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Journal of Chemical and Pharmaceutical Research

J. Chem. Pharm. Res., 2010, 2(4):635-641

ISSN No: 0975-7384
CODEN(USA): JCPRC5

Biochemical analysis of *in vitro* derived plants of *Sterculia urens* during acclimatization

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ABSTRACT

Sterculia urens is a commercially important tree with wide applications. Nodal explants were grown on MS medium supplemented with various growth regulators like 6-benzyladenine (BA), Thidiazuron (TDZ) and Kinetin (Kn). These shoots were subsequently rooted in vitro on half strength MS medium containing various concentrations of auxins like Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA). Plants were acclimatized to ex vitro conditions and established in the field. Role of peroxidase, poly phenol oxidase, total proteins, total carbohydrates and total phenols were studied during acclimatization by estimating their at an interval of 20, 40 and 60 days. Peroxidase and poly phenol oxidase level decreases from 20 to 60 days where as levels of total proteins, carbohydrates and phenols increases.

Keywords: *Sterculia urens*, Regeneration, Peroxidase, Polyphenol oxidase, Acclimatization.

INTRODUCTION

Sterculia urens is a medium sized tree of the family Sterculiaceae. The tree yields a gum known as gum karaya. A large part of the Karaya is used in the pharmaceutical Industry as a bulk laxative [1] and as a denture adhesive. The gum also has applications in petroleum, textile, Pharma, food and dairy industries [2].The natural propagation of *Sterculia urens* is through seeds. However the seed viability decreases as the time progress. Furthermore its propagation through stem cuttings poses difficulties. Destructive tapping, poor seed germination coupled with over exploitation and low seedling survival [3, 4, 5] has depleted species from its natural habitat. So there is gap between demand and supply and thus putting pressure on the species. Hence there is grave concern about the loss of wild germplasm of *Sterculia urens.*

Micropropagation has been extensively used for the rapid multiplication of many plant species. However, its more widespread use is restricted by often high percentage of plants lost or damaged when transferred to ex vitro conditions (greenhouse or field). During in vitro culture, plantlets grow under very special conditions in relatively air-tight cultivation vessels. These conditions result in the formation of plantlets of abnormal morphology, anatomy and physiology [6]. In the present study in order to know whether the in vitro derived plants of Sterculia urens exhibit same biochemical parameters like that of ex vitro plants (seed derived and grown under field conditions), we assayed the levels of peroxidase, poly-phenol oxidase and find out the concentration of total proteins, phenols and carbohydrates at 20, 40 and 60days intervals of time in both in vitro and ex vitro derived plants.

MATERIALS AND METHODS

Plant material

Seeds were collected from Kovela foundation an NGO organization, Visakhapatnam, AP India and stored in an air tight container. Healthy seeds were selected and treated with concentrated sulfuric acid for one minute, washed thoroughly with running tap water and with 5%(w/v) Teepol for 10 minutes followed by treatment with bavistin, a commercial fungicide for 5 minutes. Then the seeds were subsequently surface sterilized with 0.1% (w/v) $HgCl_2$ for 5minutes. Thoroughly washed with sterile distilled water and were soaked for 24 hours. Before inoculation the seeds were once again washed thoroughly with sterile distilled water and were surface dried on sterile filter paper and cultured on MS [7] medium with 4.4 µM BAP.

Culture Medium

Nodal explants were excised from one month old *in vitro* raised plants consisting of approximately 3 to 4 nodes below the apical meristem and were inoculated on MS basal medium and also on MS medium supplemented with different concentrations of growth regulators TDZ, BAP and Kn. TDZ at 0.45µM to 2.27µM, BAP at 4.44µM to 22.22µM and Kn at 4.65µM to 23.25µM were used respectively. The pH of all the media was adjusted to 5.7 before autoclaving. Agar at a concentration of 0.8% was added and sterilized at 121^0C for 20 minutes.

