# CHANGES IN ACTIVITY AND ISOZYMES OF PEROXIDASE DURING *IN VITRO* DEDIFFERENTIATION AND CALLUS PROLIFERATION IN DIFFERENT EXPLANTS OF SOYBEAN

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### Abstract

Total activity of peroxidase and its isozymic profiles in the explants of soybean during the process of dedifferentiation was investigated. The explants obtained from aseptically germinated seedling were transferred to callusing medium containing MS salts supplemented with 9.0 $\mu M$ (2.0 mgl$^{-1}$) 2,4-D and 6.9 $\mu M$ (1.5 mgl$^{-1}$) Kinetin. Total activity of peroxidase increased in hypocotyl (0.75 to 1.80), cotyledon (0.55 to 1.20) and internode (0.31 to 1.00) during dedifferentiation process with a peak of activity observed prior to callus initiation. In leaf however, there was a sharp decline in enzyme activity (1.42 to 0.30) after excission with a moderate increase (0.96) prior to callus initiation. Electrophoretic pattern of peroxidases were organ specific. The explant of hypocotyl internode and leaf had three band each but with different Rf values. The expant of cotyledon showed only one isozyme. In all the explants marked changes were associated with the process of dedifferentiation. Once the meristematic activity was triggered no further qualitative changes took place in the isozymes. Calluses of all the four explant had only two bands and these persisted during successive subcultures.

## Introduction

Establishment of callus from the explant can be divided in to three phases: induction, cell division and differentiation (Aitchison *et al.*, 1977). During the induction phase cells prepare to divide and undergo certain biomolecular changes. The phenomenon of quiescent cells reverting to a meristematic state is known as dedifferentiation. Since changes at cellular level must be preceded by quantitative or qualitative changes at molecular level, it is useful to study these alterations in biomolecules during ontogeny of growth and differentiation. The peroxidases have been associated with many morphogenetic processes. Changes in tissue peroxidase levels and isozyme profile have been correlated with the *in vitro* differentiation of carrot cells (Chibbar *et al.*, 1984), shoot initiation in tobacco (Thorpe & Gasper, 1978), initiation of leaf primordia in *Arabidopsis* callus (Negrutiu *et al.*, 1979), bud formation on roots of *Cichorium intybus* (Legrand & Bouazza, 1991) and root formation in *Cynara scolymus* (Moncousin & Gasper, 1983) and *Populus tremula* (Pythoud & Buchala, 1989). Peroxidase has been correlated with a wide range of oxidative processes (Kvaratskhelia *et al.*, 1997) including its interaction with other enzyme systems such as Cytochrome C Oxidase and ATP/ADP Translocase (DeSantis *et al.*, 1999). The present report describes the quantitative and qualitative variations in peroxidases in the explants of soybean undergoing *in vitro* dedifferentiation.

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**Materials and Methods**

Plant Material: Seeds of soybean [*Glycine max* (L.) Merr. cv. William I] were grown under sterile conditions at $26\pm2°C$ on semi-solid agar supplemented with 3% (w/v) sucrose. Seven day old seedling were used as explant source. The hypocotyl was removed 1-5 mm below the cotyledonary attachment and cut into 5 mm pieces. In case of internode middle portion was cut into 5 mm pieces lengths. Cotyledon was cut into square fragments of 5 mm. For leaf, 5 mm segments close to midrib were taken.

Induction of Callus: Callus was initiated from all the explant on agar solidified MS (Murashige & Skoog, 1962) medium containing 9 $\mu$M (2 mgl⁻¹) 2,4-D and 6.9 $\mu$M (1.5 mgl⁻¹) Kinetin. The cultures were grown at $26\pm2°C$ under diffuse cool-white fluorescent light (90 $\mu$ mol m⁻²S⁻¹) with a 16 h photoperiod. Callus initiation happened in 2,3,4 and 6 days from cotyledon, hypocotyl, internode and leaf explants, respectively. The dedifferentiating explants were harvested at regular intervals depending upon the lag period prior to callus induction. The explant of cotyledon was harvested after one day, hypocotyl after one and two days, internode after two and three days and leaf explant after three, four and five days of incubation.

