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BIOCHEMICAL STUDIES OF DIFFERENTIATING CALLUS CULTURES OF *OROXYLUM INDICUM* (L.) VENT.

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Abstract

Some biological parameters in respect to total sugar, protein, total amino acid, peroxidase, Polyphenol oxidase and IAA oxidase activity were determined during the process of shoot regeneration in the callus culture of *Oroxylum indicum* (L.) Vent. Following stages were taken into consideration to describe biochemical changes: callus, differentiating green callus, callus with shoot primordia, differentiating callus with multiple shoots and shoots of 6 month old plant. *In vivo* leaf used as control. Reducing sugar and amino acid found increased during initial stage and play important role during the *in vitro* growth and differentiation. Whereas, protein was found higher in differentiated stage and shows rapid structural build up. The biochemical changes in terms of enzyme activities varied during different stages of *in vitro* organogenesis.

Key words: *Oroxylum indicum*, biochemical, metabolites

Introduction

Oroxylum indicum (L.) Vent., belongs to the Bignoniaceae family grows wild in India, Sri Lanka, Phillippines and Indonesia (Anonymous, 1972), at an altitude of 1200m and found mainly in ravine and moist place in the forests (Bennet *et al*., 1992; Dey, 1980). Every parts of this plant possesses medicinal importance. For high medicinal value, it is collected from its natural habitat in indiscriminate manner, so, this plant become vulnerable in Kerala, Maharastra, M.P. and Chhatisgarh (Darshan and Ved, 2003; Jayaram and Prasad, 2008). A short span of viability and low germination rate, restrict the propagation of *Oroxylum indicum* by seeds (Dwivewdi and Boro, 2012). Various bioactive compounds like chrysin, oroxylin-A, scutellarin, baicalein are present in stem bark and leaves (Sankara and Nair, 1972-a; Sankara and Nair, 1972-b). Seeds of this plant are reported to contain ellagic acid (Vasanth *et al*., 1991). The plant is used in many Ayurvedic preparations like Dasamoola, Chyawanaprasha, Brahma rasayana, Narayana Taila, Awalwha and Dantyadarishta (Anonymous,1998).

Plant has several biochemical processes starting from germination till the end of plant life. The growth speed, types, development patterns at every stage is highly controlled by some total of biochemical pathways where it is in *ex vitro* or *in vitro* growth condition. Biochemical attributes are indicators of morphogenetic potential, growth and differentiation, representing differential gene action or expression or change in endogenous level of growth regulators in cell cultures and are used for analysis of gene function and metabolic regulation (Scandalios, 1974; Carrillo and Mata, 2000) . Many investigations have been made about the physiological changes taking place during organogenesis in callus culture (Saka and Maeda, 1974; Ross *et al*., 1973; Malik and Kumari, 1977; Santos *et al*., 2008; Cheniany *et al*., 2010). Estimation of different metabolites like sugars, protein, amino acid and oxidative enzymes are interpreted to understand of mobilization and utilization of storage reserves. Hence, present study was an attempt to observed biochemical changes during *in vitro* organogenesis of *Oroxylum indicum* (L.) Vent.

Materials and Methods

Callus cultures were derived from leaf obtained from 20-25 days old seedling on MS (Murashige and Skoog, 1962) medium supplemented with different concentration of BAP with IAA. For morphogenesis and shoot differentiation, callus was transferred on same medium with same hormonal concentration and combination. All

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the culture tubes were incubated in light with 25±2^{0}C temperature and 50-60% relative humidity of culture room. To study the biochemical changes associated with different stages of organogenesis, following five stages were selected to determine biochemical characteristics:

Stage-1 Dedifferentiated callus

Stage-2 Differentiating green callus

Stage-3 Differentiating callus with shoot primordia

Stage-4 Differentiating callus with well-developed multiple shoot

Stage-5 Young shoots of 6 month old plant derived from tissue culture.

