

## Biomolecular changes during *in vitro* organogenesis of *Asteracantha longifolia* (L.) Nees — A medicinal herb

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High frequency plant regeneration in *A. longifolia* (L.) was achieved from leaf explant implanted on MS basal medium supplemented with NAA (0.5 mg/l) + BA (2.0 mg/l) through intervening callus phase. Well-developed shoots (>3cm) were successfully rooted on MS medium supplemented with NAA (0.1 mg/l). Protein and total soluble sugar contents were maximum during organogenesis and multiple shoot induction phase compared with non-organogenic callus and root induction phase. Esterase and catalase activities were maximum during organogenic differentiation, while activities were minimum at non-differentiated callus stages. Peroxidase activities were higher during rhizogenesis. Contradiction to peroxidase activity, acid phosphatase activities were high during organogenesis and declined during rhizogenesis. SDS-PAGE analysis of total soluble proteins revealed expression of non-organogenic callus (97.9 kDa), organogenic callus (77.2, 74.1, 21.9 kDa), multiple shoot induction phase (106.6, 26.9, 11.6 kDa) and root induction phase (15.9 kDa) specific polypeptides. Esterase zymogram revealed one band (Rm 0.204) appeared in both organogenic callus and multiple shoot induction phase. Peroxidase zymogram detected two stage specific bands, one band (Rm 0.42) was specific to root induction phase, while another (Rm 0.761) was specific to multiple shoot induction. Catalase and acid phosphatase zymogram resolved one band (Rm 0.752 and 0.435, respectively) in differentiated stages including both multiple shoot induction phase and root induction phase, but absent in undifferentiated phases.

**Keywords:** *Asteracantha longifolia*, Organogenesis, Enzyme activity, SDS-PAGE, Shoot bud, Zymogram.

*Asteracantha longifolia* (L.) Nees syn. *Hygrophila auriculata* (Schum) Heine (Family- Acanthaceae), is an important medicinal herb and is extensively used in traditional system of medicine for various ailments like diuretics, jaundice, diopsy, rheumatism, hepatic obstructions and dissolution of gallstones<sup>1</sup>, kidney stones<sup>2</sup>, liver dysfunction<sup>3</sup> and diseases of urino-genital tracts<sup>4</sup>. The plant has been used in several ayurvedic preparations—Lukol, Speman, Confindo etc. by Himalaya Health Care Pvt. Ltd. (www.Himalayahealthcare.com), Breastone by Vedic Biolabs Pvt. Ltd. (www.vedicbiolabs.com), Biogest and Rasanagugul by Trihealth Care, Kerala Ayurveda Pharmacy Division (www.oilbath.com) and Microlactin by Victoria Health Care Ltd. (www.victoriahealth.com). The extensive use in several herbal preparations pose a positive threat for the plant, to the extent of being extinct. *In vitro* culture technique provides an alternative way of plant propagation and germplasm conservation<sup>5</sup>. Plant

regeneration via organogenesis has already been reported using leaf, node and internode explants<sup>6</sup>. Mishra *et al.*<sup>6</sup> have reported that non-organogenic callus grows in unorganized manner, but the organogenic callus grows very fast and gives rise to multiple shoot buds. The different morphological appearance and regeneration potential of non-organogenic and organogenic callus might be either due to differences in gene expression or due to different bio-molecules composition. The biochemical changes have been observed during *in vitro* morphogenesis in different plant species<sup>7-10</sup>. Iso-esterase<sup>11,12</sup>, iso-peroxidase<sup>13-15</sup>, catalase<sup>10,16,17</sup> and acid phosphatase<sup>10,18</sup> activities are most commonly used in several species as parameter to monitor the differentiation pathway.

