyacrylamide Gel Electrophoresis

The vertical slab gels were prepared as described by van Edlic *et al*., (1980) for all the Isozymes. The stacking gel was made with 4% polyacrylamide and the separating gel was made with 12% polyacrylamide. The gel was polymerized with ammonium per sulphate. 25 mM Tris-glycine (pH 7.5) was used as tank buffer. The isozymes were separated under a constant voltage (200 volts) and current (30 mA) for 6 hours at 4°C in the vertical gel electrophoresis unit.

Staining of Gels for Isozyme Activity

After electrophoresis, the gels were individually stained for determining the acid phosphatase, esterase, peroxidase and polyphenol oxidase activities.

Peroxidase

The gel was stained for 5 minutes in the staining solution (Benzidine, Acetic acid, 3% hydrogen peroxide). After the bands were sufficiently stained, the reaction was arrested by immersing the gel in 70% acetic acid solution for 10 minutes.

Esterase

The gel was incubated at 37°C for 30 minutes at dark using the staining solution (NaH_2PO_4, Na_2HPO_4, Fast blue RR salt, a-naphthyl acetate) and then treated with a mixture of methanol : water : acetic acid : ethyl alcohol in the ratio of 10 : 10 : 2 : 1 to terminate the reaction.

Polyphenol oxidase

The gel was equilibrated for 30 min in 0.1% p-phenylenediamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10mM catechol in the same buffer.

Acid Phosphatase

The gel was washed 3-4 times in 0.1 M acetate buffer (pH 5.0) by changing the buffer every 15 minutes to lower the pH of the gel. Then the gel was incubated at 37°C for 2-3 hours in the staining solution (1-naphthyl phosphate, fast blue, NaCl, MgCl2 and 0.5% acetate buffer with pH 5). After sufficient staining the gel was fixed with 50% ethanol.

Results and discussion

The post translational growth of the micropropagated plants obtained from different explants based on visual observation was uniform. However, the current problem facing the regeneration system in plant tissue culture is the occurrence of somaclonal variations, which are undesirable in any clonal propagation and conservation programmes. It has been established that regenerants raised from resident meristem would be true to type (Hu and Wang, 1983; Bajaj *et al.*, 1988). However in some reports, variations were detected even when shoot meristems were used (Swartz *et al.*, 1983; Pramanik and Datta, 1986). Isozymes are considered to be the direct expression of the gene function of cells during differentiation and their variations are often associated with the somaclonal variations. A detailed analysis of their changing patterns during development may lead to the understanding of the basic mechanism of cellular differentiation to obtain efficient plant regeneration *in vitro*. In the present investigation, four different isozymes namely peroxidase, esterase, polyphenol oxidase and acid phosphatase have been employed to verify the clonal uniformity of *in vitro* regenerated plants (both direct and indirect), and compared with the samples of parental field grown plants.

a) Peroxidase

Results with peroxidase showed no detectable variation in the banding pattern among the parent field grown plant (FP) and the plants regenerated from axillary buds of nodal explants (TP). But a variation was observed i.e., two new bands were observed in callus regenerated plants (CP). This change in isozyme profile in indirect regenerated plants is due to somaclonal variations that occurred during organogenesis. The intensity of staining in isozyme bands in parent plant (FP) were slightly thick than other samples. This slight variation between the mature plant and callus regenerated plants may be due to variation aroused from sexual hybridization but not due to somaclonal variations.

b) Esterase

In the present investigation the results with esterase isozyme showed no detectable variation in the banding pattern among the parent plant (FE) and the plants regenerated from axillary buds of nodal explants (TE) and from callus regenerated plants. This change in esterase profile in indirect regenerated plants is due to somaclonal variation. The intensity of staining for esterase bands in parent plant sample (FE) were slightly thick than other samples.

c) Polyphenol Oxidase

Results with peroxidase showed no detectable variation in the banding pattern among the parent plant (FPO) and the plants regenerated from axillary buds of nodal explants (TPO). But the variation was observed in callus regenerated samples (CPO).

d) Acid Phosphatase

Results with acid phosphatase showed no detectable variation in the banding pattern among the parent plant (FAP) and the plants regenerated from axillary buds of nodal explants (TAP). But the variation was observed in indirect regenerated plants (CAP).