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 extract was centrifuged at 10,000 RPM for 30 minutes at 4^{0}C in refrigerated centrifuge. Enzyme extract thus prepared was assays for peroxidase, polyphenol oxidase and IAA oxidase activities. Polyphenol oxidase was determined using method of Shinshi and Noguchi, (1975). Peroxidase was determined by the method given in Worthington enzyme manual, (Anonymous, 1973). The IAA oxidase activity was determined using 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 William, 1948).

Results and Discussion

Result of Metabolites assays

Estimation of sugars

Highest sugar content was measured in control (*in vivo* leaf) followed by stage-1, with a gradual decline during subsequent stages of organogenesis. A sharp decline was recorded when cultures differentiated in to multiple shoots. During different stages highest content was recorded at differentiating callus and were utilized on following stages while lowest content was recorded in 6 months old plant. (Fig. 1).

Estimation of proteins

Protein content showed a gradual and significant increase starting with differentiated callus culture through different stages of redifferentiation. The protein content showed decrease at stage-4 for regeneration of multiple shoots while minimum protein content was recorded in 6 months old plant. A sharp increase in protein content was recorded when shoots were initiated for the formation of shoot primordial via organogenesis. With the progress towards development of shoots the protein content showed decline during different stages (Fig. 2).

Estimation of amino acids

The highest concentration of total amino acid was observed in callus at 1st stage. The amino acid content in differentiated and redifferentiated tissue showed significant difference. As protein content increase amino acid content was decrease through the different stages. Amino acid content was decrease during redifferentiation. A secondary increase was recorded when green shoots started differentiating in to complete plant (Fig. 3).

Significant changes in various biochemical parameters were observed when different stages of *Oroxylum indicum* were analyzed. A detailed study on physiological, biochemical and molecular aspects is necessary to understand the potential of morphogenesis and make it accessible as a tool for plant production (Thorpe and Meier, 1974; Litz and Gray, 1995). There was a rapid accumulation of total sugar and amino acid in dedifferentiated callus than there was gradual depletion in subsequent stages. Whereas, protein level were higher in differentiating callus. Jeyaseelan and Rao, (2005) reported similar result in *Cardiospermum halicacabum*. A steep fall in sugar content was associated with utilization of sugars for growth and differentiation process (Chatrah *et al*., 1996). Whereas, Kumar and Maherchandani, (1988) reported more soluble carbohydrates in the differentiating callus tissues as compared to non-differentiating ones. Thus, a clear correlation exists between differentiating stages and carbohydrate utilization. The protein content was maximum at stage-3, callus with shoot primordial followed by differentiated callus and dedifferentiated callus. This might be due to synthesis of certain amino acids or polypeptides required to initiate shoot bud formation (Panigrahi *et al*., 2007).

Result of enzyme assays

Estimation of Polyphenol oxidase

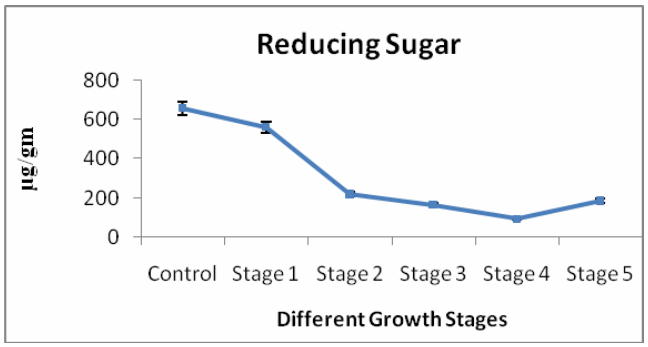

Fig.1: Reducing Sugar content during *in vitro* growth stages.

