International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:4, No:12, 2010

Callusing in *Stevia rebaudiana* (Natural Sweetener) for Steviol Glycoside Production

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Abstract**—***Stevia rebaudiana* Bertoni (natural sweetener) belongs to *Asteraceae* family and can be used as substitute of artificial sweeteners for diabetic patients. Conventionally, it is cultivated by seeds or stem cutting, but seed viability rate is poor. A protocol for callus induction and multiplication was developed to produce large no. of calli in short period. Surface sterilized nodal, leaf and root explants were cultured on Murashige and Skoog (MS) medium with different concentrations of plant hormone like, IBA, kinetin, NAA, 2,4-D, and NAA in combination with 2,4-D. 100% callusing was observed from leaf explants cultured on combination of NAA and 2,4-D after three weeks while with 2,4-D, only 10% callusing was observed. Calli obtained from leaf and root explants were shiny green while with nodal explants it was hard and brown. The present findings deal with induction of callusing in *Stevia* to achieve the rapid callus multiplication for study of steviol glycosides in callus culture.

Keywords**—**2,4-D, Callusing, NAA, *Stevia,* Steviol glycosides

I. Introduction

STEVIA rebaudiana Bertoni (natural sweetener) is a perennial plant belonging to the Asteraceae family. It is sweet herb of Paraguay, which contains natural non-caloric sweetener and also referred as 'the sweet herb of Paraguay'. *Stevia* leaves contain a number of diterpene steviol glycosides (SGs) which are about 300 times sweeter than sucrose at their concentration of 4% (w/v) [7]. The nine types of steviol glycosides found in *Stevia* are; Stevioside, Rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside E, Rebaudioside F, Steviolbioside A and Dulcoside A. It was reported that these glycosides are non-toxic, non- mutagenic and low calorie compounds and unlike traditional sugar substitutes such as xylitol or sorbitol [9]. Nowadays, *Stevia* plants and Stevioside are being used as sweeteners in South America, Asia, Japan, China, and in some countries of Europe. *Stevia* is more frequently used in the food industry of Japan, Korea and Brazil. The production of Steviol glycosides in the *in vitro* raised cultures is poorly understood, and the results obtained by different authors are contradictory. However, regeneration through micropropagation of *Stevia* was developed by many scientists in India [2], [5], [11] and [13] as well as in other countries. Plants can also be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators but very few reports are available for the regeneration of *Stevia* plant through callus culture [1], [6], [10], [12], [16]. The composition of culture medium plays an important role in determining the morphogenetic pathway. A cytokinin (usually BAP) and an auxins (mostly Indole acetic acid (IAA), α-Napthalene acetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4D) are normally included in the primary culture medium for callus formation followed by incorporation of a lower auxin to cytokinin ratio for shoot induction and a higher ratio for rooting in the subsequent media.

Development of seedlings suitable to India and production of required quantity of biomass is the first requirement. Therefore the development and phytochemical characterization of new variety of *Stevia rebaudiana* with higher level of steviol glycoside is a primary aim of research groups and industries concerned with the improvement and utilization of this source of natural sweetener. Hence the objective of this work was to develop a tissue culture protocol for the induction and development of callus from different explants (leaf, node and root) of *Stevia* to study the production of steviol glycosides in callus culture.

II. Materials and Method

A. Collection of Plant Material

Stevia plants were procured from Ritnand Balved Education Foundation Amity Education valley, Panchgaon (Manesar, Gurgaon) for study and are presently maintained in the Micromodel complex of IIT Delhi, India.

B. Explants Preparation and Culture Conditions

The nodal and leaf explants were cultured on MS medium to choose the best performing explants for callus production. These explants were initially washed with running tap water and thereafter surface sterilized with 70% alcohol for 2 min followed by treatment with 0.1% mercuric chloride (10 minutes for nodal explants and 6-7 minutes for leaf explants) under aseptic conditions. Finally, they were washed with sterile distilled water successively for 3-4 times and inoculated on MS medium supplemented with different concentrations of phytohormones like 2,4-D, IBA, NAA, and Kinetin. Murashige and Skoog (MS) medium was prepared with 3% (w/v) sucrose and solidified with 0.8% Agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121ºC and

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1.2–1.3 kg/cm^2 pressure for 20 min. All the cultures were incubated in growth chamber, at 24±1ºC temperature and 1600 lux light intensity provided by white fluorescent tubes for 16 hr.

