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Biochemical Changes During *In Vitro* Organogenesis of *Tylophora indica* (Burm. F.) Merrill

KEYWORDS: Tylophora indica (Burm. F.) Merrill, Differentiation, Peroxidase, IAA oxidase, Protein, Reducing sugar

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ABSTRACT *Biochemical changes in respect to Peroxidase, Polyphenol oxidase, IAA oxidase, Total amino acid, Reducing sugar and Protein content were studied during the process of shoot regeneration in the callus culture of Tylophora indica (Burm. F.) Merrill. The following stages: dedifferentiated callus, differentiating green callus, differentiating callus with shoot primordial, differentiating callus with well-developed multiple shoots were selected for present study. Mature leaf was used as control. From the present investigation, it was proved that the process of differentiation had high metabolic activity. High peroxidase and polyphenol oxidase and IAA oxidase activities were recorded in differentiating green callus. Protein content along with reducing sugar and total amino acid contents were decreased significantly during differentiation as compared to control. Increased metabolic rate during in vitro organogenesis help Tylophora for rapid growth and multiplication.*

INTRODUCTION

Tylophora indica (Burm. F.) Merrill. is an important medicinal climber, belongs to family Asclepiadaceae. Traditionally, this plant has been widely used for the treatment of bronchial asthma, bronchitis, rheumatism, inflammation, allergies and dermatitis (Kaur *et. al.,* 2011). Multiple uses and medicinal values raised a need for development of new multiplication protocol for this plant. *In vitro* organogenesis has been achieved in large number of plant species through plant tissue culture. The structural changes that take place in organogenesis are indication of proceeding physiological and biochemical events (Thorpe, 1990). Biochemical changes that precede the onset of organogenesis can serve as markers of differentiation processes that bring about morphological, developmental and functional specialization (Thorpe, 1980). During morphogenesis certain enzymes and proteins are responsible for callus proliferation and differentiation in to shoot buds (Chawala, 1991). Regulation and control of differentiation *in vitro* and the relationship with enzymes like peroxidase, catalase and protein synthesis during cellular development in cell culture have been reported in Pea nut (Verma and Van Huystee, 1970). Tissue culture studies showing *in vitro* organogenesis through callus have been reported in *Feronia limonia* L. (Purohit *et. al.,* 1996) and *Tylophora indica (Burm. F.)* Merr. (Kalimathu and Jeyaraman, 2012). Biochemical changes associated with morphogenesis was also reported in Saffron (Sharma *et. al.,* 2009). The present study was under taken to describe biochemical changes occurring during the process of *in vitro* regeneration via callus culture in *T. indica* with special reference to Enzymes and Metabolites.

MATERIALS AND METHODS

Leaf explants were collected from mature and healthy field grown plants of *T. indica* from Botanical garden of Hemchandracharya North Gujarat University, Patan, Gujarat. Collected explants were washed under running tap water for 30 minutes followed by washing in liquid detergent for 5 minutes and then washed with tap water. After these treatments explants were taken inside the laminar air flow for further sterilization. The explants were then surface sterilized with 0.1 % (w/v) $HgCl_2$ for 2 minutes. and finally rinsed 4-5 times with sterile distilled water. The sterilized leaves were cut into 1 cm^2 pieces and inoculated on MS medium supplemented with BAP (1, 2 mgl^{-1}) and the combination of 2,4-D (1 mgl^{-1}) + Kin. (1 mgl^{-1}), 2,4-D (2 mgl^{-1}) + Kin. (2 mgl^{-1}) and 2,4-D (1 mgl^{-1}) + BAP (1 mgl^{-1}) were maintained through regular subculture after every 4 weeks. For morphogenesis and shoot differentiation, callus was transferred on MS medium supplemented with 3 mgl^{-1} BAP + 0.2 mgl^{-1} IAA. All the cultures were incubated in light with 2000 lux, 25±2°C temperature and 50-60 % relative humidity of culture room. Biochemical analyses were done through the different stages of organogenesis. Following four stages were selected to determine biochemical parameters:

Control – Mature Leaf
Stage 1 – Dedifferentiated callus

Stage 2 – Differentiating green callus

Stage 3 – Differentiating callus with shoot primordia

Stage 4 – Differentiating callus with well-developed multiple shoots

Enzyme assays:
Plant material (1 g) was grounded in 10 ml chilled phosphate buffer (0.2 M, pH 6.0) in chilled pestle and mortar. The ex-

Volume : 4 | Issue : 1 | Jan 2014 | ISSN - 2249-555X

tract was centrifuged at 10,000 RPM for 30 minutes at 4°C in refrigerated centrifuge. Enzyme extract thus prepared was assays for peroxidase, polyphenol oxidase and IAA oxidase activities. Polyphenol oxidase was determined using Shinshi and Noguchi, 1975. Peroxidase was determined by the method given in Worthington enzyme manual, 1972. The IAA oxidase activity was determined using method of Srivastava and Van Huystee, 1973.

Metabolite assays:
Plant material (1 g) was grounded in 10 ml 80% ethanol in pestle and mortar. The extract centrifuged at 5000 RPM for 15 minutes. Supernatant was collected and used for estimation of reducing sugar and total free amino acid. Remaining pellet was resuspended with 10 ml 1N NaOH and centrifuged at 5000 RPM for 15 minutes. Supernatant was collected and used for estimation of Protein. Total Protein content was estimated by Lowry method (Lowry *et. al.,* 1951). Reducing Sugar content was measured by the Di-nitro Salicylic Acid (DNS) Method (Miller, 1972). Estimation of Total Free Amino Acid was done by Moore, and Stein's protocol (Moore and Stein, 1948). All the samples were analyzed using three replicates and data represented as Mean ± SE.

RESULTS AND DISCUSSION

Callus induction Effect of single concentration of BAP (1, 2 mgl^{-1}) and the combination of 2,4-D (1 mgl^{-1}) + Kin. (1 mgl^{-1}), 2,4-D (2 mgl^{-1}) + Kin. (2 mgl^{-1}) and 2,4-D (1 mgl^{-1}) + BAP (1 mgl^{-1}) on callus induction and proliferation from leaf explants were shown in Table. 1.

Shoot multiplication Effect of combinations of BAP (1,2,3 mgl^{-1}) + IAA (0.2 mgl^{-1}) and Kin. (1,2,3 mgl^{-1}) + IAA (0.2 mgl^{-1}) on shoot multiplication from leaf derived callus was shown in Table. 2.

Peroxidase activity Different *in vitro* stages showed a significant peroxidase activity. The maximum peroxidase activity was showed in Stage 2 (differentiating green callus) which is supposed to highest meristematic activity whereas *in vivo* leaf showed lower peroxidase activity than other stages. Towards development of organized structure at Stage 3 (differentiating callus with shoot primordia) the peroxidase activity showed little decline (Fig. 1).

Similarly maximum peroxidase activity at rooting stage and minimum activity was obtained in organogenic callus in *Asteracantha longifolia* L. (Panigrahi *et. al.,* 2007). Highest peroxidase activity recorded at rooting stage of hybrid between *Populus termula* and *P. tremuloides* (Hausman, 1993). *In vitro* shoot bud differentiation is accompanied by an increase of the peroxidase activity in *Albizia odoratissima* (L.f.) Benth (Rajeshwari and Paliwal, 2008).

IAA oxidase activity Highest IAA oxidase activity was recorded in tissues showing dedifferentiated callus tissues (Stage 1) as compared to *in vivo* leaf taken as control. Decrease in IAA oxidase activity was registered in differentiating green callus tissues of stage 2 (Fig. 2). Similarly little increase was observed in Stage 4 and higher IAA oxidase activity recorded in the cultures in earlier study, which showed a sign of organogenesis and when green shoots started differentiating into complete plantlets in saffron (Sharma *et. al.,* 2009).