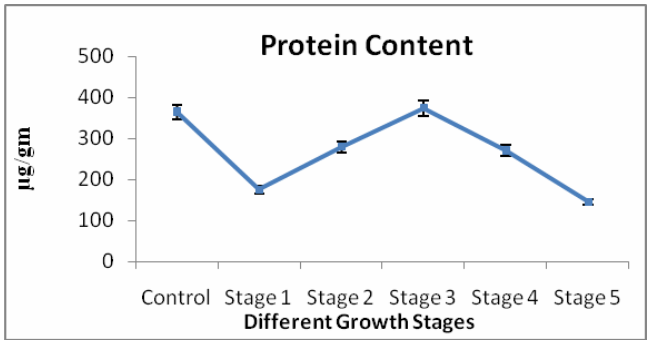

Fig. 2: Protein content during *in vitro* growth stages.

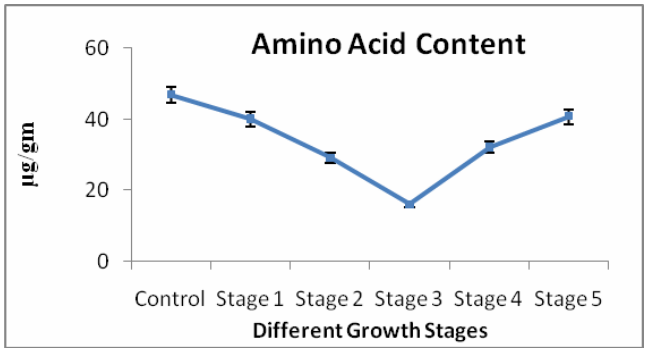

Fig. 3: Amino acid content during *in vitro* growth stages.

Note: Control- *in vivo* leaf, Stage 1 –Dedifferentiated callus, Stage 2- Differentiating callus, Stage3 – Differentiating callus with shoot primordial, Stage 4- Differentiating callus with well developed multiple shoot, Stage 5 – well developed shoot after 6 month

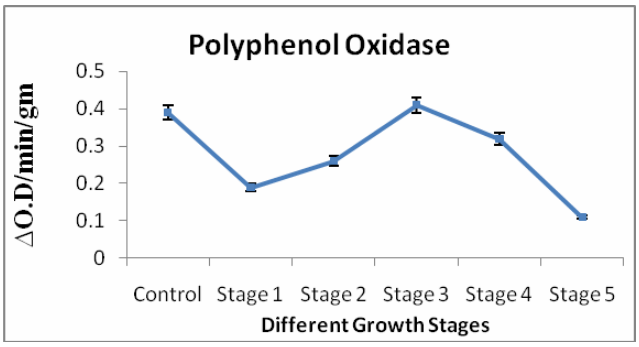

Fig. 4: Polyphenol Oxidase activities during *in vitro* growth stages

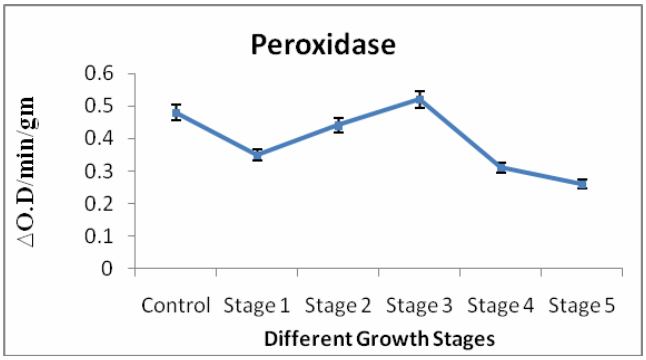

Fig. 5: Peroxidase activities during *in vitro* growth stages.