Polypeptide expression varies depending upon the different developmental stages due to differential gene expression of concerned structural or regulatory gene(s), which are under the control of master gene. The expression is *albeit* less influenced by media composition, explant type and culture environment. Deletion or mutation of structural genes coding for polypeptides or their regulatory loci results in the

inhibition of transcription or translation of polypeptides<sup>19</sup> leading to lack of expression of concerned polypeptides. Polypeptides, polymorphic for presence or absence could be potentially used as marker to decipher the differentiation pathway and selection of organogenic potential callus or tissue<sup>20,21</sup>. Polypeptide variation also been used to monitor the organogenesis and embryogenesis in several species<sup>9,22</sup>. Isoenzymes are multiple molecular forms of enzymes catalyzing identical reactions within tissues of an organism<sup>23</sup>. Isoenzymes provide a natural in-built marker system for biochemical, developmental and genetic studies. Isoenzymes may also be used as marker for differentiating non-embryogenic callus from the embryogenic callus<sup>24</sup>, non-organogenic callus from the organogenic callus or shoot initiating culture<sup>14</sup>.

Therefore, the present study was initiated to decipher the changes of bio-molecular activity during *in vitro* organogenesis of *A. longifolia*.

### Materials and Methods

**Plant material**—Seeds of *A. longifolia* were collected from paddy fields of Narayanapatna, Orissa and the plants were maintained in the experimental garden of Division of Biotechnology, MITS, Rayagada, Orissa, India. Seeds washed with 5% (v/v) Teepol for 2 min were surface sterilized with 70% alcohol for 45 sec followed by 5 min of soaking in 0.1% (w/v) HgCl<sub>2</sub> (E-Merck, India). The sterilized seeds were washed thoroughly with sterilized double distilled water and implanted on Murashige and Skoog's (MS) medium<sup>25</sup>. Leaves were excised from 12 days old seedlings used as explant, size ranged from 20-25 mm<sup>2</sup>.

**Culture medium**—The culture medium consisted of MS medium with 100mg/l meso-inositol (Hi-media, India) and 3% (w/v) sucrose (Qualigen, India). The medium was augmented with different concentrations and combinations of N<sup>6</sup>-benzyladenine (BA), kinetin (Kn) and  $\alpha$ -naphthalene acetic acid (NAA); (Hi-media, India). The pH of the medium was adjusted to 5.8 prior to gelling with 0.8% agar-agar (bacteriological grade, Hi-media, India). One explant was implanted in each tube and cultures were incubated at 25 $\pm$ 1 $^{\circ}$ C, 60-70% relative humidity and 16 hr photoperiod of 35  $\mu$  Em<sup>-2</sup>s<sup>-1</sup> irradiance level provided by cool white fluorescent tubes (Philips, India).

**Induction of rooting and transplantation**—Regenerated shoots with 5-6 leaves (>3cm long) were

transferred to MS medium with different concentrations of NAA and indole butyric acid (IBA). Cultures were incubated as described previously. Plantlets with well-developed roots were transferred to a plastic cup containing sand and soil (1:1) and acclimatized following routine protocol<sup>6</sup>.

**Data and statistical analysis**—Visual observations were made every week and data on explant response, number of shoot buds/shoots per explant and number of roots per shoot were recorded at the end of 8<sup>th</sup> week. Each phytohormone treatment consisted of five replications and repeated thrice. Data on shoot bud regeneration, multiple shoot production and rooting were statistically analyzed using completely randomized block design and means were evaluated at  $P=0.05$  level of significance using Duncan's multiple range test<sup>26</sup>. For this SPSS 8.0.1 software used with parameters — one way ANOVA and homogeneity of variance.

**Biomolecular analysis**—Tissue samples were collected at four different developmental stages— at non-organogenic callus (after 4 weeks of culture from the callusing medium), organogenic callus (after 5-6 weeks of culture), organogenic callus with multiple shoot initials (after 8 weeks of culture from the differentiating medium) and at rooting stages (after 1 week of culture in rooting medium) for both quantitative and qualitative analysis. Total soluble sugar was estimated using phenol-sulphuric acid method<sup>27</sup>.

