BIOLOGIA PLANTARUM 43 (2): 167-171, 2000

Biochemical and histological changes during *in vitro* organogenesis in *Jatropha integerrima*

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Abstract

Biochemical changes associated with adventitious shoot regeneration during *in vitro* culture of hypocotyl explants of *Jatropha integerrima* were determined. Histological and biochemical studies were undertaken at 7-d intervals, up to four weeks on hypocotyl explants cultured on basal Murashige and Skoog's medium supplemented with 0.5 mg dm^{-3} N^6-benzyladenine and 1.0 mg dm^{-3} indole-3-butyric acid. Initial cell proliferation occurred within one week of culture; meristemoid differentiation within two to three weeks and shoot development after four weeks. Peak activities of alkaline phosphatase, peroxidase and polyphenol oxidase was observed at day 14 indicating their involvement in the formation of meristematic centers. Protein accumulation and acid phosphatase activity were maximum at day 28.

Additional key words: alkaline phosphatase, peroxidase, polyphenol oxidase, tissue culture.

Introduction

The progress in *in vitro* regeneration of many plant species is remarkable. Nevertheless, regeneration of lot of them is still not easy achievable. Hence, there is a need to study the structural and biochemical aspects underlying initiation of organized development *in vitro*.

Jatropha integerrima (*Euphorbiaceae*) is one of the most important ornamental species cultivated in the tropics and subtropics. A very rapid shoot regeneration has been accomplished through adventitious organogenesis from seedling and mature plant parts (Sujatha and Dhingra 1993). Many parameters including the activities of a number of enzymes have been reported to change during shoot differentiation (Brown and Thorpe 1986). The present study was undertaken to determine the enzymatic changes associated with shoot regeneration from hypocotyl explants of *J. integerrima* and correlate them with the ontogenesis during *in vitro* culture.

Materials and methods

Plants: Hypocotyls from 15-d-old seedlings of *Jatropha integerrima* Jacq. were washed thoroughly under tap water and surface sterilized with sodium hypochlorite for 15 min, followed by four rinses with sterile distilled water. Explants were cut into 0.5 cm segments and aseptically cultured on Murashige and Skoog (1962) medium containing 3.0 % sucrose and 0.7 % agar (*Hi-media*, Mumbai, India). The medium was supplemented with 0.01 - 2.0 mg dm^{-3} BA in combination with 0.5 - 1.0 mg dm^{-3} IBA. The pH of each medium was adjusted to 5.8 prior to autoclaving at 104 kPa at 120 °C for 20 min. The cultures were incubated at temperature 26 ± 2 °C, relative humidity 55 ± 5 %, a 16-h photoperiod and irradiance of 30 μmol m^{-2} s^{-1} (cool fluorescent tubes).

Histology: Hypocotyl segments cultured on medium supplemented with 0.5 mg dm^{-3} BA and 1.0 mg dm^{-3} IBA were fixed at 3 d intervals up to 30 d after culture in FAA (formalin:acetic acid:alcohol 6:1:14). For correlation with changes in enzymatic activities, the histological changes at weekly intervals were taken into consideration.

Received 4 February 1999, *accepted* 7 July 1999.

Abbreviations: BA - N^6-benzyladenine; EDTA - ethylenediamine-tetra-acetic acid; FAA - formalin - acetic acid - alcohol; IBA - indole-3-butyric acid; TBA - tertiary butyl alcohol.

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Customary methods for dehydration in TBA series and embedding in paraffin wax were followed (Johansen 1940). Sections of 10 - 12 μm thickness were cut on a rotary microtome followed by staining with 0.1 % toluidine blue O.

Enzyme assays: Hypocotyl explants were harvested at 7 d interval (0 d up to 28 d of culture). The enzyme assays were carried out according to Sadasivam and Manickam (1992). Each assay was done in 3 replicates and each replicate had 5 culture tubes with 5 explants in each. Means of the enzyme activities were determined and the standard error was calculated.

Acid phosphatase (EC 3.1.3.2): Tissue was homogenized in ice-cold 50 mM citrate buffer (pH 5.3) (1:10 m/v) and filtered through four layers of cheese cloth. The homogenate was centrifuged at 10 000 *g* for 10 min (4 °C) and the resultant supernatant was used as enzyme source. 3 cm^3 of substrate solution (0.4 mM EDTA, 0.43 mM citric acid, 0.03 % *p*-nitrophenyl phosphate) were incubated for 5 min at 37 °C. To the substrate, 0.5 cm^3 of enzyme extract was added and further incubated for 15 min at 37 °C. Reaction was terminated at 0 (control) and 15 min with 0.085 M NaOH and the absorbance was measured at 405 nm. Specific activity was expressed in mmoles *p*-nitrophenol released per mg of protein by extrapolating on the standard curve.