Polyphenol oxidase activity Significant increase in rate of polyphenol oxidase activity was recorded in tissues showing dedifferentiation and redifferentiation indicating its active role. Highest polyphenol oxidase activity was recorded in differentiating green callus tissues (Stage 2) as compared to *in vivo* leaf tissues (Control). In Stage 4 Polyphenol acivity was higher than Stage 3 (differentiating callus with shoot primordia) and stage 1 (Fig. 3). Change in the level of enzymes in cultures before onset of organogenesis reflects the ability of the tissue to degrade endogenous auxin and may possibly be due to the promotion of enzyme activity (Galaston and Davies, 1969).

Metabolic changes
The protein concentration varied among different developmental stages (Fig. 4). The significant increase in protein contents was observed in control (*in vivo* Leaf) and there was decrease in protein content from Stage 1 to Stage 4. It has been observed that mature leaf contained good amount of protein because of dedifferentiation and redifferentiation whereas in the stages of metabolic activity, amount of proteins was decreased. With the development of shoot formation protein content was minimum at Stage 4. Similarly results obtained in *Feronia limonia* L. (Purohit *et. al.,* 1996). This might be due to synthesis of certain amino acids/polypeptides required to initiate shoot bud formation and their depletion lead to rhizogenesis. Highest concentration of reducing sugar was observed in Control (*in vivo* mature leaf) (Fig. 5). The lowest concentration of reducing sugar was observed in Stage 4 than the other stages. During organogenesis accumulation of starch and sugars also plays an important role as osmotic agents (Kavi Kishore and Mehta, 1989). All the developmental stages of *in vitro* regeneration showed the significant accumulation of total amino acid (Fig.6). The highest concentration of total amino acid was observed in Control. The mechanisms of amino acid influence on *in vitro* organogenesis are poorly understood, although it is common to use media supplemented with amino acids or hydrolyzed proteins to promote explant proliferation (Hamasaki *et. al.,* 2005). Glutamine and glutamate are known to be the main endogeneous amino acid involved in plant metabolism, providing nitrogen for the biosynthesis of amino acids, nucleic acids and other N-compounds (Coruzzi and Last, 2000).

CONCLUSION

In vitro regeneration technique is an important tool for conservation of rare or threatened plants and micropropagated plants can be used to supplement the natural stock of this plant. The results of the present study will provide better protocol for the conservation and propagation of this species much easier. The results of biochemical changes during *in vitro* developmental stages indicated the periodic quantitative changes in the various metabolites like increase in protein content in *in vivo* leaf and differentiating green callus stage whereas decrease at differentiating callus with shoot primordia stage, decrease in reducing sugar content at the stage of differentiating callus with well-developed multiple shoots, but it increases in *in vivo* leaf and decrease in endogenous amino acids levels at differentiating green callus stage but it increases in *in vivo* leaf. The developmental stages even differ greatly in their enzyme activities like there is a great increase in peroxidase, polyphenol oxidase and IAA oxidase activities during differentiating green callus stage. The significant variation in the stress related enzyme during the *in vitro* organogenesis and metabolites turnover in various stages shows the hormonal mediated growth and differentiation in the plant.

ACKNOWLEDGMENT

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Table: 1 Effect of different hormones on Callus formation of *Tylophora*

Explant	Hormones in mg/l	Total no. of Explants	No. of Explants In which Callus Form	Frequency growth response (in %)
Leaf	BAP(1)	10	2	20
	BAP(2)	10	-	-
	2,4-D(1)+ Kinetin(1)	10	8	80
	2,4-D(2)+ Kinetin(2)	10	3	30
	2,4-D(1)+ BAP(1)	10	6	60

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Table: 2 Effect of Cytokinins along with Auxins on shoot multiplication of *Tylophora*