**Extraction of protein and gel electrophoresis**—Tissue samples were collected as described previously. The proteins were extracted by homogenizing the tissue sample in pre-chilled mortar and pestle using 2.5 ml of extraction buffer consisting of 0.2M, tris hydroxymethyl aminomethane (Tris); 0.001M ethylene diamine tetra acetic acid (Na<sup>2+</sup>EDTA); 12%, glycerol; 0.01M, dithiothreitol (DTT); and 0.05mM phenyl methyl sulfonyl fluoride (PMSF). The samples were centrifuged at 15000  $\times$  g for 15 min, and the supernatant was used for determination of total protein content and sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). The conventional UV method<sup>28</sup> was used for estimation of protein using bovine serum albumin (BSA) as standard protein. The protein sample was added with an equal volume of cracking buffer (0.125M, Tris.Cl; pH 6.8; 4%, SDS; 20%, glycerol; 10%,  $\beta$ -mercaptoethanol and 0.01%, bromophenol blue) and was denatured by boiling in waterbath at

90°C for 3 min. Protein samples (~500µg) were electrophoresed in a discontinuous SDS-polyacrylamide gel following Laemmli<sup>29</sup> using a 12% resolving gel (0.375 M, Tris. Cl; pH 8.8) and 4% stacking gel (0.125M, Tris-Cl; pH 6.8) in Tris-glycine buffer (0.025M, Tris; pH 8.3; 0.192M, glycine; 0.1%, SDS) for 16 hr, constantly at 20mA. Staining of the gel was done using 0.2% (w/v) Commassie Brilliant Blue R-250 in 12.5% (w/v) trichloroacetic acid (TCA). The position of the protein band in the gel was expressed to compare with standard protein markers with known molecular weight.

### Enzyme extraction and assay

**Esterase**—Fresh tissue samples (100mg each) at four different phases, as mentioned earlier, were homogenized in pre-chilled mortar and pestle using 1ml of chilled extraction buffer containing 0.2 M, Tris-Cl; pH. 7.0; 0.001M, EDTA; 1M, sachharose; 0.056 M,  $\beta$ -mercaptoethanol<sup>30</sup>. The crude homogenate was centrifuged at 12500× g for 30 min at 4°C to remove the cellular debris and the supernatant was used for both enzyme assay and electrophoresis. The esterase activity was determined using indo-phenyl acetate as substrate at 625nm. One unit of esterase activity was expressed as absorbance change of 1.0 unit per min.

**Peroxidase**—Fresh tissue samples (100mg each) at four different stages, as mentioned earlier, were homogenized in pre-chilled mortar and pestle using 2ml of pre-chilled 0.1 M, phosphate buffer containing 1% (w/v) of polyvinyl pyrrolidone (PVP) and 0.5% of sodium ascorbate<sup>10</sup>. The crude homogenate was filtered through four layers of cheese cloth and centrifuged at 12500× g for 30 min at 4°C. The supernatant was used for both enzyme assay and electrophoresis. The assay mixture contained 0.1M, phosphate buffer (pH 6.1); 4mM, guaicol as donor; 3mM, H<sub>2</sub>O<sub>2</sub> as substrate; and 0.4 ml, crude enzyme extract. The total reaction volume was 1.6 ml. The rate of change in absorbance (OD) at 420 nm was measured using UV-vis spectrophotometer (UV1601, Shimadzu, Japan). The enzyme activities were expressed as OD difference/minute/mg protein<sup>31</sup>.