Alkaline phosphatase (EC 3.1.3.1): Enzyme extraction and assay procedures were the same as that for acid phosphatase except for the extraction buffer (50 mM glycine-NaOH) and substrate solution (0.5 mM glycine, 0.01 % magnesium chloride, 0.17 % *p*-nitrophenyl phosphate in 0.042 M sodium hydroxide - pH 10.5).

Peroxidase (EC 1.11.1.7): Enzyme was extracted by homogenizing tissue in (1:3 m/v) 0.1 M phosphate buffer (pH 7.0, 4 °C) and obtaining the supernatant by centrifuging at 18 000 *g* for 15 min (4 °C). Phosphate buffer (pH 7.0; 3 cm^3), 0.05 cm^3 of 20 mM guaiacol and 0.03 cm^3 of 12.3 mM hydrogen peroxide were taken in a cuvette and the absorbance was set at 436 nm. The enzyme activity was calculated as the time required to increase the absorbance by 0.1 (Sadasivam and Manickam 1992).

Polyphenol oxidase (EC 1.14.18.1): Tissue (1 g) was homogenized in 0.1 M phosphate buffer (pH 7.0, 4 °C) and the homogenate was centrifuged at 5 000 g for 15 min (4 °C). The residue was re-extracted with fresh buffer and the final volume of the supernatant was made up to 10 cm^3. 1.5 cm^3 of freshly prepared buffered catechol (0.01 M in 0.1 M phosphate buffer, pH 6.0) was taken in a cuvette and the absorbance was set to zero at 495 nm. To this 0.5 cm^3 of enzyme extract was added and the absorbance was recorded after every 15 s. Change in absorbance per unit time was calculated from linear phase of the initial reaction.

Proteins were estimated according to Lowry *et al.* (1951).

Results

The hypocotyls swelled at the cut ends after 3 d of culture initiation and produced compact green calli from the swollen regions within 10 to 12 d after incubation. Fromthese calli protrusion of leafy shoots was observed within 20 d of culture. These leafy shoot-like structures developed into well formed shoots within a week after their appearance. Among the various combinations of BA-IBA tested, shoot proliferation was observed on medium supplemented with 0.1 to 2.0 mg dm^{-3} BA in combination with 1.0 mg dm^{-3} IBA (Fig. 1*A*). Hypocotyl segments cultured on medium supplemented with 0.01 - 0.05 mg dm^{-3} BA with 1.0 mg dm^{-3} IBA failed to respond and necrosis occurred. Medium fortified with 2.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA promoted only callus formation. Subculture of hypocotyls with leafy protrusions on to medium supplemented with 0.5 dm^{-3} of BA and IBA resulted in the development of well organized shoots (Fig. 1*B*). Elongated shoots rooted within 7-10 d on medium containing 1.0 mg dm^{-3} IBA (Fig. 1*C*). Rooted shoots were successfully acclimatized and transferred to field conditions (Fig. 1*D*).

Histological examination of *J. integerrima* hypocotyls showed zones of preferential cell division activity in the sub-epidermal and cortical regions at day 6 in culture which led to callus formation (Fig. 2*A*). The localized cell division resulted in the organization of meristematic centers, termed promeristemoids at day 9 (Fig. 2*B*). The formation of these meristematic zones resulted in the enlargement and degeneration of interspersed parenchyma cells leading to the formation of air spaces (Fig. 2*C*). The promeristemoids continued to develop, leading to the formation of meristemoids after 12 - 14 d which gave the hypocotyl segments a nodular appearance. Cells in each meristematic group were small, isodiametric, densely cytoplasmic with a prominent nucleus and compactly arranged without intercellular spaces. Three weeks after culture initiation, shoot bud differentiation was observed from the meristematic regions situated at the periphery (Fig. 2*D*). Shoot meristems were multicellular in origin and shoot bud formation followed the typical stages of differentiation as seen in other *in vitro* systems.

The enzyme activities and total protein were higher in cultured hypocotyls when compared to hypocotyls in time 0 (Table 1). However, the peak activity of the different enzymes varied with the stage of differentiation. Acid phosphatase activity increased steadily up to 28 d. Activity of alkaline phosphatase was higher on day 14 and declined there after. The peak activities of peroxidase and polyphenol oxidase were recorded in 14-d-old cultures associated with rapid differentiation. The total protein increased rapidly up to 28 d. Correlation studies indicated a negative correlation of polyphenol oxidase activity with acid phosphatase activity and total protein while all other correlations were positive. Correlations between acid phosphatase-protein and alkaline phosphatase-peroxidase were highly significant.