C. Culture Establishment

1. Callus Induction from Leaf Explants

Callus cultures were established from surface sterilized nodal and leaf explants cultured on the MS medium with different concentrations of phytohormones. Leaf explants were cut into small pieces (Approx.1.0 cm^2) and placed on MS medium with dorsal surface in contact with the medium supplemented with different concentrations of IBA (0.25 mg/l to 3.0 mgl^{-1}), kinetin (0.25 to 3.0 mgl^{-1}), NAA (1.0 to 3.0 mgl^{-1}), 2,4-D (1.0 to 5.0 mgl^{-1}) and NAA in combination with 2,4-D (0.5+1.0, 0.75+1.0, 1.0+1.0 and 2.0+ 1.0 mgl^{-1} respectively).

2. Callus Induction from Nodal Explants

Establishment of culture was done by culturing approx. 1.0-1.5cm of nodal explants on MS medium supplemented with kinetin (1 to 5.0 mgl^{-1}), BAP alone (1.0 and 2.0 mgl^{-1}) and BAP in combination with NAA (1.0+0.5, 1.0+1.0, 1.0+ 2.0, 2.0+ 1.0 and 2.0+2.0 mgl^{-1} respectively).

3. Callus Induction from Root Explants

Root explants were collected from the rooted *Stevia* shoots cultured on 1/2 strength MS with 0.5 mgl^{-1} IBA. For callus induction these root explants were further sub-cultured on MS medium supplemented with different concentration of IBA (0.5, 1.0 and 2.0 mgl^{-1} respectively).

D. Callus Development / Callus Multiplication

Callus culture obtained from the leaf, nodal and root explants were sub-cultured on full and half strength of MS medium supplemented with different phytohormones (NAA alone, IBA, and NAA+2, 4-D) for the development of callus. The amount of callus was estimated by a 0-4 Scale scoring system (Table I) [3] and the number of shoots developed from the callus was counted.

TABLE I
A '0-4 SCALE' SCORING SYSTEM DEVELOPED FOR MEASURING THE AMOUNT OF CALLUS OBTAINED FROM LEAF, NODE AND ROOT EXPLANTS

Score	Description
0	No visible callus
1	Small proliferation at cut ends only
2	5 mm callus at cut ends
3	5-10 mm callus from all over the explants
4	> 10 mm callus from all over the explants

Experiments were repeated many times, each with fifteen replicates, and callus and shoot formation were recorded after 30 days of culture initiation. Analysis of variance was carried out and the differences between the treatments were determined by Duncan's Multiple Range Test (DMRT) at 5% level of significance using SPSS (SPSS ver. 16.0).

III. RESULTS AND DISCUSSIONS

A. Callus Induction

1. Callus Induction from Stevia Leaves

Callus induction has been reported from different plant parts (leaf, node and root etc.) of *Stevia*. Auxins are a class of growth regulators which causes cell elongation, apical dominance and root initiation and Cytokinins promote cell division and proliferation of tissue.