**Catalase**—Fresh tissue samples (100mg each) at four different stages, as mentioned earlier, were homogenized in pre-chilled mortar and pestle using 1ml of chilled extraction buffer<sup>10</sup> containing 0.2M, Tris-Cl (pH 8.5); 5mM, Na<sup>++</sup>-EDTA; 5mM, DTT; 10%, (w/v) PVP; and 0.5mM, PMSF. The crude

homogenate was filtered through four layers of cheese cloth and centrifuged at 12,500 g for 30 min at 4°C. The supernatant was used for both enzyme assay and electrophoresis. The catalase activity was determined by floating discs method<sup>32</sup>. Crude supernatant (10µl) was applied to Whatman 42 filter paper disc (6mm) and placed in a vial containing 5 ml of 30 mM H<sub>2</sub>O<sub>2</sub> at 25°C. Ten discs were used separately for each crude extract. The activity of catalase in each phase was calculated according to the activity of Bovine liver catalase ( $\sigma = 11,000$  U/mg protein). One unit of catalase activity was expressed as micromole of H<sub>2</sub>O<sub>2</sub> degraded per min at 25°C.

**Acid phosphatase**—Fresh tissue samples (100mg each) at four different stages, as mentioned earlier, were homogenized in pre-chilled mortar and pestle using 1ml of pre-chilled 0.05 M Tris maleate buffer (pH 5.2) under ice box and kept at 5°C for 30 min. The crude homogenate was filtered through four layers of cheese cloth and centrifuged at 12500× g for 20 min at 4°C. The supernatant was used for both enzyme assay and electrophoresis. The assay mixture contained 0.05M of Tris-maleate buffer (pH 5.2), 10mM of *p*-nitrophenyl phosphate (PNPP) as substrate and 0.1 ml of crude enzyme extract. Total volume of the mixture was 1.0 ml. The mixture was incubated at 37°C. The rate of change in absorbancy (OD) at 405 nm was measured using UV-Vis spectrophotometer (UV1601, Shimadzu, Japan) for both zero time assay and full time assay. The enzyme activities were expressed as 10<sup>-7</sup> mole of *p*-nitrophenol released per min per mg protein<sup>33</sup>.

**Iso-enzyme marker analysis**—All the isoenzymes were separated into discrete bands by discontinuous native polyacrylamide gel electrophoresis (PAGE) using 7.0% resolving gel (0.375M Tris-Cl; pH. 8.8) and 4% stacking gel (0.125M Tris-Cl; pH 6.8) in Tris-glycine buffer (0.025M Tris; pH 8.3; 0.192M glycine). Gels were precooled at 4°C prior to electrophoresis. Extracts were prepared by using 1/10<sup>th</sup> vol of tracking dye (sucrose 10%; bromophenol blue 0.01%). Electrophoresis was performed in dark at 4°C and 150v for 180 min. After electrophoresis, the gels were stained for esterase, catalase, peroxidase and acid phosphatase activity separately. For esterase activity, the gel was incubated at 37°C using 0.2 M phosphate buffer (pH 6.1) containing 1.31mM,  $\alpha$ -naphthyl acetate; 0.17mM, acetone; and 2.79mM, Fast blue RR salt<sup>30</sup>. For peroxidase activity the gel was stained at room temperature (in dark) using 0.2 M sodium

acetate buffer (pH 5.0) containing 1.3 mM, benzidine and 1.3 mM, H<sub>2</sub>O<sub>2</sub><sup>34</sup>. For catalase activity, the gel was incubated in substrate solution containing 3.3mM H<sub>2</sub>O<sub>2</sub> followed by staining solution containing equal proportion of 1% potassium ferric cyanide and 1% ferric chloride<sup>35</sup>. For acid phosphatase activity the gel was incubated at room temperature (in dark) using 0.1M sodium acetate buffer (pH 5.2) containing 0.1%,  $\alpha$ -naphthyl acid phosphate; and 0.1%, Fast blue BB salt. The position of isoenzyme band in the gel was expressed as relative mobility (Rm) by measuring the distance traveled by the particular band in comparison to tracking dye font.