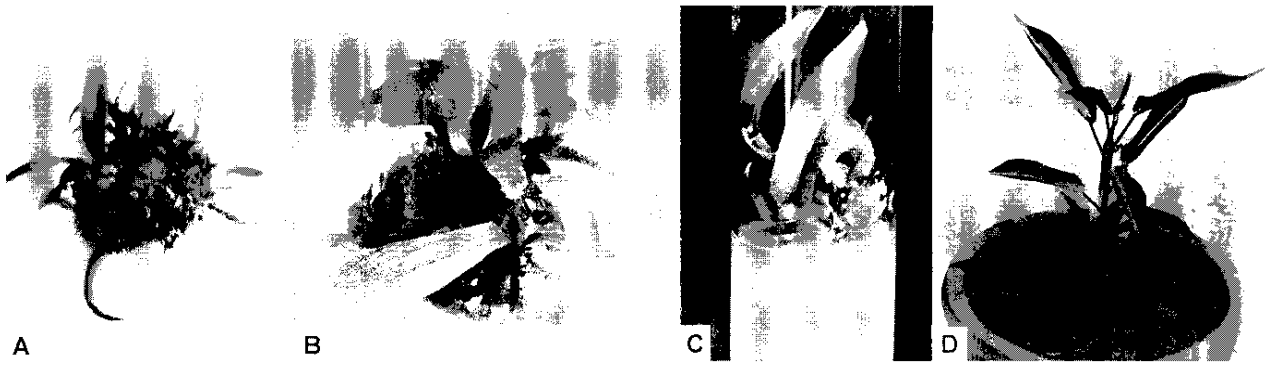

Fig. 1. Shoot regeneration from hypocotyl explants of *Jatropha integerrima*: *A* - shoot proliferation in 4-week-old hypocotyl on MS medium with 1.0 mg dm^{-3} BA and IBA; *B* - well developed shoots after two weeks of subculture on to MS medium with 0.5 mg dm^{-3} BA and IBA; *C* - rooted shoot on MS medium with 1.0 mg dm^{-3} IBA; *D* - transplanted plantlet after 5 weeks of transfer to vermiculite.