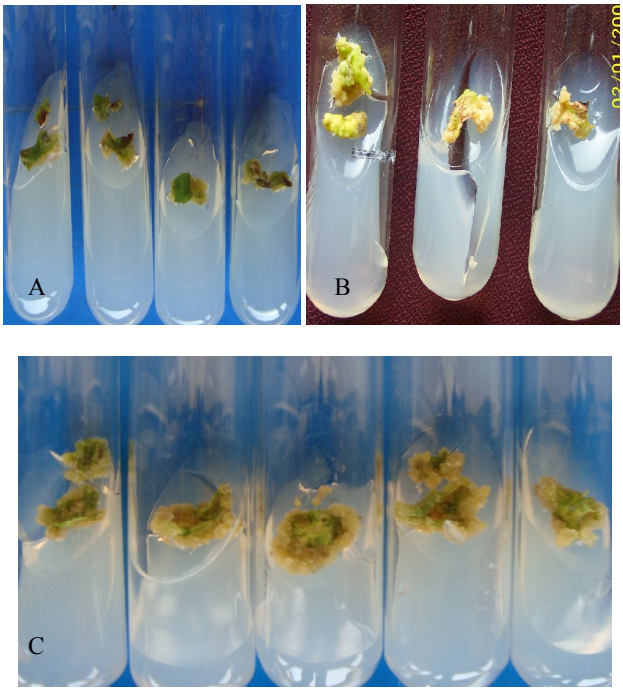

Fig. 1 Callusing from leaf explants on MS medium supplemented with 1.0 mgl^{-1} IBA (A), 0.5 mgl^{-1} kinetin (B) and 0.75 mgl^{-1} NAA+ 1.0 mgl^{-1} 2,4- D (C) respectively.

The most frequently used auxins are 2,4-D, NAA, IAA, IBA etc. of which IAA occurs naturally in plants. Leaf pieces were used as a primary explants for callus induction. Callus initiation was observed from cut surface of leaves after 3 to 4 week of culture initiation.

Maximum amount of callusing was observed on the medium supplemented with combination of NAA and 2,4-D (0.75 mgl^{-1} NAA+ 1.0 mgl^{-1} 2,4-D) after 3 weeks of culture initiation. It was found that leaf explants cultured on lower conc. of NAA with 2,4-D (0.75 mgl^{-1} NAA+ 1.0 mgl^{-1} 2,4-D) showed 100% response while at other higher concentrations of NAA, the callus induction response was only 33.3% (Fig. 1). Calli obtained from this conc. were soft, globular and whitish green in colour. Such growth hormones concentrations were further used for callus induction from *Stevia* leaves. The results pertaining to induction medium for callusing are given in Table II.

TABLE II
EFFECT OF AUXIN AND CYTOKININ ON LEAF EXPLANTS OF *STEVIA* FOR CALLUS DEVELOPMENT

Conc. of GRs (mgl^{-1})				% of tubes showing callusing#	Description of Callus	Nature of callus
2,4-D	IBA	NAA	Kinetin			
0	0	0	0	$0.0^{a*} \pm 0.00$	0	0
0.5-2.5	0	0	0	$0.0^{a} \pm 0.00$	0	0
3.0	0	0	0	$0.47^{cd} \pm 0.52$	0	0
3.5	0	0	0	$0.40^{bcd} \pm 0.51$	0	0
4.0	0	0	0	$0.33^{bc} \pm 0.49$	0	0
4.5	0	0	0	$0.27^{ab} \pm 0.46$	0	0
5.0	0	0	0	$0.33^{bc} \pm 0.49$	0	0
0	0.25	0	0	$0.33^{bc} \pm 0.49$	1	Whitish green
0	0.5	0	0	$0.40^{bcd} \pm 0.51$	2	Whitish green
0	1	0	0	$0.67^{de} \pm 0.49$	4	White and fragile
0	1.5	0	0	$0.00^{a} \pm 0.00$	1	0
0	2	0	0	$0.00^{a} \pm 0.00$	1	0
0	2.5	0	0	$0.00^{a} \pm 0.00$	1	0
0	0	0.5	0	$0.00^{a} \pm 0.00$	2	0
0	0	1.0	0	$0.13^{ab} \pm 0.35$	2	0
0	0	1.5	0	$0.27^{ab} \pm 0.46$	2	0
0	0	2.0	0	$0.40^{bcd} \pm 0.51$	2	0
1.0	0	0.5	0	$0.80^{e} \pm 0.41$	4	Whitish green & globular
1.0	0	1.0	0	$0.93^{f} \pm 0.26$	4	Whitish green & globular
0	0	0	0.5	$0.53^{cd} \pm 0.52$	3	White and fragile
0	0	0	1	$0.13^{ab} \pm 0.35$	2	White and fragile

± Standard Error of 15 replicates in each treatment and each experiments were repeated three times,
Mean % of nodal explants showing callusing
* Different alphabets represent significant variance at $p \leq 0.05$ level by Duncan's Multiple Range Test (DMRT).