Culture Conditions

All cultures were incubated at 25 ± 2ºC under 16-h photoperiod with 60% relative humidity. Light intensity of 40-50 µmol m^{-2} s^{-1} was provided by using cool white fluorescent tubes.

Rooting

The shoots of 2-3cm height were taken after elongation and thoroughly washed under running tap water in order to remove agar and transferred to quarter strength MS medium. The above media was supplemented with different concentrations of IBA at 4.92µM to 24.63µM, NAA at 5.36µM to 26.88µM and IAA at 5.71µM to 28.57µM.

Plantlets with well developed roots were removed and transferred to plastic cups containing autoclaved garden soil mixed with vermiculite and sand (1:1:1) and covered with perforated polythene bags to maintain humidity (70%) and irrigated every other day with Hoagland's (1950) solution and were kept under culture room conditions till the new leaf appeared. Later polythene bags were removed and placed under green house conditions for about four weeks. Once the plants were adjusted to environmental conditions they were planted under normal garden conditions.

Biochemical analysis

About 1 gram of leaf segments of 20, 40 and 60 days old in vitro derived plants which were grown under green house conditions were washed thoroughly with double distilled water, air dried and homogenized separately with 20 ml of pre-chilled phosphate buffer (0.1M, P^H 7.6), containing 0.1mM EDTA in chilled pestle and mortar. The homogenates were squeezed through double layered cheese cloth and centrifuged (Sorvall Instrument RC5C, Rotor SS-34) at 16,000 rpm for 15minute at 4^0 C. The supernatant was used for the assay of peroxidase, polyphenol oxidase and also for phenols, total proteins and total carbohydrates.

Peroxidase activity was assayed spectrophotometrically (Shimadzu UV-265, UV-visible recording spectrophotometer) with O-dianisidine as a hydrogen donor (8, 9). Polyphenol oxidase activity was measured by the modified method of Bateman [10], Sadasivam and Manickam [9]. Enzyme activity was expressed in terms of change in absorbance per unit time per mg protein. Total protein was estimated by the method of Lowry et al [11] with Bovine Serum Albumin as a standard. Total phenols were estimated by the method described by Sadasivam and Manickam [9]. Total carbohydrates were determined by the phenol sulfuric acid [12] method. As controls leaves of seed germinated plants (20, 40 and 60 days old) were taken. Each experiment has three replicates and the experiment was repeated twice.

Statistical analysis:

The levels of peroxidase, polyphenol oxidase and the concentration of total proteins, total carbohydrates and total phenols estimated for ever 20 days and the data was subjected to oneway analysis of variance (ANOVA). A significance level of 0.05 was used for all statistical analysis.

RESULTS AND DISCUSSION

Table 1: Effect of different cytokinins on adventitious shoot multiplication of nodal explants of *Sterculia urens* after four to six weeks. Means within a column followed by the same letter are not significantly different from each other at α =0.05 according to Duncan's multiple range test

Concentration of cytokinin (µM)	Percentage of nodal explants producing shoots	Mean number of shoots per explant (±SE)
TDZ 0.0	80.00	1.0 ± 0.00 f
0.45	88.00	5.02 ± 0.13 b
0.90	92.00	8.68 ± 0.17 a
1.36	85.30	4.89 ± 0.10 b
1.81	77.30	3.28 ± 0.11 c
2.27	64.00	2.94 ± 0.10 c
BAP 4.44	82.67	2.69 ± 0.16 d
8.88	85.33	3.78 ± 0.19 c
13.33	90.67	7.51 ± 0.19 a
17.77	80.00	4.67 ± 0.15 b
22.22	76.00	2.82 ± 0.14 d
Kn 4.65	77.33	1.91 ± 0.13 b
9.30	80.00	2.87 ± 0.14 a
13.95	72.00	2.00 ± 0.14 b
18.60	69.33	1.69 ± 0.08 bc
23.25	57.33	1.44 ± 0.08 c