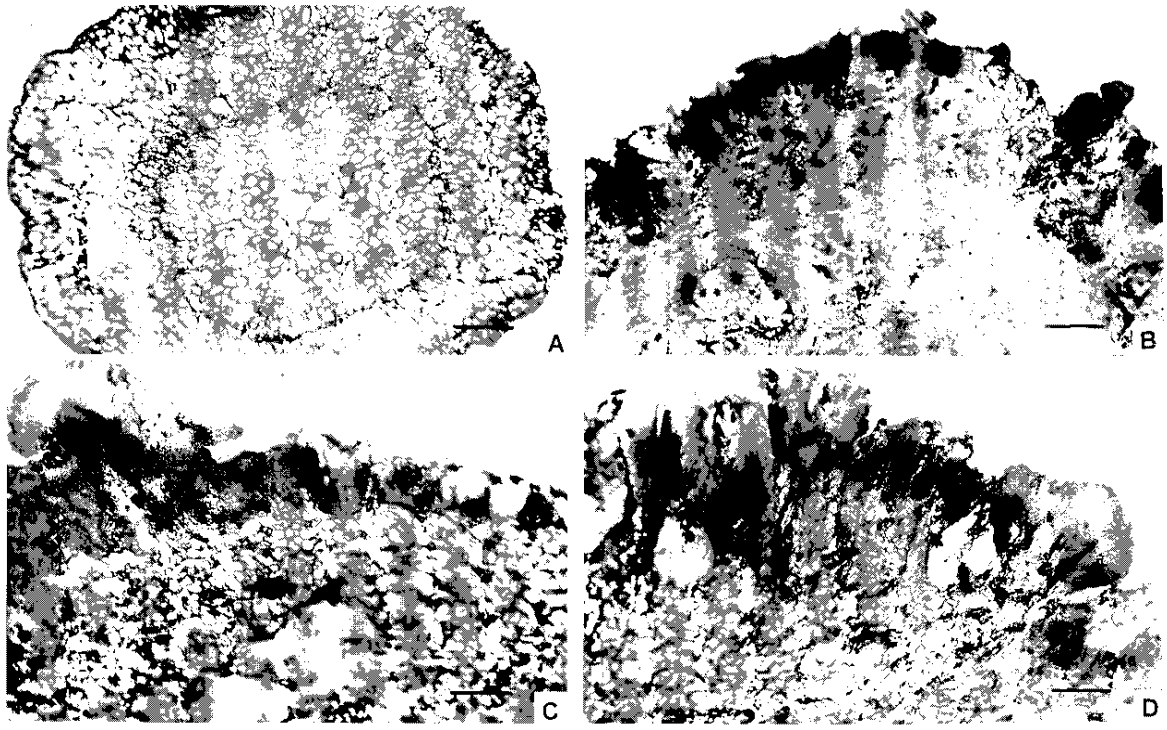

Fig. 2. Histological changes during organogenesis in hypocotyl explants of *Jatropha integerrima* (*bar* = 0.45 mm): *A* - sub-epidermal and cortical regions at day 6; *B* - promeristemoid formation at day 9; *C* - meristematic zones leading to formation of air spaces at day 14; *D* - shoot buds at the periphery at day 21.

Table 1. Activities (means ± SE) of acid phosphatase [mmol(*p*-nitrophenol released) min^{-1}], alkaline phosphatase [mmol(*p*-nitrophenol released) min^{-1}], peroxidase [U dm^{-3}], polyphenol oxidase [U mg^{-1}(protein)] and protein [mg g^{-1}(f.m.)] at weekly intervals in cultured hypocotyls of *Jatropha integerrima*.

Enzyme	0 d	7 d	14 d	21 d	28 d
Acid phosphatase	6.80 ± 0.20	8.17 ± 0.29	8.13 ± 0.03	9.31 ± 0.05	16.80 ± 0.20
Alkaline phosphatase	0.58 ± 0.02	1.29 ± 0.01	2.39 ± 0.01	2.30 ± 0.02	2.16 ± 0.03
Peroxidase	0.18 ± 0.01	0.23 ± 0.01	0.41 ± 0.01	0.34 ± 0.01	0.38 ± 0.02
Polyphenol oxidase	0.26 ± 0.01	0.30 ± 0.02	0.74 ± 0.02	0.47 ± 0.02	0.26 ± 0.01
Protein	19.10 ± 0.10	19.70 ± 0.20	21.60 ± 0.20	25.40 ± 0.20	30.02 ± 1.02

Discussion

Tissue/callus cultures have been extensively used for investigating factors regulating organogenesis (Brown and Thorpe 1986). In the present study, attempts were made to correlate morphogenic, histological and biochemical data in relation to shoot differentiation in cultured hypocotyls of *J. integerrima*.

Increase of peroxidase activity and alterations of its isozymes have been correlated with adventitious shoot formation and somatic embryogenesis (Kochba *et al.* 1977, Thorpe and Gaspar 1978, Kay and Basile 1987). Enzymes of the shikimate pathway were stimulated by culture variables promoting shoot formation (Beaudoin-Eagen and Thorpe 1983). In most of these cases, studies were undertaken to distinguish organogenic cells from non-organogenic cells. Furthermore, the studies were confined to one particular stage of development. However, in the present study detailed investigations were made to observe the biochemical changes during various stages of shoot differentiation. The activities of alkaline phosphatase, peroxidase and polyphenol oxidase were maximum at day 14 during meristemoid development while that of acid phosphatase was maximum at day 28 during shoot formation. Thus, it is evident that the peak activities of different enzymes vary with the development stage and are associated with specific changes during the morphogenic processes.

In cultured hypocotyls of *J. integerrima*, protein accumulation increased gradually from day 0 to 28 d indicating active protein synthesis during all stages of organo-genesis. Dougall (1962) and Syono (1965) suggest a definite role for protein synthesis in the organogenetic processes. Thorpe and Murashige (1970) also observed denser staining for protein in shoot forming regions just before and during the formation of meristemoids and shoot primordia. In the studies of Patel and Thorpe (1984) protein accumulation was reflected in the changes in succinic dehydrogenase, peroxidase, acid phosphatase and ATPase in shoot forming regions. In the present study, positive correlations existed between total protein content and activities of acid phosphatase, alkaline phosphatase and peroxidase, and especially of acid phosphatase.

Transverse sections of *J. integerrima* hypocotyls indicated a direct origin of the buds from subepidermal and adjoining cell layers. Similarly in *Euphorbia pulcherrima*, some cortical cells of hypocotyls divided actively and developed into shoot buds (Nataraja *et al.* 1973). This clearly indicates that although all plant cells are totipotential, the expression is limited to relatively few cells which respond to variations in milieu to differentiate into shoots, roots or embryos. Changes in activities of a wide range of enzymes indicates multiple metabolic processes involved in cell division and differentiation. Thus, manipulations are possible only with meristemoids or cells that could be readily transformed into meristemoids and it would be difficult to manipulate non-totipotent cells by varying the culture environment thereby, limiting the scope for regulating the process of shoot organogenesis.

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