Only 55 % response for callus induction was observed from leaf explants cultured on lower concentrations of IBA and kinetin after six weeks of culture initiation. The calli obtained on these growth hormone concentrations were white and fragile. No callusing was observed on the medium supplemented with either 2,4-D or NAA. It was observed that leaf explants cultured on lower concentration of 2,4- D (1.0 - 2.5 mgl^{-1}) died during first week of culture initiation while the leaf explants cultured on higher concentrations of 2,4-D (3.0-5.0 mgl^{-1}) showed good response (leaf explants expand and green in colour) upto the three weeks of culture initiation but before start callusing all leaf explants gradually changed their colour from green to yellow then brown and finally died.

The effect of 2,4-D on callus culture was found in contrast to as [14], who developed the callus on MS with 0.1mgl^{-1} 2,4-D. Variations in the results may be due to the endogenous phytohormone contents in plants, their uptake, type of Auxins and Cytokinins used and their mode of action. Reference [4] induced the callusing from leaf explants cultured on MS medium supplemented with 2.0 mgl^{-1}NAA and 2.0 mgl^{-1} kinetin. Reference [12] and [13] also reported the callus from leaf explants cultured on MS medium supplemented with 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP and 0.9% Agar.

2. Callus Induction from Nodal Explants of Stevia

Surface sterilized nodal explants cultured on MS medium showed callusing from the cut end (base of nodal explants which is dipped in nutrient medium) and nodal part (Table III, Fig 2). It was observed that nodal explants cultured on 2.0 mgl^{-1} BAP+ 0.8 mgl^{-1} NAA showed 60% response while the nodal explants cultured on higher conc. of BAP + NAA showed only 10% callusing after six weeks of culture initiation. The callus obtained from the cut end was brown and hard and they were died during second weeks of sub culturing. The nodal explants cultured on higher concentrations of kinetin (3.0, 4.0 and 5.0 mgl^{-1}), showed maximum amount of callusing from their nodal part. At 5.0 mgl^{-1} of kinetin 70 % callusing was observed and callus obtained was white and hard.

TABLE III
EFFECT OF AUXIN AND CYTOKININ ON NODAL EXPLANTS OF *STEVIA* FOR CALLUS DEVELOPMENT

Conc. of GRs (mgl^{-1})			Nodal Explants showing Callusing (%) #	Amount of Callus	Nature of callus
BAP	NAA	Kinetin			
0	0	0	0.00 ± 0.00	0	No callus
2.0	0.8	0.00	0.60^{c} ± 0.51	2	Brown & hard
2.0	2.0	0.00	0.47^{bc} ± 0.52	1	Brown & hard
2.0	4.0	0.00	0.20^{ab} ± 0.41	0	No callus
4.0	4.0	0.00	0.33^{abc} ± 0.49	0	No callus
0	0	3.00	0.40^{bc} ± 0.51	1	White and compact
0	0	4.00	0.53^{bc} ± 0.52	1	White and compact
0	0	5.00	0.60^{c} ± 0.51	2	White and compact

± Standard Error of 15 replicates in each treatment and each experiments were repeated three times,
Mean % of nodal explants showing callusing
* Different alphabets represent significant variance at p≤ 0.05 level by Duncan's Multiple Range Test (DMRT).

The auxin 2, 4-D alone or in combination with cytokinins is widely used to enhance callus induction and maintenance. As reported by Morel (1948) NAA, 2,4-D; 2,4-T were the most effective auxin to initiate callus.

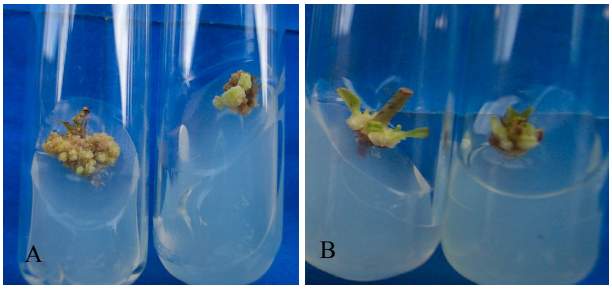

Fig. 2 Callus induction from nodal explants of *Stevia* on MS medium supplemented with 2.0 mgl^{-1} BAP+ 0.8 mgl^{-1} NAA (A) and 5.0 mgl^{-1} Kinetin (B).