### Result and Discussion

*In vitro organogenesis*— Surface sterilized seeds were cultured directly on MS basal medium without any growth regulators in the culture flasks showed 35-50% germination after seven days and 90-95% germination after ten days. The leaf explants (20-25mm<sup>2</sup>) were excised from the aseptic seedlings and used for *in vitro* studies. The implanted leaf segments, enlarged and produced protuberances from the cut ends within 7-10 days of culture (Fig. 1A) on semisolid MS medium supplemented with BA (0.5-2.5mg/l). By the end of 5<sup>th</sup> week, these protruded structures developed into shoot buds without intervening callus phase and 80±2% of them were flanked by green leaves (Fig. 1B). By the end of 8<sup>th</sup> week, well-developed shoots (Fig. 1D) were obtained on the same medium. Maximum number of healthy shoot/tubes (17.4±0.24) was obtained in MS medium with BA (2.0mg/l) and NAA (0.2mg/l) (Table 1). Similar result with combination of NAA and BA in inducing multiple shoot buds has been observed earlier in several species<sup>36-40</sup>.

The leaf explants implanted on MS medium with other combinations of hormones (Table 1) gave rise to multiple compact callus by the end of 6<sup>th</sup> week (Fig. 1C). By the end of 8<sup>th</sup> week, the number of shoot initial/explants was maximum (22.8±0.37) in MS medium with BA (2.0mg/l) and NAA (0.5mg/l; Fig. 1E). Similar result has been obtained on *Centella asiatica*<sup>41</sup>.

For root induction, healthy individual shoots (>3cm long) were separated and transferred to MS medium with NAA (0.1, 0.2 & 0.5mg/l) and IBA (0.1, 0.2 & 0.5mg/l). Root induction was observed in all combinations (Table 2). However, the average number of root and root length was better on half strength MS medium containing 0.1mg/l of NAA

(Fig. 1F). After two weeks the rooted plants were transferred to plastic cups containing sand and soil mixture (1:1) and were maintained in culture room for two weeks (Fig. 1G) and then transferred to shade and then to soil. The survival rate is 80±4%.

*Changes in biomolecular composition during in vitro organogenesis*—Biomolecular analysis has been carried out at four different stages of regeneration,

Table 1—Effect of plant growth regulators on multiple shoot bud regeneration from leaf explant of *A. longifolia* after four weeks\*

[Values are mean ± SE of 3 replications]

Medium code	Plant growth regulator conc. (mg/l)		No. of shoot buds
	BA	NAA	
M <sub>1</sub>		Control	0.0±0.0 <sup>a</sup>
M <sub>2</sub>	0.5	-	9.6±0.24 <sup>f</sup>
M <sub>3</sub>	1.0	-	10.4±0.24 <sup>fg</sup>
M <sub>4</sub>	1.5	-	11.2±0.2 <sup>g</sup>
M <sub>5</sub>	2.0	-	3.4±0.24 <sup>c</sup>
M <sub>6</sub>	2.5	-	3.8±0.2 <sup>c</sup>
M <sub>7</sub>	0.5	0.2	3.8±0.2 <sup>c</sup>
M <sub>8</sub>	1.0	0.2	3.4±0.24 <sup>c</sup>
M <sub>9</sub>	1.5	0.2	9.6±0.24 <sup>f</sup>
M <sub>10</sub>	2.0	0.2	17.4±0.24 <sup>h</sup>
M <sub>11</sub>	2.5	0.2	4.8±0.37 <sup>d</sup>
M <sub>12</sub>	0.5	0.5	10.8±0.4 <sup>g</sup>
M <sub>13</sub>	1.0	0.5	18.2±0.2 <sup>hi</sup>
M <sub>14</sub>	1.5	0.5	19.0±0.32 <sup>i</sup>
M <sub>15</sub>	2.0	0.5	22.8±0.37 <sup>j</sup>
M <sub>16</sub>	2.5	0.5	10.8±0.49 <sup>g</sup>
M <sub>17</sub>	0.5	1.0	7.6±0.4 <sup>e</sup>
M <sub>18</sub>	1.0	1.0	6.8±0.37 <sup>e</sup>
M <sub>19</sub>	1.5	1.0	10.4±0.24 <sup>fg</sup>
M <sub>20</sub>	2.0	1.0	2.2±0.44 <sup>b</sup>
M <sub>21</sub>	2.5	1.0	0.0±0.0 <sup>a</sup> (callus)