The above isozyme result shows that the variation was observed only in callus regenerated plants. This change in isozyme profile in indirect regenerated samples is due to somaclonal variations that occurred during organogenesis. Propagation through indirect organogenesis carries a risk that the regenerated plant will differ genetically from each other and from the mother plant (George, 1993). In recent years, the application of isozymes as markers in morphological and regeneration studies has been reported by several workers (Franszet *al.*, 1989; Bapat*et al.*, 1992). The current problem facing the regenerating system in plant tissue culture is the occurrence of uncontrollable somaclonal variations which are undesirable in any clonal propagation and conservation programmes. The observations obtained with isozymes in the present investigation states the uniformity of the clonal plants in nodal explants derived multiple shoots, which is most desirable in any micropropagation system. This was supported by the work of Mallikarjuna (2003) in *Holarrhenaantidysentrica*, Chakradhar (2004) in *Wattakakavolubilis*. These findings also support the view that biochemical traits such as isozymes provide an evidence to study the extent of somaclonal variations in a manner analogous to their use in elucidating genetic variation in natural population (Bhaskaran*et al.*, 1987).

Summary

In vitro propagated plants need to be genetically stable so that no unwanted variation in secondary metabolite yield can occur. Therefore, our major concern was to determine the clonal purity of the *in vitro* raised *A. curassavica* plants. In the present study, the extent of clonal purity of the *in vitro* regenerated plants at the biochemical level was studied by employing isozymes as markers, in order to get an insight into the impact of somaclonal variations in the course of regeneration. Clonal uniformity of the micropropagated plants was thus substantiated through uniformity in isozyme profiles between seedling explants and nodal explant derived samples. As reported in many cases, variations in isozyme profiles were observed in callus regenerated plants.

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Figures

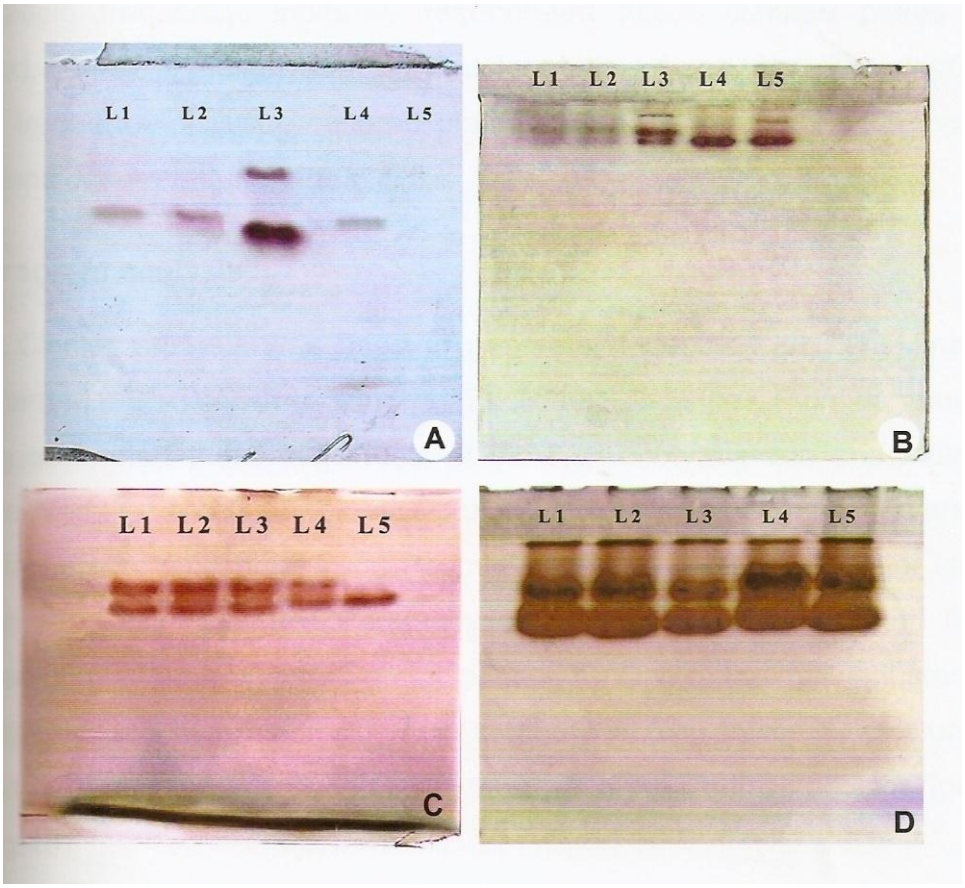

Figure 1: Isozyme banding patterns of (A). Peroxidase, (B). Esterase, (C). Polyphenol Oxidase and (D). Acid phosphatase on Native PAGE. (L1, L2 -Nodal Regenerated clone, L4, L5- Callus Regenerated clone, L3- Parent Plant).