The most widely used cytokinins are kinetin, BAP, 2 ip and adenin sulphate. Reference [8] reported that auxins were more effective when combined with low BAP levels (0.05mgl^{-1}) for callus production. However early callus formation and maximum callus production from nodal explants of *Stevia rebaudiana* using MS medium with 13.56 µM 2,4-D was reported [15], whereas in the present study leaf explants produced better results than that study.

B. Callus Development / Callus Multiplication

To choose the best nutrient medium for callus development a various umber of experiments were conducted. For callus development, callus culture (obtained from leaves, node and root explants) were further sub-cultured on full and half strength of MS medium supplemented with different phytohormones (NAA alone, IBA, and NAA+2, 4-D). The results for developing medium are given in Table IV, Fig 3.

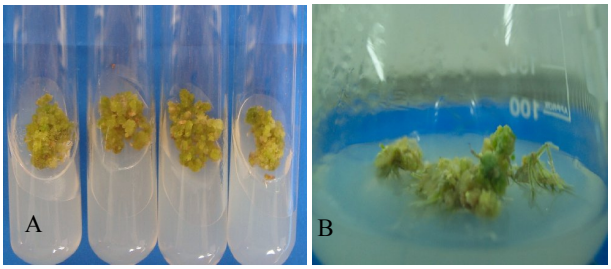

Fig. 3 Callus developed from leaf explants (A) and root explants (B) cultured on MS medium supplemented with 2.0 mg l^{-1} NAA and 0.5 mgl^{-1} IBA respectively.

Maximum amount of calli were found with leaf callus sub cultured on full strength MS medium supplemented with 2.0 mgl^{-1} NAA while the callus obtained from nodal part were brown and soft which were died after two weeks. Roots (obtained from *in-vitro* plants) of *Stevia* were also sub cultured on developing medium (half strength MS with different concentration of IBA). It was observed that the calli obtained from all the concentrations were shiny and greenish white and showed 100% response.

IV. CONCLUSION

It is important to develop an efficient protocol for callus proliferation in order to start *in vitro* selection for salt tolerance, and to broaden the opportunities for genetic manipulation of *Stevia* through tissue culture, using various explants and media having different composition of growth hormones.

TABLE IV
EFFECT OF GROWTH REGULATORS AND NUTRIENT MEDIUM ON CALLUS CULTURE OBTAINED FROM DIFFERENT EXPLANTS OF *STEVIA* (AFTER THREE WEEKS OF CULTURE)

S.N.	Explants	Growth regulators (GRs)	Conc. of GRs (mgl^{-1})	Nutrient medium		% of tubes showing response*	Nature of callus	Multiplication fold
				MS	½ MS			
		NAA	1.0	-	+	80	White & fragile	2
1.	Leaf			+	-	70	Light green & globular	3
			2.0	-	+	90	Whitish green & globular	2
				+	-	100	Dark green & globular	4
		NAA+ 2,4-D	1.0	-	+	80	Brown & fragile	2
			2.0	-	+	70	Brown & fragile	2
2.	Node	NAA	1.0	-	+	90	White & soft	1
			2.0	-	+	70	White & soft	1
		NAA+ 2,4-D	1.0	-	+	50	Brown &soft	1
			2.0	-	+	70	Brown &soft	1
3.		IBA	0.5	-	+	100	Shiny & greenish white	2
	Root		1.0	-	+	70	Shiny & greenish white	2
			2.0	-	+	80	Shiny & greenish white	2

*10 tubes in each experiment, (- sign) Absent, (+ sign) Present

Present findings on *Stevia* plants indicated that the leaf explants could serve as a best planting material for callus production. The protocol developed using leaf explants cultured on MS medium supplemented with 0.75 mgl^{-1} NAA & 1.0 mgl^{-1} 2,4-D could be a suitable medium and noble approach to produce maximum amount of callus within short time period.

ACKNOWLEDGMENT

The authors would like to thanks Council for Scientific and Industrial Research (CSIR), India to provide Travel grant and Science and Society Division, Department of Science and Technology, Government of India for the financial support.

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