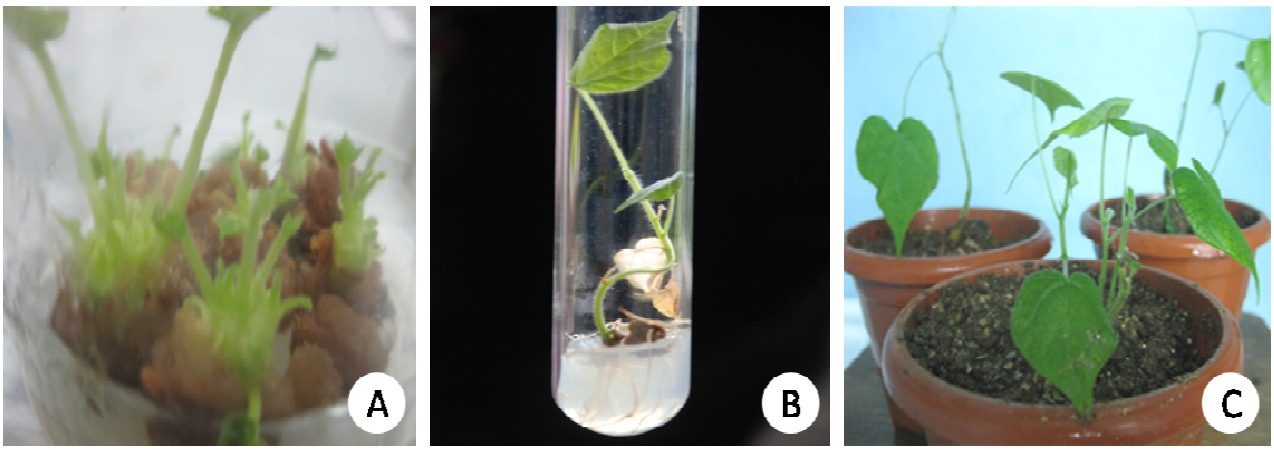
In the absence of growth regulators a single shoot emerged from the leaf axil of nodal explant. However, in the presence of growth regulators the mean number of shoots per explant varied significantly with the type and concentration of cytokinin in the medium. Our results showed that TDZ was able to induce the maximum number of shoots when compared to BAP and Kn.

Among the various concentrations of TDZ tested, 0.90µM TDZ resulted in formation of highest number of shoots (8.68±0.17) followed by BAP at a concentration of 13.33µM (7.51±0.19) and Kn at 9.30µM (2.87±0.14) per node after four weeks of culture (shown in table I and Figure 1A). At higher concentration more callusing response was observed for all the three hormones.

Individual shoots with 4 or 5 leaves were excised and cultured on full strength, half strength and quarter strength MS medium with and without growth regulators. Of the three auxins we used, treatment with IAA failed to stimulate rooting at any concentration however IBA and NAA were effective. Maximum of 78% shoots rooted on ¼ MS+IBA (19.70µM) within eight to ten weeks and maximum of 73% shoots rooted on ¼ MS+NAA (16.12µM) within eight to ten weeks (shown in table 2 and Figure 1B and 1C).

Table 2: Effect of different auxins on adventitious rooting of shoots of *Sterculia urens* after 8 - 10 weeks. Means within a column followed by the same letter are not significantly different from each other at α =0.05 according to Duncan's multiple range test.

Concentration of auxin (µM)		Percentage of cultures producing roots	Mean number of roots per shoot (±SE)
IBA	0.0	0.00	0.00 ± 00
	4.92	46.67	2.71 ± 0.20 c
	9.85	53.33	3.09 ± 0.20 bc
	14.77	65.00	3.90 ± 0.21 b
	19.70	78.33	5.49 ± 0.39 a
	24.63	70.00	3.95 ± 0.27 b
NAA	5.37	51.00	3.13 ± 0.20 cd
	10.75	60.00	3.78 ± 0.23 bc
	16.12	73.33	5.18 ± 0.38 a
	21.50	65.00	4.36 ± 0.23 b
	26.88	55.00	2.85 ± 0.14 d



Figure

1A. Formation of multiple shoots from nodal explants of one month old plants of *S.urens* cultured on MS medium with TDZ (0.90 µM)

1B. Rooting of in vitro raised shoots on half-strength MS medium with IBA (19.70 µM).

