IN VITRO STUDIES ON *STEVIA REBAUDIANA* FOR STEVIOL GLYCOSIDE PRODUCTION

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By

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Dedicated
to
My family

CERTIFICATE

This is to certify that the thesis entitled "***In vitro* studies on *Stevia rebaudiana* for Steviol glycoside Production**" submitted by Pratibha Gupta has been prepared under our guidance in accordance to the rules and regulation of Indian Institute of Technology Delhi, India. The research report and results presented in thesis have not been submitted for any degree or diploma in any other institute or university.

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(Pratibha Gupta)

ABSTRACT

Stevia rebaudiana (Bertoni) has attracted economic and scientific interests due to its sweetness and therapeutic properties present in leaves. The sweetening compounds found mainly in the leaves are Steviol glycosides (SGs), with Stevioside being the most abundant, followed by Rebaudioside A. These SGs are non-fermentative, low-calorific, non-toxic and flavor enhancing sweeteners. This shows the advantages of *Stevia* over other artificial sweeteners as an ingredient for the food industry, thereby making *Stevia* a more suitable substitute for saccharine in different drinks, beverages and bakery products. *Stevia* also offers therapeutic benefits having anti-hyperglycemic, anti-hypertensive, and immuno-modulatory effects. These beneficial effects focused the importance of *Stevia*, however, plants raised through seeds varied widely due to its heterozygous nature, therefore, affecting the overall Stevioside content.

To avoid such problems and to improve the yield of Stevioside, it is necessary to propagate a genetically homogeneous population from a selected elite plant of desirable characters. For this, plant tissue culture (PTC) is found to have potential alternative approach for the production of advantageous medicinal compounds from plants and cell culture. Keeping all these facts in consideration, the present study was conducted on "*In vitro* studies on *Stevia rebaudiana* for SGs production". The work was focused on; (1) to develop an efficient protocol for micropropagation using axillary branching (2) the development and optimization of culture conditions for callus and suspension culture (3) the effect of elicitors (NaCl, Na_2CO_3, Proline and Polyethylene glycol (PEG)) on *in vitro* cultured shoots, callus and suspension culture for their growth (biomass yield i.e., fresh weight and dry weight) and on SGs production (4) evaluation of Stevioside and Rebaudioside A in *in vitro* cultured shoots, callus and suspension culture using HPLC technique.

For micropropagation studies, the application of growth regulators (cytokinin alone and in combination with auxin) showed a promoting effect on shoot multiplication and rooting. It was noticed that cytokinins especially kinetin (*Kn*) produced best results. Hence, a protocol was standardized taking different concentrations of *Kn* (1-10 mgl^{-1}) and it was observed that nodal explants cultured on Murashige and Skoog (MS) medium + 4.0 mgl^{-1} *Kn* was the best medium for shoot multiplication. Also, for rooting ½ strength MS medium + 0.05 mgl^{-1} IBA + 0.05% activated charcoal produced best results. For hardening, mixture of soil and agropeat (3:1) was found to be best. Among different explants (leaves, nodes and *in vitro* raised roots) tested for callus induction, leaves explant showed better response. However, combination of auxins (0.75 mgl^{-1} NAA + 1.0 mgl^{-1} 2,4-D) with MS media were found to be suitable for callus induction while for development and multiplication, MS medium with 2.0 mgl^{-1} NAA (i.e. maintenance media) showed superior results. Further, suspension culture developed through calli obtained from maintenance media demonstrated good growth followed by S-shaped growth curve.

The effect of different elicitors (NaCl, Na_2CO_3, Proline and PEG) on *in vitro* cultured shoots, callus and suspension culture were studied. *Stevia* shoots showed maximum stress with PEG followed by Na_2CO_3. However, lower concentrations of NaCl and Proline could be tolerated by explants with shoot growth. A significant reduction in shoot growth (i.e., shoot length, number of shoots/node and multiplication fold) was noticed with increased concentrations of elicitors tested. Similarly, a significant reduction in callus biomass (i.e., fresh weight and dry weight) and growth index (GI) was observed with different concentrations of elicitors tested. However, in case of salts (NaCl, Na_2CO_3), continuous decline in biomass and GI was observed with their increased concentrations. In contrast, with optimum concentrations of Proline (5 mM) both biomass and GI were increased while at further higher concentrations (≥7.5 mM) negative effect was seen. However, in case of PEG, only fresh weight content increased upto 5% PEG, whereas dry weight and GI reduced

significantly at all concentrations of PEG. In case of suspension culture, all parameters studied (i.e., fresh weight, dry weight and moisture content) were affected with increased concentrations of salts (NaCl and Na_2CO_3). Proline upto 5 mM concentration increased the fresh weight, dry weight and moisture content and after that negatively affected the results. Similarly, fresh weight increased upto 5% PEG while at further higher concentrations significant reduction was observed. Also, with all concentrations of PEG moisture content increased while dry weight decreased.

SGs analysis of *in vivo* and *in vitro* grown plants, callus and suspension culture (with and without elicitors) was done. In case of field grown plants, maximum production of total SGs (Stevioside and Rebaudioside A) were found in the cultivar procured from Maharashtra (9.26%) followed by Haryana A (8.74%), Haryana B (7.82%) and Uttarakhand (6.33%) plants. Leaves obtained from *in vitro* shoots showed maximum amount of SGs (2.60%) with 0.025% Na_2CO_3 followed by 5 mM Proline (1.65%), 0.10% NaCl (1.25%) and 5% PEG (1.15%), which were 3.3, 2, 1.6 and 1.5 times higher than control (0.79%), respectively. Further, in case of callus, optimum concentrations of each salt i.e., 0.10% NaCl and 0.025% Na_2CO_3 produced 5.3 and 5.6 times higher SGs (1.43% and 1.51%) while 2.5 mM Proline and 5% PEG produced 4.0 and 7.0 times higher SGs (1.09% and 1.83%) than control (0.27%), respectively. Similarly, in case of suspension culture, with optimum concentrations of each, 0.10% NaCl and 0.025% Na_2CO_3, 5 mM Proline and 5% PEG produced 2.61%, 5.14% 5.03% and 6.38% SGs respectively, which were 1.9, 3.8, 3.7 and 4.7 times higher than control, respectively.

Hence, it can be concluded that protocol developed for mass propagation and callusing will be helpful for high frequency regeneration, somaclonal variation and genetic engineering experiments. Also, it was found that the application of abiotic elicitors and appropriate culture conditions can enhance the production of secondary metabolites in *in vitro* raised *Stevia* shoots, callus and suspension culture.

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