\*Means within a column having the same letter are not statistically significant ( $P=0.05$ ) according to Duncan's multiple range test (SPSS V 8.0.1)

Table 2—Influence of two auxins on rooting of in regenerated shoots of *A. longifolia* after 3 weeks\*

[Values are mean ± SE of 3 replications]

Media code	Plant growth regulator conc. (mg/l)		No. of roots developed per shoot
	NAA	IBA	
R <sub>1</sub>		Control	10.8±0.58 <sup>a</sup>
R <sub>2</sub>	0.1	-	32.4±1.96 <sup>d</sup>
R <sub>3</sub>	0.2	-	24.8±1.16 <sup>c</sup>
R <sub>4</sub>	0.5	-	20.4±0.24 <sup>b</sup>
R <sub>5</sub>	-	0.1	9.8±0.49 <sup>a</sup>
R <sub>6</sub>	-	0.2	27.8±2.52 <sup>c</sup>
R <sub>7</sub>	-	0.5	20.6±0.4 <sup>b</sup>

\*Values within a column having the same letter are not statistically significant ( $P=0.05$ ) according to Duncan's Multiple range test (SPSS V 8.0)



Fig. 1—Plant regeneration in *Asteracantha longifolia*. (A)—Protuberances at the cut end of leaf explant implanted on MS+ 1.5 mg/l BA; (B)—Multiple shoot initials after 5<sup>th</sup> week on MS BA (1.5mg/l); (C)—Greenish, hard, compact calli developed after 6<sup>th</sup> week on MS + BA (2.0mg/l) + NAA (0.5mg/l); (D)—Well developed multiple shoots after 8<sup>th</sup> week; (E)—Shoot bud regeneration from the callus after 8<sup>th</sup> week; (F)—Shoot showing profuse rooting on MS + NAA (0.1mg/l); and (G)—A set of regenerated plantlets in plastic cups containing sand and soil mixture

including non-organogenic callus, organogenic callus, organogenic callus with multiple shoot initials and rhizogenesis, to analyse the role of biomolecules content during regeneration. The protein content was maximum at organogenic callus with multiple shoot initials followed by organogenic callus, non

organogenic callus and rooting (Table 3). This might be due to synthesis of certain amino acids / polypeptides required to initiate shoot bud formation and their depletion led to rhizogenesis. The total soluble sugar content was maximum at organogenesis as compared with those at callusing and root



induction which might be due to the hydrolysis of reserve polysaccharides and depletion of non-reducing sugars to meet the energy requirement during organogenesis<sup>10,42</sup> (Table 3).

*Changes in enzyme activities during in vitro organogenesis*—Enzymes, which are known as metabolic markers change during development and differentiation. During morphogenesis certain proteins and enzymes are responsible for callus proliferation and differentiation into shoot buds<sup>43</sup>. The results showed that esterase and catalase activities were maximum in differentiated stages, while the activities were minimum at non-differentiated callus stages

Table 3—Analysis of biochemical components (protein and total soluble sugar) and enzyme activity (esterase, peroxidase, catalase and acid phosphatase) during organogenesis of *A. longifolia*\*