Enzyme Analysis: Two gram of each sample was homogenised in 4 ml ice-cold extraction buffer (0.1 M phosphate buffer, pH 7.2) and a pinch of acid-washed sand in a chilled mortar. The slurry was squeezed through four layers of cheese cloth and centrifuged at 10,000 g for 20 minutes in a refrigerated centrifuge. The supernatant was used for quantitative estimation as well as qualitative analysis. Peroxidase activity in fresh (0-day control) and dedifferentiating explant was assayed by the procedure of David & Murray (1965). A discontinuous SDS system of polyacrylamide gel electrophoresis described by Laemmli (1970) was used for electrophoretic separation of isoperoxidases. Completely run gel was stained by a modified procedure of Siegel & Galston (1967) where the gel was flooded with 0.5M guaiacol and 0.05M $H_2O_2$ in 0.2M phosphate buffer pH 5.8. The peroxidase activity was indicated by distinct brown bands.

**Results**

Total Activity of Peroxidase (units/mg tissue) During Dedifferentiation and Callus Proliferation: In hypocotyl explant the enzyme activity increased from 0.75 (0-day) to 0.85 (+13%) after one day of culture (Table 1). A sharp increase to 1.80 (+140%) was noted on 2nd day of culture. The enzyme activity remained at this level on 3rd day when callus was initiated and during the proliferation of main (primary) callus. The activity decreased by 22, 33 and 44% in 1st, 2nd and 3rd subcultures, respectively. At 4th subculture the enzyme activity dropped further to 0.90 (-50%), which was maintained during 5th and 6th subcultures. In cotyledon peroxidase activity increased from 0.55 (0-day) to 1.20 (118%) after one day of culture. The activity remained the same on 2nd day when callus became visible on the explant. The total activity of enzyme showed a moderate increase (16%) upto 2nd subculture in relation to the main (primary) culture. There was a sharp decline in enzyme activity during 3rd subculture (-68%) and then a steady level of fairly low activity at 0.30 (-75%) compared to the main callus was maintained in the subsequent subcultures. The internode explant had a low level of

Table 1. Total activity of peroxidases (units/mg tissue) in control and *in vitro* dedifferentiating explants, primary calluses and successive subcultures of soybean.

| Source | Explant (0-Day) control | Dedifferentiating explant | | Main culture | Subcultures | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 2 | 3 | 4 | 5 | 6 |
| Hypocotyl | 0.75 | 0.85(1)* (+13)** | 1.80(2) (+140) | 1.80 | 1.40 -22*** | 1.20 -33 | 1.00 -44 | 0.90 -50 | 0.90 -50 | 0.90 -50 |
| Cotyledon | 0.55 | 1.20(1) (+118) | | 1.20 | 1.40 +17 | 1.40 +17 | 0.38 -68 | 0.30 -75 | 0.30 -75 | 0.30 -75 |
| Internode | 0.31 | 0.64(2) (+110) | 1.00(3) (+223) | 1.00 | 1.10 +10 | 0.50 -5- | 0.25 -73 | 0.25 -73 | 0.25 -73 | 0.25 -73 |
| Leaf | 1.42 | 0.30(3) (-79) 0.96(5) (-32) | 0.80(4) (-44) | 0.96 | 0.72 -25 | 0.60 -37 | 0.45 -53 | 00.38 -60 | 0.38 -60 | 0.38 -60 |

\* Days after culture

\*\* Percentage increase or decrease in comparison with explant

\*\*\* Percentage increase or decrease in comparison with main culture

peroxidase activity (0.31) relative to hypocotyl and cotyledon explant. However, it also increased to 0.64 (110%) and 1.00 (223%) on 2nd and 3rd day of culture, respectively. The same level of activity was maintained on 4th day when callus masses covered the explant. The 1st subculture showed a slight increase (10%) in the total activity in relation to the main callus. There was however, a sharp decline in enzyme activity during 2nd subculture (-50%). The enzyme activity decreased further (-73%) in 3rd subculture and this low level was maintained during subsequent subcultures. The total activity of peroxidases in the leaf explant was highest (1.42) compared to other explants. However, a sharp decrease in activity was noted after 3 days in culture (-79%). There was an increase in peroxidase activity on 4th (0.80) and 5th (0.96) day of culture. The enzyme activity was still lower (-32%) compared to its explant on 6th day when callus appeared on the explant. The main callus of leaf had a low peroxidase activity (0.96) compared to other calluses and there was a further decrease in activity in successive subcultures. Minimum enzyme activity (-60%) compared to the main callus was recorded in the 4th and subsequent subcultures.