Hormones (mg/l)			% Frequency of growth response	No. of Shoots/ callus (Mean ± SD)
BAP	KIN.	IAA		
1	-	0.2	20	1.4±2.9
2	-	0.2	80	8.1±5.7
3	-	0.2	40	4.7±6.2
-	1	0.2	10	1.1±3.4
-	2	0.2	50	5±5.6
-	3	0.2	20	2±4.3

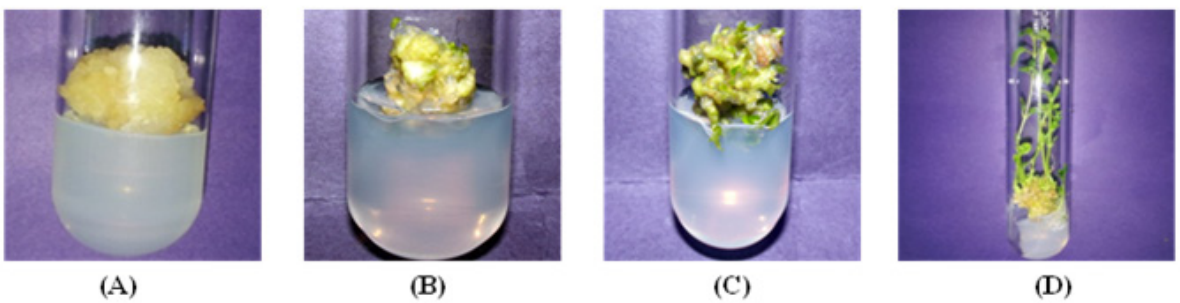

Fig. A Stage 1 (Dedifferentiated Callus)
Fig. B Stage 2 (Differentiating green Callus)
Fig. C Stage 3 (Differentiating Callus with shoot primordia)
Fig. D Stage 4 (Differentiating Callus with well-developed multiple shoots)

Enzymes

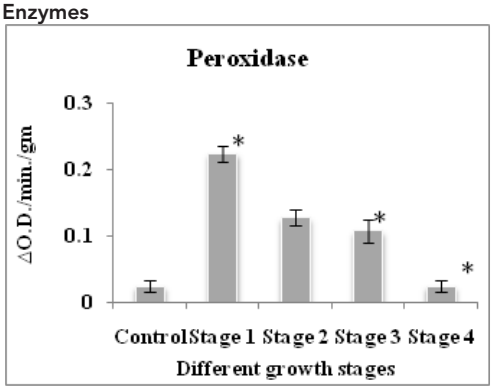

Fig. 1 Peroxidase activity during Different growth stages of callus culture of *Tylophora*

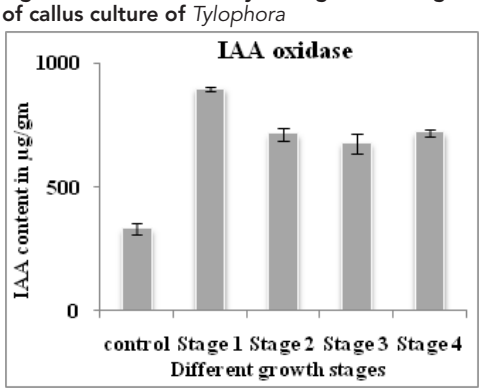

Fig. 2 IAA oxidase activity during Different growth stages of callus culture of *Tylophora*

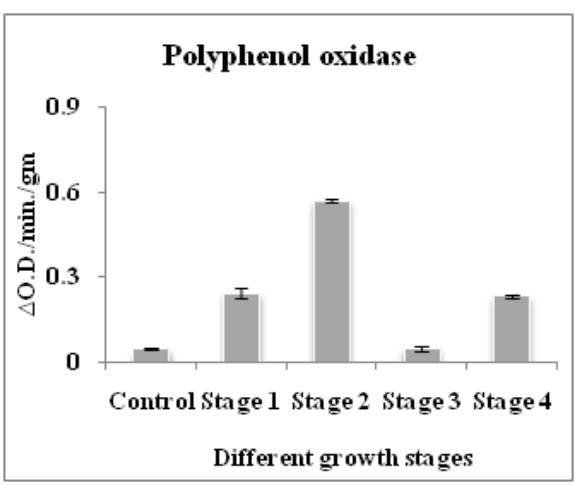

Fig. 3 Polyphenol oxidase activity during Different growth stages of callus culture of *Tylophora*