Parameters	Stages of differentiation#			
	A	B	C	D
Total protein ( $\mu\text{g/g}$ )	0.79 $\pm$ 0.32	2.39 $\pm$ 0.3	2.41 $\pm$ 0.2	0.68 $\pm$ 0.32
Total soluble sugar ( $\mu\text{g/g}$ )	12.46 $\pm$ 0.3	20.34 $\pm$ 0.4	21.36 $\pm$ 0.3	11.22 $\pm$ 0.3
Esterase activity	2.72 $\pm$ 0.4	1.54 $\pm$ 0.2	5.78 $\pm$ 0.2	5.38 $\pm$ 0.4
Peroxidase activity	3.82 $\pm$ 0.3	2.35 $\pm$ 0.2	7.62 $\pm$ 0.3	8.32 $\pm$ 0.2
Catalase activity	1.68 $\pm$ 0.1	2.39 $\pm$ 0.1	8.95 $\pm$ 0.2	8.72 $\pm$ 0.3
Acid phosphatase activity	4.87 $\pm$ 0.96	3.08 $\pm$ 0.8	5.36 $\pm$ 0.96	1.92 $\pm$ 0.4

\*Callus and shoot initials are derived from leaf explants.

A= Non-organogenic callus; B= Organogenic callus; C= Organogenic callus with shoot initials; and D= Rhizogenesis

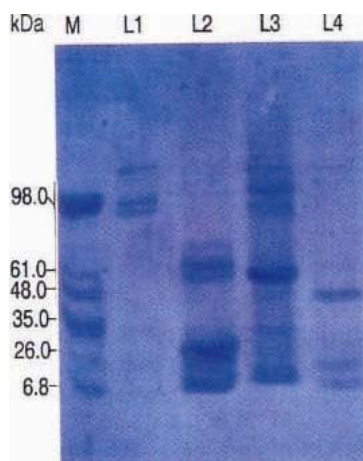


Fig. 2—Electrophoregram exhibiting changes polypeptide banding pattern at four different stages during organogenesis of *A. longifolia*. Lane (M)— MWM in KDa; Lane (L1)—Non-organogenic callus; (L2)—Organogenic Callus; lane (L3)—Organogenic Callus with multiple shoots; and Lane (L4)—Rhizogenesis.

(Table 3). Peroxidase activities were maximum during rooting and minimum at organogenic callus stage (Table-3). Contradiction to the peroxidase activity, the acid phosphatase activities were minimum during rooting and were maximum during shoot bud induction (Table 3). This variation in enzyme level can be used as indicator to monitor the differentiation pathway. Peroxidase activity seems to be sensitive to plant hormones and its activity may be important in controlling of auxin degradation, lignification, growth and differentiation<sup>13,44,45</sup>. Peroxidase activity can be used as indicator for organogenic differentiation in several species<sup>10,46,47</sup>. The increased peroxidase activities during differentiation might be due to accumulation or synthesis of high phenolic substances<sup>47</sup>.

*Expression of polypeptides during in vitro organogenesis*—SDS-PAGE analysis revealed 13 distinct polypeptides with molecular weight 110.9 to 6.8 kDa (Fig. 2). Out of these, one polypeptide 97.9 kDa was specific to the non-organogenic callus phase, while three polypeptides (77.2, 74.1, 21.9 kDa) were

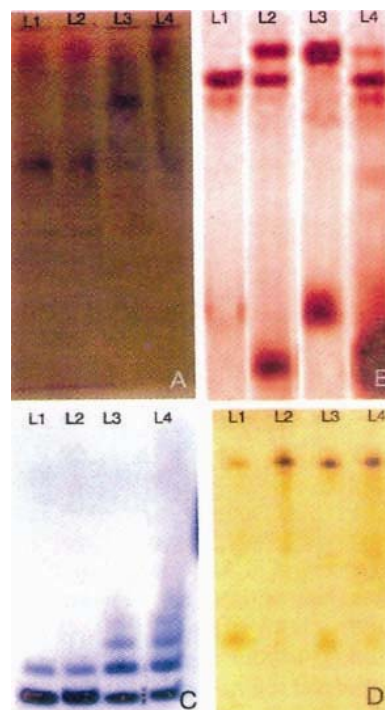


Fig. 3 A-D—Zymograms exhibiting changes banding pattern (A)—Isoesterase; (B)—Isoperoxidase; (C)—Catalase; and (D)—Acid phosphatase at four different stages. Lane (L1)—Non-organogenic callus; Lane (L2)—Organogenic callus; Lane (L3)—Organogenic callus with multiple shoots; and Lane (L4)—Rhizogenesis.