Changes in Pattern of Peroxidase Isozymes: Polyacrylamide gel electrophoresis profile for peroxidases from the hypocotyl explant (0-day) had three bands with Rfs 0.15, 0.45 and 0.65 (Fig.1). After two days of culture the band with Rf 0.15 disappeared while the other two bands greatly increased in intensity. This isozymic profile persisted during callus initiation in the main callus as well as in subcultures (Fig.1). In cotyledon, the peroxidases had a very low mobility in the gel. In the fresh explant only one weak band with Rf 0.15 was visualized (Fig.2). After one day of culture i.e., one day prior to
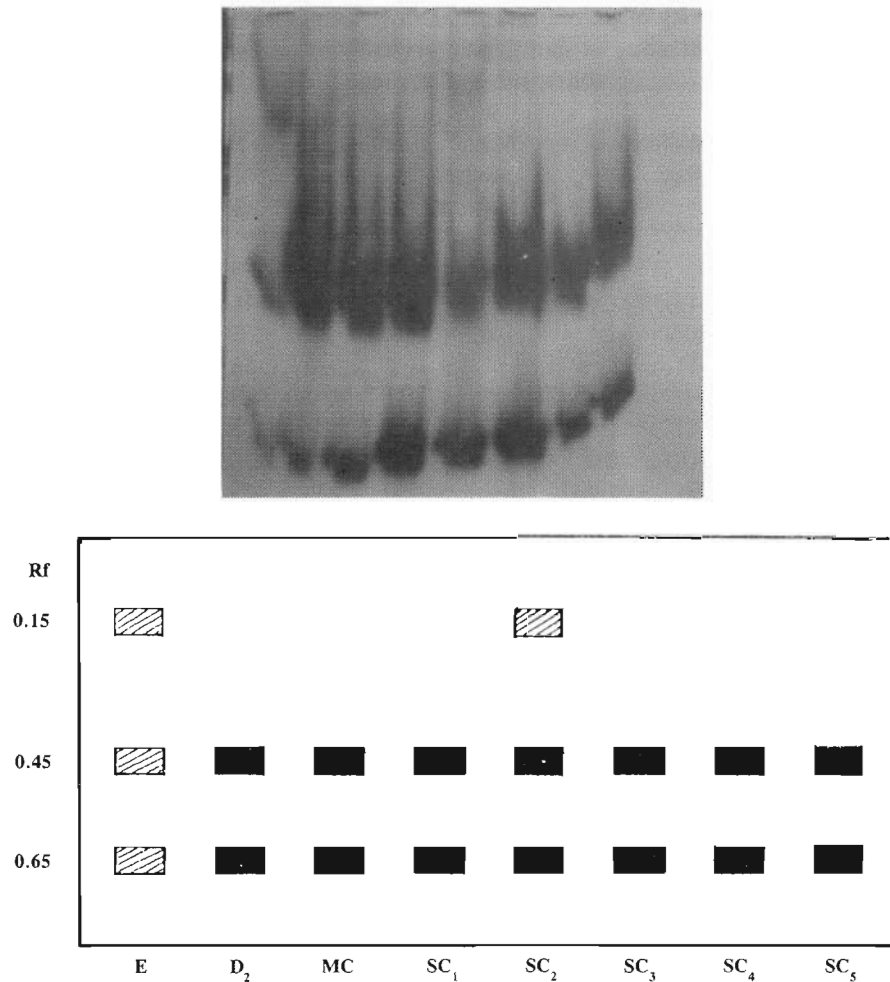
Fig.1. Electrophoretic pattern of peroxidases from hypocotyle explant before and during dedifferentiation, main callus and subcultures (E: Explant; $D_2$: Explant after 2 days in culture; MC: Primary callus; SC1-SC5: Subculture 1 to subculture 5; ▮ Dark bands; ▯ Light bands).