1C. Potted plants under green house conditions.

Biochemical analysis of in vitro derived plants revealed the presence of essential compounds for growth, nutrition, higher resistance to pathogens and exhibit normal development. In vitro derived plants showed similar chemical composition with respect to control (seed derived plants) and therefore presents 90-100% establishment. Peroxidase activity varied from 2.50 to 0.97

units/min/mg protein with respect to invitro derived plants, where as 1.81 to 0.57 units/min/mg protein in case of control. Peroxidases (EC 1.11.1.7) are the most studied plant enzymes due to their abundance in plant tissues. These are most directly involved in lignin biosynthesis. Our study showed optimum peroxidase activity in the invitro derived plants which gives stability for acclimatization after field transfer. This can also be correlated to the survival percentage of in vitro derived plants (shown in table 3 and graph 1).

Poly phenol oxidase activity varied from 0.093 to 0.023 units/min/mg protein with respect to invitro derived plants, where as 0.076 to 0.023 units/min/mg protein in case of control. PPO levels were high in the invitro derived plants compare to control indicating PPO is required for acclimatization which is supported by previous reports where highest PPO expression levels are usually associated with young tissues (leaves, flowers, fruits, tubers) and in meristematic regions, which are particularly vulnerable to diseases and insect pests, and gene expression generally declines during development and maturation of plant tissues [13]. Polyphenol oxidase (PPO; EC 1.14.18.1) catalyses the oxidation of polyphenols and the hydroxylation of monophenols [14] and lignification of plant cells. Recent studies have indicated that phenol-oxidizing enzymes may participate in response to the defense reaction and hypersensitivity in inducing resistance of plants to biotic and abiotic stress [15, 16] (shown in table 3 and graph 2).

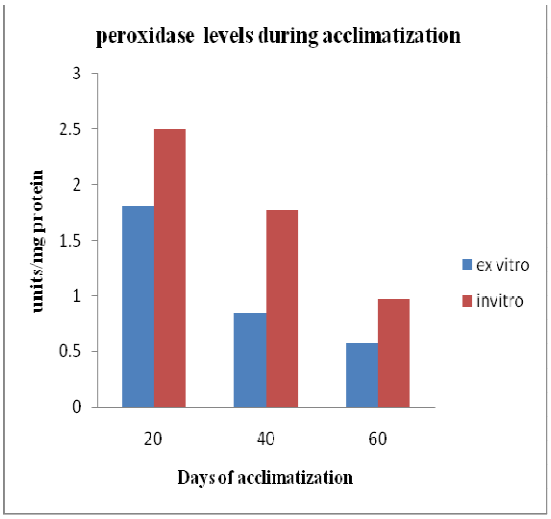
Table 3: Biochemical analysis of control and in vitro derived plants of *Sterculia urens*

Each value represents the mean of three replicates ± S.E.