specific to organogenic callus phase. Similarly, one polypeptide (15.9 kDa) was expressed only during root induction phase and three polypeptides (106.6, 26.9, 11.6 kDa) were expressed only at multiple shoot induction phase. Expression of specific polypeptides at these stages may be used as markers to characterize differentiation pathway and to augment the selection of regenerating potential callus for rapid *in vitro* propagation.

*Changes in zymogram profile during in vitro organogenesis* — Isoenzymes are catalyzing identical reactions during growth and differentiation and it has been regulated by application of growth regulators. It follows codominance mode of inheritance. Thus, it can be used as potential genetic marker system to characterize developmental pathway.

*Esterase* zymogram revealed homogenous banding pattern during four developmental stages in the present study. Four distinct bands with Rm values ranging from 0.09 to 0.57 resolved (Fig. 3A). One band (Rm 0.204) appeared at both organogenic callus and multiple shoot induction phase, but the intensity of this band (enzyme fraction) was more during multiple shoot induction phase. This fraction of enzyme expression might be associated with shoot bud regeneration and it may be used as marker to tag the redifferentiation pathway. Another two bands (Rm 0.376 and 0.57) appeared at all stages but these two bands exhibited intensity polymorphism. Similar trend of isoesterase enzymes has been detected in *Brassica* spp.<sup>14</sup>, sugar beat and horse radish<sup>12</sup>.

*Peroxidase* zymogram revealed quite heterogenous banding pattern consisting of six distinct bands (Rm value ranges from 0.113 to 0.897; Fig. 3B). Out of which one band (Rm 0.42) was specific to root induction phase, while another (Rm 0.761) was specific to multiple shoot induction. These bands may be used as marker to characterize differentiation pathway—caulogenesis and rhizogenesis in *A. longifolia*. Similar observation has also been reported in several species including *Brassica* spp.<sup>14</sup>, *Xanthomonas sagittifolium*<sup>15</sup> and *Nothofagus antarctica*<sup>48</sup>. Isoperoxidase system has also been used as genetic marker in *Cereus peruvianus* to test the genetic uniformity of callus and the plants regenerated from the callus cultures<sup>49</sup>.

*Catalase* zymogram resolved quite homogenous banding pattern consisting of three distinct bands towards anode end having Rm value ranges from 0.752 to 0.921 (Fig. 3C). One band (Rm 0.752) was

only present in differentiated stages including both multiple shoot induction phase and root induction phase, but absent in the undifferentiated phases. This band may be organogenesis specific and its expression triggered by critical auxin to cytokinin ratio. The other two bands exhibited intensity polymorphism. One among them (Rm 0.831) was more intense during organogenesis in comparison to undifferentiated callus phase, while other one (Rm 0.921) showed *vice versa* pattern of expression. However, no report is available for catalase zymogram study during *in vitro* propagation of medicinal plants.

*Acid phosphatase* zymogram resolved quite homogenous banding pattern consisting of three distinct bands having Rm value ranges from 0.153 to 0.705 (Fig. 3D). One band (Rm 0.435) was only present in differentiated stages including both multiple shoot induction phase and root induction phase, but absent in the undifferentiated phases. This band might be organogenesis specific and its expression triggered by critical auxin to cytokinin ratio. The other two bands exhibited intensity polymorphism. However, no report is available for acid phosphatase zymogram studies *in vitro* propagation of medicinal plants.

The present study indicated that an efficient protocol on shoot bud regeneration from callus culture of *A. longifolia* was dependent on growth regulators levels and expression of specific proteins and enzymes. Esterase, catalase and acid phosphatase may be used as marker to depict organogenesis pathway in *A. longifolia*.

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