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REGULAR ARTICLE

# METABOLIC CHANGES DURING DIFFERENTIATION IN CALLUS CULTURES OF *STEVIA REBAUDIANA* (BERTONI)

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## SUMMARY

*Stevia rebaudiana* (Bertoni) is an economically important medicinal plants act as an option for artificial sweetening agents. The plant regeneration protocol under *in vitro* conditions has been developed for this natural sweetener herb. Callus was efficiently induced when leaf segments were cultured on MS medium supplemented with 1.0 mg/l 2, 4-D + 1.0 mg/l Kinetin. The above callus was sub cultured on MS medium + 0.5 mg/l BAP + 0.3 mg/l NAA for root differentiation and on MS medium + 0.5 mg/l BAP + 0.1 mg/l NAA for shoot differentiation. Biochemical changes occurring during the callus differentiation were adopted to quantify the metabolites. Metabolites like starch, total soluble sugars and total phenols decreased while total soluble proteins increased in callus culture during the process of root and shoot differentiation.

**Key words:** Callus, differentiation, *in vitro*, metabolites, *Stevia rebaudiana*

**Abbreviations:** BAP Benzyl amino purine; 2,4-D 2,4-Dichlorophenoxyacetic acid; MS Murashige and Skoog (1962) basal medium; NAA α-naphthalene acetic acid; IBA Indole-3-butyric acid; IAA Indole acetic acid

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## 1. Introduction

*Stevia rebaudiana* Bertoni, belongs to the Asteraceae family is a natural sweetener perennial herb commonly known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf". The leaves of this plant are estimated to be 300 times sweeter than sucrose and the sweetness is due to glycosides of which the most abundant is stevioside (Kinghorn, 1992).

The increasing consumption of sugar (sucrose) has resulted in several nutritional and medical problems, such as obesity. Therefore, low caloric sweeteners have been investigated to substitute sugar. The refined extracts of leaves of this plant are officially used as high potency natural-source, low calorie (nonsucrose) sweetener in processed foods, artificial diets and pharmaceuticals (Mizutani & Tanaka, 2002). The sweet compounds pass through the digestive process without chemically breaking down, making it safe for diabetic and obese people.

The plants grow better in the temperature range of 0-40°C (Carneiro *et al.*, 1997). The seeds of this plant show a very low germination percentage and vegetative propagation is limited by lower number of individuals. So, Tissue culture is the only rapid process for the mass propagation of this plant because the species is becoming rare in natural habitat condition due to habitat destruction and over exploitation.

The process of growth and morphophysiological specialization of cells from unorganized mass of callus cells i.e. differentiation, is a prerequisite for the application of biotechnology for crop improvement. Differentiation of organized structures in tissue culture is controlled by growth regulators such as cytokinins and auxins along with other components of the culture medium. Differentiation through callus cultures involves changes in some of the biochemicals (Kumar *et al.*, 2009). But very little is known about the biochemical

events occurring in the cultured cells. Analysis of various cellular metabolites and enzyme activities provides a reasonable and promising approach towards an understanding of the biochemical basis of the developmental pathway (Singh *et al*., 2009).

There are few reports available on biochemical studies related to shoots differentiation in callus cultures of another plant species (Singh *et al.,* 2006).The present study was therefore, undertaken to determine the changes in the levels of metabolites during shoot and root differentiation from callus cultures of *S. rebaudiana.*

## 2. Materials and Methods

MS basal medium was used without additives and supplemented with different concentrations of BAP, KIN, 2,4-D, IAA or NAA alone or in different combinations with different concentrations for callus induction.

The pH of the medium was adjusted to 5.8, prior to addition of agar and autoclaving at 121˚C, 1.2 kg $cm^{-2}$ pressure for 15 min.

**Plant material and callusing**

The leaf explants were obtained from mature plant of *S. rebaudiana* growing in Herbal Garden of Department of Botany, Kurukshetra University, Kurukshetra. These were washed with liquid detergent under running tap water to remove dust particles. The explants were then treated with 0.1% (w/v) mercuric chloride for 3-5 minutes under aseptic conditions. These explants were then thoroughly washed 4-5 times with sterilized double distilled water to remove the traces of mercuric chloride. Surface sterilized explants were inoculated onto the medium aseptically. Two explants were placed in each culture bottles.

The cultures were incubated at a temperature of 25±2$^{o}$C and a photoperiod of 16hrs light (intensity of 2000 lux) and 8hrs of dark.