callus initiation, the explant again had only one band but with different Rf value (0.25) (Fig.2). The main callus derived from the explant had two bands of high intensity with Rf 0.15 and Rf 0.25. This isozyme pattern persisted during successive subcultures. The isozyme forms of peroxidases in the explant of internode (Fig.3) revealed three very weak bands with Rfs 0.50, 0.63 and 0.72. After two days of culture the explant again showed three bands but with different pattern (Fig.3). A weak band with Rf 0.25 appeared *de novo*. The band with Rf 0.50 showed a sharp increase in intensity while the band with Rf 0.63 disappeared. After three days in culture the enzyme with Rf 0.63 reappeared while the band with Rf 0.25 and 0.72 indicated a sharp increase in activity.
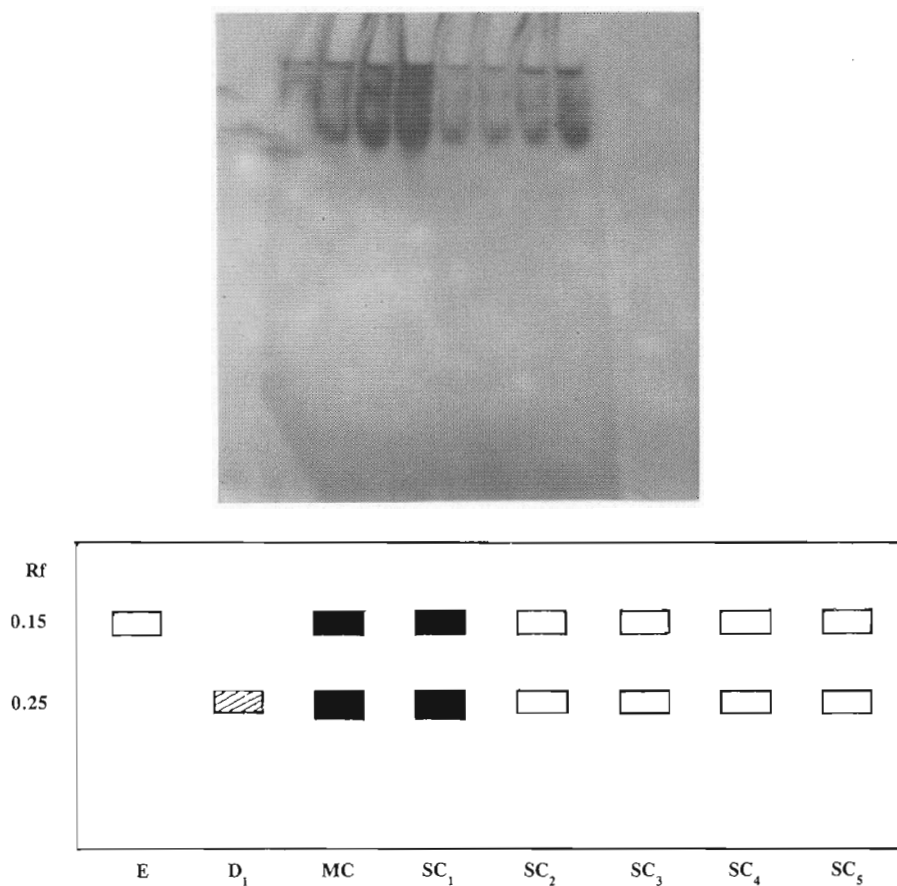
Fig.2. Electrophoretic pattern of peroxidases from cotyledonary explant during dedifferentiation, primary callus and subcultures (E: Explant; $D_1$: Explant after 1 day in culture; MC: Primary callus; SC1-SC5: Subculture 1 to subculture 5; ■ Dark bands; ▨ Light bands; ▢ Weak bands).