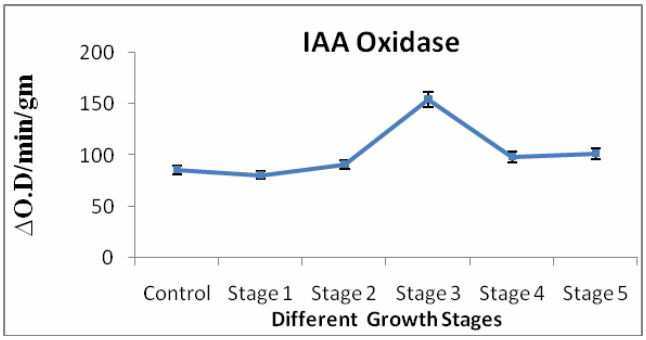

Fig. 6: IAA Oxidase activities during *in vitro* growth stages.

Note: Control- *in vivo* leaf, Stage 1-Dedifferentiated callus, Stage 2- Differentiating callus, Stage 3-Differentiating callus with shoot primordial, Stage 4-Differentiating callus with well developed multiple shoot, Stage 5-well developed shoot after 6 month.

Highest Polyphenol oxidase activity was observed during stage-3. This stage corresponds to the shoot appearance of shoot primordial. A gradual decrease in Polyphenol activities was detected during the stage – 4 and 5. Tissues obtained from 6 months plant showed minimum activity of Polyphenol oxidase. Enzyme activity recorded in control condition was low as compared to stage-3, but increased during dedifferentiation and redifferentiation. Decrease was measured during the process of green shoot in to multiple shoot (Fig. 4).

Estimation of peroxidase

Significant increase in peroxidase activity was recorded during callus and organogenesis phases. Maximum peroxidase activity was measured during stage-3, stage of shoot differentiation which is supposed to have highest meristematic activity. Towards development of organized developed structure a decline was registered. Peroxidase activity measured in callus did not significantly differ then shoot primordial (Fig. 5).

Estimation of IAA- oxidase

IAA oxidase activity was recorded high in *in vivo* leaf samples as compared to callus. A secondary increase in enzyme activity was observed during redifferentiation stage which showed a sign of developing shoot primordial. Further decrease in activity was recorded during the next two stages, in stage-4 and stage-5. IAA-oxidase activity minimum in 6 months old plant among all the stage (Fig. 6).

In present study, high peroxidase activity was observed in differentiating stages. Similar result was observed by Purohit *et al*., (1996) in *Feronia limonia* L. T. Günes (2000) reported peroxidase activity increased during rooting or primordium formation periods of *Populus nigra* and *Populus tremula*. Changes in the levels of peroxidase activity in relation to auxin treatment have been reported in many plant species (Rout *et al*., 2000). Nieves *et al.,* (2003) reported higher peroxidase activity in non embryogenic callus.

In the present study, the activities of Polyphenol oxidase were high during differentiating stages but declined during organogenesis. Similarly, Trivedi (2014) reported the activities of POD and PPO were high during the early stages of *in vitro* morphogenesis which could be correlated with the acquisition of competence, dedifferentiation, division and induction which occurred during organogenesis. The enhanced polyphenol acivity might result in the augmented rate of oxidation of phenolics substance that participates in the defense reaction of host (Meena *et al*., 2008).

Higher IAA oxidase activity parallel to peroxidase and Polyphenol oxidase during differentiating tissue. Similar result was observed by Purohit *et al*., (1996). Previous studies have shown that IAA-O is localised in the tissue, where root initials are first formed, and promotes rooting (Mancousin et al., 1989; Srivastava and Haystee, 1977). Higher accumulation of various enzymes during the initial stages of cell divison and multiplication is cumulative result of endogeneous and exogenously applied hormones which promotes the *in vitro* organogenesis in *Oroxylum indicum* (L.) Vent. Tissue culture technique reported here offer a powerful technique for mass multiplication, of this endangered medicinal plant.

Conclusion

The present study indicate that callus culture provide a important tool to study biochemical changes. The result of biochemical profiles during *in vitro* developmental stages indicated the expression of specific proteins, amino acids and reducing sugars levels in a particular stage and enzyme activities i.e. peroxidase, Polyphenol oxidase and IAA oxidase probably plays a significant role in *in vitro* organogenesis.

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