**Differentiation**

After four-five weeks, the developed calli from leaf tissues were separated from explants and transferred to fresh medium of the same composition as induction medium (MS + 1.0 mg/l 2, 4-D + 1.0 mg/l Kn) thus using it also as 'callus proliferation medium'. For biochemical studies during root and shoot differentiation, the calli were further subcultured for root differentiation on MS medium + BAP (0.5 mg/l) + NAA (0.3 mg/l) and for shoot differentiation on MS medium+ BAP (0.5 mg/l) + NAA (0.1 mg/l).

The content of various metabolites, viz. starch, total soluble sugars, total soluble proteins and total phenols were studied on day 0, 5, 10, 15 and 20 after inoculation in callus on root induction medium, as well as on shoot induction medium.

**Estimation of metabolites**

Extraction of metabolites was done by the modified method of Barnett and Naylor (1966). For each sample, 100 mg of dry callus was homogenized in 80 per cent ethanol (v/v). The homogenate was refluxed for 15 min on a water bath at 60°C and centrifuged for 10 min at 5000 rpm. This procedure of refluxing and centrifuging was done three times with residue and collect supernatant each time. The supernatants were pooled and used for estimation of starch, total soluble sugars, total soluble proteins and total phenols by using methods of Yemm and Willis (1954), Hassid and Neufeld (1964), Bradford (1976), Amorim et al. (1977) respectively.

Various metabolites (starch, total soluble sugars, reducing sugars, total soluble proteins and total phenols) was assayed during roots differentiation as well as shoots differentiation at 5$^{th}$ , 10$^{th}$ , 15$^{th}$ and 20$^{th}$ day after inoculation of callus. The visual appearance of root formation was observed after 10$^{th}$ day of subculture. Before the appearance of shoot formation, green patches were formed. The visual appearance of shoots were observed after 15$^{th}$ day of subculture.

The callus was harvested on 5$^{th}$, 10$^{th}$, 15$^{th}$ and 20$^{th}$ day after inoculation. The undifferentiated callus before keeping on differentiating media was served as control.

**Statistical data analysis**

For the above experiments, 24 replicates were used for each treatment and each experiment was repeated thrice. The results are expressed as a Mean ± S.E. of three independent experiments.

Statistical analysis was done by using the formula:

$$SE = \pm \sqrt{\frac{(X^2)}{n(n-1)}}$$

SE = Standard error

X = deviation of mean

n = number of replicates (Snedecor, 1956).

## 3. Results and Discussion

The basal medium without growth regulators failed to respond for callus induction from leaf explant. Supplementation of growth regulators to MS basal medium resulted in callus induction but the frequency varied with the type and concentration of growth regulators used.

MS basal medium supplemented with auxins (IBA and NAA) alone failed to induce callus. However, when supplemented with 2, 4-D, 26-87% callusing was observed at different concentrations (0.5 - 2.0 mg/l) with poor growth of callus. Presence of 2,4-D has been shown to be essential for callus formation in *Momordica charantia* (Agrawal and Kamal, 2004). On adding different concentrations of Kn to MS medium, the callus growth was poor

On MS medium supplemented with 2,4-D (0.5 mg/l) + Kn (0.5 - 1.5 mg/l), per cent callus induction was upto 100% but visual growth of callus was poor to moderate. Good callus growth was observed when concentration of 2, 4-D was increased i.e. 1.0 mg/l along with Kn (0.5 and 1.0 mg/l). Best growth of callus was observed on 2, 4-D (1.0 mg/l) along with Kn (1.0 mg/l). So, this combination was selected for callus maintenance and for biochemical studies during root and shoot differentiation. The above callus was subcultured for root differentiation on MS medium + BAP (0.5 mg/l) + NAA (0.3 mg/l) and for shoot differentiation on MS medium+ BAP (0.5 mg/l) + NAA (0.1 mg/l). The role of auxins and cytokinins in callus induction was also advocated by Kumar and Singh (2009) in S*tevia rebaudiana,* Goel and Singh (2009) in *Peganum harmala*, Kumar and Singh (2009) in *Prosopis cineraria*, Lal and Singh (2010) in *Celastrus paniculatus* and Yadav and Singh (2010) in *Spilanthes acmella*.

To understand the differentiation events in callus tissues on regeneration medium, metabolites associated with organ differentiation in callus tissue were studied.

**Metabolites: Starch**

In the undifferentiated calli, starch content was high but on 5th and 10th day after subculture to rooting medium, the content increased further. The starch content decreased during root formation (10th and 15th days) but a little increase was observed on 20th day (**Fig. 1**).

The status of starch content in the calli during shoot differentiation showed a zig-zag trend i.e. the content increased on 5th day, then it decreased on 10th and 15th day but again it increased on shooting. Thus, it was observed that the undifferentiated calli contained more starch content than the differentiated ones (**Fig. 1**).