Thus there were four zones of peroxidase activity one day prior to the appearance of callus. The main callus and subcultures derived from it had only two bands. The explant of leaf (Fig.4) had three isozymes (Rfs 0.15, 0.30, 0.50). After three days of culture the band with Rf 0.15 disappeared and remaining two bands persisted during lag period (4th and 5th day) prior to callus initiation as well as during callus proliferation in the main callus and subcultures.
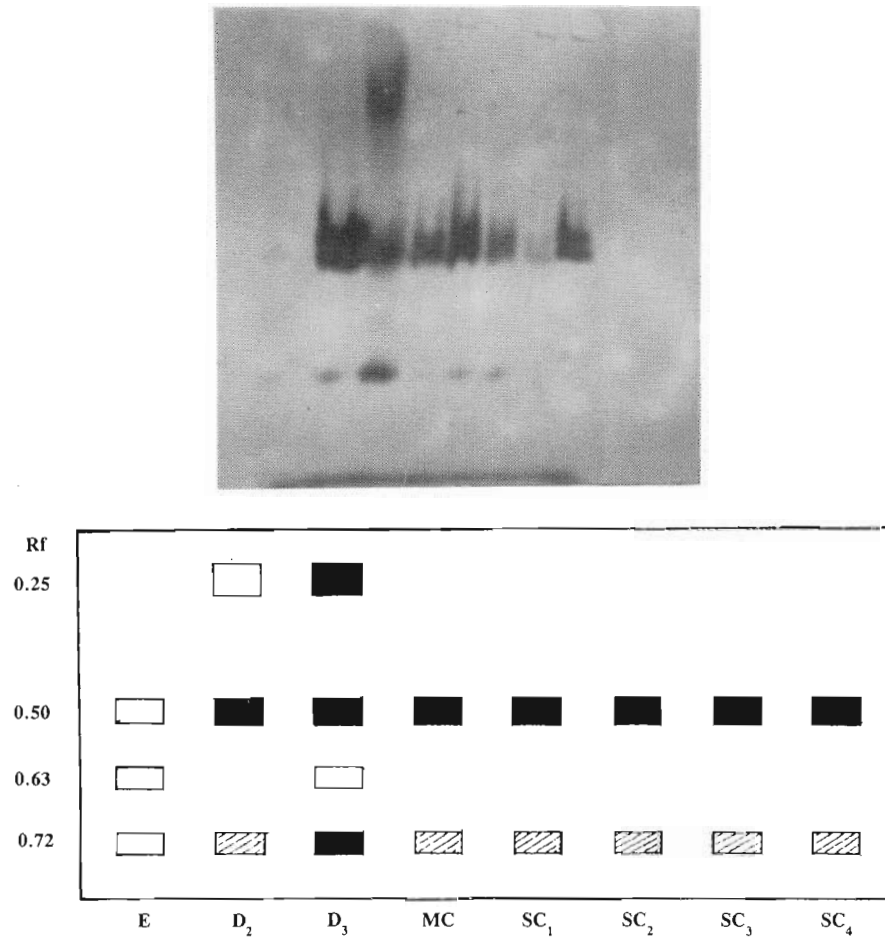
Fig.3. Eectrophoretic pattern of peroxidases from explant of internode before and during dedifferentiation, primary callus and subcultures (E; Explant; $D_2$: Explant after 2 days in culture; $D_3$: Explant after 3 days in culture; MC: Primary callus; SC1-SC5; Subculture 1 to subculture 4; ■ Dark bands; ▨ Light bands; [] Weak bands).

## Discussion

Total activity of peroxidase registered a sharp increase in hypocotyl, cotyledon and internode prior to callus initiation. In contrast, the total activity in leaf explant was highest compared to other explant and it showed a sharp decrease after 3 days of culture. The pattern of increase in total activity of peroxidase in explants of soybean other than leaf are similar to those observed in *Medicago* spp.,

Kawaoka *et al.*, (1994) have studied wound induced peroxidase activity in horse radish. Total peroxidase activity was increased by wounding in cell wall fractions
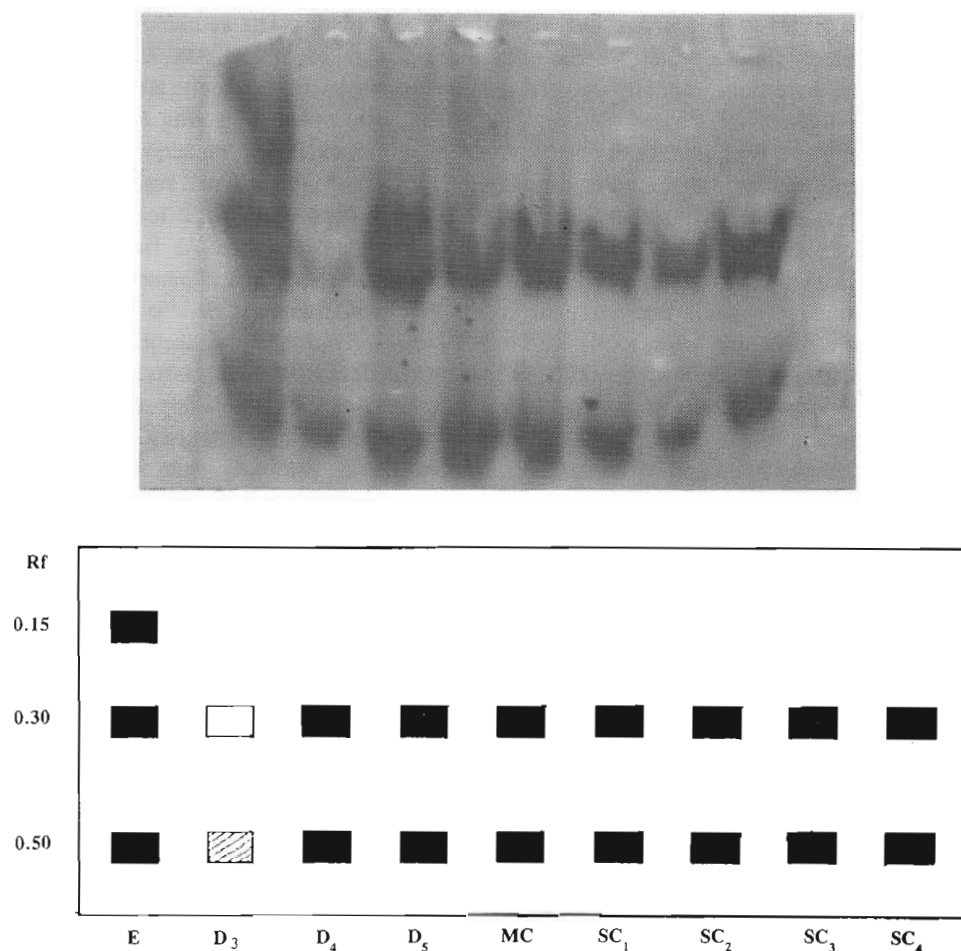
Fig.4. Electrophoretic pattern of peroxidases from leaf explant before and during dedifferentiation, primary callus and subcultures (E: Explant; $D_3$: Explant after 3 days in culture; $D_4$: Explant after 4 days in culture; $D_5$: Explant after 5 days in culture; MC: Primary callus; SC1-SC5: Subculture 1 to subculture 4; ▮ Dark bands; ▤ Light bands; [] Weak bands).