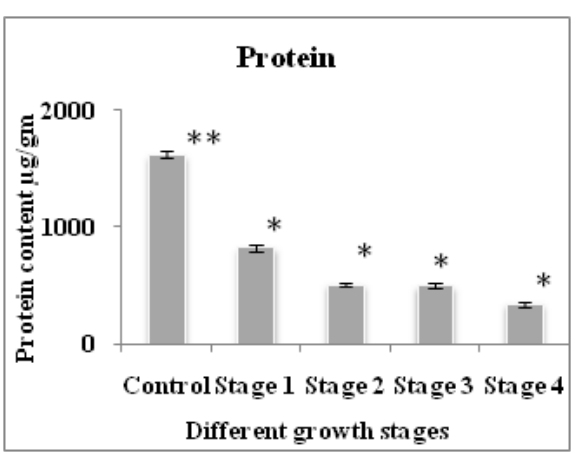

Fig. 4 Changes in Protein content during Different growth stages of callus culture of *Tylophora*

Volume : 4 | Issue : 1 | Jan 2014 | ISSN - 2249-555X

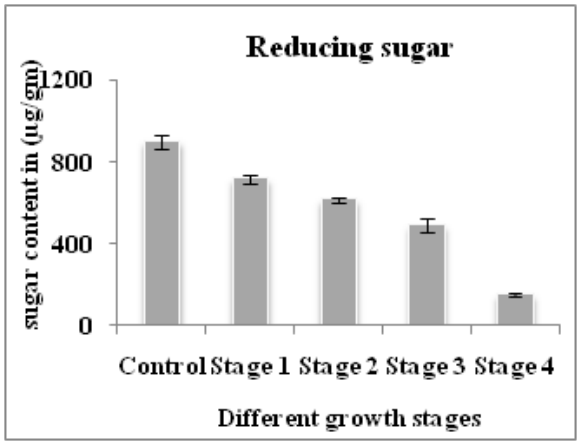

Fig. 5 Changes in sugar content during Different growth stages of callus culture of *Tylophora*

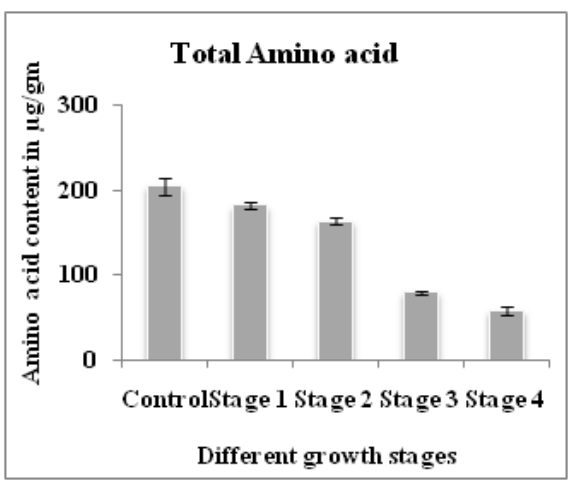

Fig. 6 Changes in Amino Acid content during Different growth stages of callus culture of *Tylophora*

Vertical bar indicates error bars. Probability *=significant at the 5% ($\alpha \leq 0.05$); ** = significant at the 1% ($\alpha \leq 0.01$);Control = *in vivo* **Leaf, Stage 1 - (Dedifferentiated Callus), stage 2 - (Differentiating green Callus), Stage 3 - (Differentiating Callus with shoot primordia), Stage 4 - (Differentiating Callus with well-developed multiple shoots)**

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