In the present study, it was found that starch content in callus declined during the process of root and shoot differentiation and increased only after shoot initiation. These results concide with the findings in *Chlorophytum borivilianum* (Singh *et al*., 2006). The decrease in starch content could be due to decrease in the activity of synthesizing enzymes or increase in hydrolyzing enzymes.

Fig. 1. Changes in the concentration of starch at different intervals on root and shoot differentiating medium

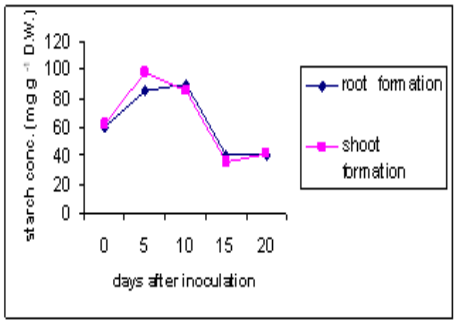


Fig. 2. Changes in the concentration of total soluble sugar at different intervals on root and shoot differentiating medium

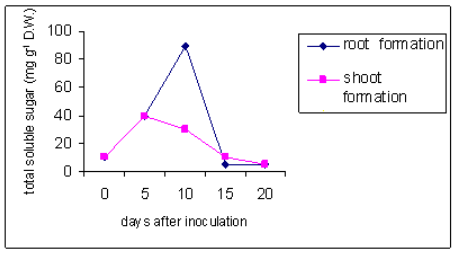



Fig. 3. Changes in the concentration of total soluble proteins at different intervals on root and shoot differentiating medium

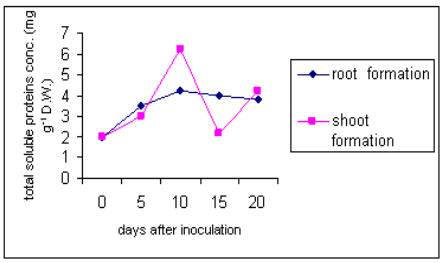
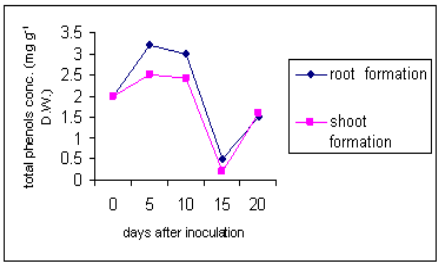



Fig. 4. Changes in the concentration of total phenols at different intervals on root and shoot differentiating medium




**Total soluble sugars**

A steady rise in total soluble sugars was observed from undifferentiating callus i.e. 0 day to 10th day old calli kept on root differentiation medium. But after that, a marked decrease was observed in total soluble sugars content in callus from root initiation to root appearance i.e. 15th and 20th day (**Fig. 2**).

Unlike root differentiating calli, marked increase in total soluble sugar content was observed only upto 5th day then the sugar content in callus decreased steadily upto shoot differentiation. Overall results show that the differentiated calli had less soluble sugars than undifferentiated calli (**Fig. 2**).

The decline in total soluble sugar content was associated with utilization of sugars for growth and differentiation process. Similar trend was also reported in *Cardiospermum halicacabum* (Jeyaseelan and Rao, 2005).

**Total soluble proteins**

In root differentiating calli, a steady increased in protein content was observed upto 15th day, but the content decreased a little after 15th day (**Fig. 3**).

While in case of shoot differentiating calli, the content increased slowly upto 5th day but a sharp increase was observed by 10th day. Thereafter, the protein content decreased on 15th day. There was again an increase in content on 20th day (**Fig. 3**).

It was observed that total soluble proteins in root and shoot forming calli were higher during root and shoot differentiation than in controlled callus. Since during differentiation the cells are quantitatively changing their activities, new proteins have to be synthesized, thus the protein concentration is high before differentiation. Similar observation was also reported by Mohapatra and Rath (2005).

**Total phenols**

The phenol content followed a zig-zag path from non-differentiated callus to differentiated one i.e. increased on 5th day, decreased during 10th and 15th day and then again increased on 20th day (**Fig. 4**).

The same trend was followed in shoots differentiating calli. Total phenols increased upto 5th day, decreased on 10th and 15th day and then again increased on 20th day. Thus, it showed that the amount of total phenols was higher in non-differentiated calli than in differentiated ones (**Fig. 4**).

Present investigation showed gradual decrease in phenolic content during differentiation. Phenols participate in formation of cross-linking of cell wall constituents which is catalyzed by peroxidase (Mader and Fussel, 1982).

Changes in the levels of metabolites and enzymes during differentiation from callus culture may be helpful in our understanding of the biochemical basis of developmental pathway of *S. rebaudiana.*

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