extracted from root, stem and leaf of horse radish. In soybean as well, the increase in total activity of peroxidase was probably due to stress of excision of cells and influence of *in vitro* plant growth hormones, the genes responsible for peroxidase synthesis are triggered into action. Kawaoka *et al.*, (1994) have isolated genomic clones for four peroxidase genes. Northern blot analysis using gene-specific probes showed that mRNA of $prxC_2$ which encodes a basic isozyme accumulated by wounding. It would suggest that dedifferentiation is basically an expression of totipotency of such cells that have not lost their mitotic machinery irreversibly. The stimulus of injury, phytohormone receptor function of an auxin-binding protein (Scherer & Andre, 1993), binding of 2,4-D to the chromatin protein (Yajima *et al.*, 1980) and finally gene action could be responsible

for enhanced levels of peroxidase activity in the three explants. The anomalous situation in leaf becomes simple when we consider the findings of Wakamatsu & Takahama (1993) where peroxidase activity in carrot tissue decreased immediately after excision and increased after 7-10 day of culture. In soybean, the explant of young and actively growing leaf had a high peroxidase activity (1.42) and on excision, the activity dropped drastically and increased again when the main factor i.e., wound induced reactivation of gene in highly specialized cells of mesophyll was achieved. This took longer period compared to parenchymatous cells of other three explants. Because the fresh explant had high peroxidase activity and it decreased after excision and then increased after 4-5 days of culture, it appears that isozymic forms involved in normal *in vivo* growth are different from those that are responsible for *in vitro* reactivation of quiescent cells.

Electrophoretic pattern of peroxidases during dedifferentiation supports this supposition. In hypocotyl, there were three bands, the band at Rf 0.15 disappeared during dedifferentiation, while other two bands greatly increased in their intensity. Explant of cotyledon had one band and its main callus had two bands. Similarly, internode explant had three very weak isozymes of peroxidases while there were four isozymes in dedifferentiating explant one day prior to callus initiation. Leaf explant had three isozymic forms, one of these (Rf 0.15) disappeared immediately after explanting. The other two became very faint and regained their intensity 1-2 days prior to callus initiation. The soybean plant contains at least 15-20 peroxidases (Huangpu *et al.*, 1995). Variations detected in the peroxidases during this study are similar to those reported in *Asimina triloba* (Hongwen *et al.*, 1997; Keming *et al.*, 1997).

A stable isozyme profile during subcultures despite decrease in total activity of peroxidase indicates a definite correlation between these two isozymic forms and continued meristematic activity. It is concluded that isozymes of peroxidase are specific at organ and tissue level and undergo dynamic changes during dedifferentiation while a uniformity exists between isozyme pattern of calluses of the different origin.

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