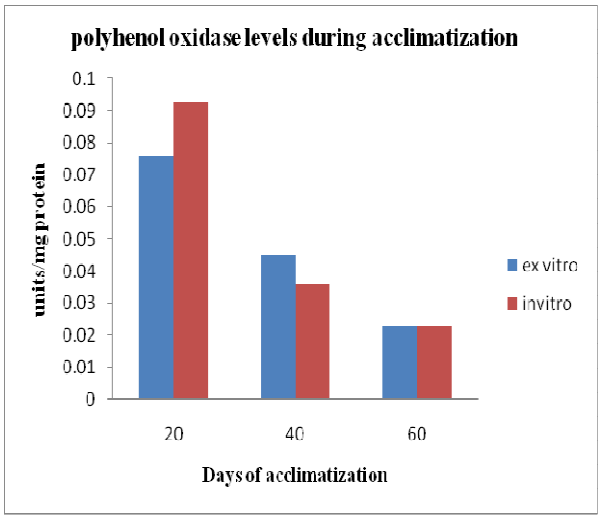
Control					
Period of collection	peroxidase	Polyphenol oxidase	Phenol content	Protein content	Carbohydrate content
Days	units/mg protein	units/mg protein	mg/g	mg/g	mg/g
20	1.81±0.75	0.076±0.00	0.68±0.04	14.50±1.32	3.86±0.11
40	0.85±0.30	0.045±0.00	0.98±0.02	20.33±1.15	5.53±0.30
60	0.57±0.17	0.023±0.00	1.37±0.28	31.66±1.52	8.40±0.91
Invitro derived plants					
20	2.50±0.15	0.093±0.02	0.33±0.06	7.50±0.00	3.20±0.20
40	1.77±0.25	0.036±0.00	0.48±0.04	12.66±1.25	5.86±0.61
60	0.97±0.15	0.023±0.00	0.66±0.16	19.50±2.64	8.20±0.20

Further phenol content increased from 0.33 to 0.66 mg/g tissue in the invitro derived plants and 0.68 to 1.37 mg/g tissue in control. Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants with a broad spectrum of physiological activities. They are the main substrates of peroxidases. They participate in redox processes, in mechanical support, in absorbing harmful UV radiation and in plant defense reaction [17].

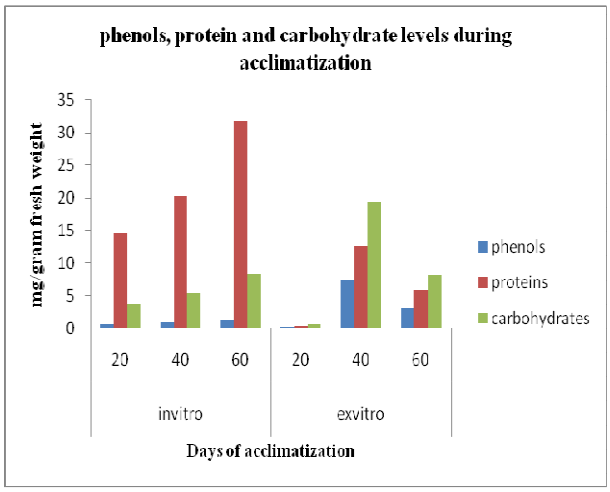
Quantitative estimation of protein showed 7.50 to 19.50 mg/g tissue in the invitro derived plants and 14.50 to 31.66 mg/g tissue in the control. Total carbohydrate levels were 3.20 to 8.20 mg/g tissue in the invitro derived plants and 3.86 to 8.40 mg/g tissue in the control. In general, during morphogenesis, cellular proteins differ not only with respect to their function but also in terms of their timing and extent of expression during the process [18, 19] (shown in table 3 and graph 3).



Graph 1 peroxidase levels during acclimatization



Graph 2 polyhenol oxidase levels during acclimatization



Graph 3 phenols, protein and carbohydrate levels during acclimatization

CONCLUSION

From the present study it was concluded that in vitro derived plants from nodal explants of Sterculia urens exhibit increased levels of enzyme activities and protein, carbohydrate and phenols with respect to control plants and therefore showed higher percentage of survival rate. In general in vitro derived plants exhibit lower percentage of establishment in the field conditions, this may be due to several reasons one of the reason is due to altered chemical composition. Optimum content of peroxidase, poly phenol oxidase, phenols, proteins and carbohydrates can be used as markers for the normal development of in vitro derived plants.

Acknowledgements
The author great fully acknowledge the financial assistance from Andhra University, Visakhapatnam, Andhra Pradesh, India under Starter Research Grant.

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