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Research Article

Regenerative callus induction and biochemical analysis of *Stevia rebaudiana* Bertoni

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Abstract: Stevia Leaves are the principal source of stevioside, which is estimated to be 100-300 times sweeter than table sugar. Stevioside has clinical significance as they are reported to maintain glucose levels in human blood. Owing to the difficulties in propagation of stevia through seeds and vegetative methods, callus culture has been an efficient alternative for generation of stevioside. The aim of this study is to develop an efficient and standardized protocol for maximum induction and multiplication of callus from a leaf. Callus culture was established from leaves in MS basal media fortified with various combinations (BAP, NAA, 2,4-D, KN, IBA) and concentrations of phytohormones. The best callusing (100%) was recorded in MS media supplemented with (2,4-D 1.0mg/l + NAA 1.0mg/l). The callus was harvested after 4 weeks and screened for the presence of various bioactive compounds. The qualitative results showed that the extracts of callus contained bioactive compounds like flavonoids, glycosides, phenol, tannins, sterols and saponins thereby making callus one of the sources for extraction of various secondary metabolites.

Keywords: *Stevia rebaudiana*, callus, 2,4-D, phytochemical screening, plant growth regulators (PGR).

1. Introduction

Stevia rebaudiana Bertoni is one of the 154 members of the genus *Stevia* (Pande & Khetmalas, 2012). It is a perennial herb and belongs to the family Asteraceae. It is a native of certain regions of South America, particularly Paraguay and Brazil (Anbazhagan *et al.*, 2010). *S. rebaudiana* has its name after Dr. M.S. Bertoni who officially discovered *Stevia* during the early 20th century (Das *et al.*, 2006). It is otherwise popularly known as candy leaves, non-caloric sweet plant, *Stevia*, sugar substitute, sweet weed, honey leaf and sweet herb of Paraguay (Das *et al.*, 2006; Anbazhagan *et al.*, 2010).

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) (Kinghora & Soejarto, 1986). Along with the steviol glycosides, Stevia leaf constituents include volatile oil components, sterols, triterpenes, flavonoids, coumarins, and non-glycosidic diterpenes (Talha *et al*., 2012). Particularly, leaves of stevia possess high protein, carbohydrate, and some active chemical. It has high Potassium, Calcium, Magnesium, Phosphorous, Sodium and Sulphur content in leaves and Cobalt, Copper, Iron, Manganese, Zinc, Selenium, Molybdenum are found in trace amounts.

Vegetative propagation of stevia is limited by the low number of individuals that can be obtained simultaneously from a single plant. On the other hand, tissue cultured platelets offer excellent quality of foliage production in disease free condition and callus masses can sometimes yield the highest amount of secondary metabolites (Das *et al.*, 2010). Thus, tissue culture is the only alternative for rapid mass propagation, conservation, and enhancement of the natural levels of valuable compounds of *Stevia* plants (Chan *et al*., 2005). Parts like leaves, nodes and shoot tips from *Stevia* can be used to raise plants *in vitro* (Naz *et al*., 2008).

The objective of the present study was to develop an efficient protocol for the development of

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regenerative callus from leaf segments and to assess the biochemical constituents of callus and leaf extracts.

2. Materials and Methods

2.1 Plant material and callus regeneration

Young twigs of *Stevia rebaudiana* were taken from the Nepal Microplant Limited, Kusunti, Nepal. The plant materials were thoroughly washed for 45 minutes under running tap water and were surface sterilized in 70% (v/v) ethanol for 1 minute and 0.1% (v/v) Mercuric chloride solution for 3 minutes before being washed three times with sterile distilled water (Table 2). Explants (leaf segment) were inoculated aseptically onto MS medium fortified with phytohormones alone or in combinations with different concentrations like BAP, 2,4-D, IBA, NAA, and Kinetin for callus induction (Table 1). Callus is routinely subcultured (every 4 weeks) in fresh medium with the respective PGRs. For the establishment of callus, 25 different types (C1 to C25) of media were prepared which were grouped into 5 types. First (C1 to C5) contained BAP (1-2mg/l) and NAA (1-3mg/l); second (C6 to C10) comprised Kinetin (2-6mg/l); third (C11 to C16) consisted 2,4-D (1-2mg/l); fourth (C17 to C20) contained and 2,4-D (1-2mg/l) and NAA (1-1.5mg/l); fifth (C21 to C25) contained 2,4-D (1mg/l) and IBA (0.5-2.5mg/l). The pH of the media was adjusted to 5.8 with 1N NaOH or 1N HCl before it was dispensed into culture vessels and autoclaved at 121^0C for 15 minutes. The temperature of culture room was maintained at 25 $\pm$ 2^0C with 16 hours photoperiod provided by 40W cool-white fluorescent tubes.

2.2 Biochemical Analysis

5g of the sample (powdered leaf and callus) was dissolved in 250ml sterile water and then boiled at 50-60°C on water bath. The solution was filtered and stored at 4°C for further use. The phytochemical analysis (Tannins, saponins, sterol, flavonoids, lignin, phenol and glycosides content) was determined by using the methods of Edeoga *et al*., (2005).

3. Results and Discussion

3.1 Surface Sterilization

Among various combinations tested for surface sterilization of explants, the best treatment was found to be the dipping of explants in 70% ethanol for 1 minute followed by sterilization with $HgCl_2$ (0.1%) for 3 minutes as it prevented browning of tissues and microbial contamination (Table 2).

Table 1. Different Media Compositions for Callus Induction.

S. No.	Medium code	BAP (mg/l)	NAA (mg/l)	KN (mg/l)	2,4-D (mg/l)	IBA (mg/l)
1	C1	1.0	1.0	0.0	0.0	0.0
2	C2	1.0	2.0	0.0	0.0	0.0
3	C3	2.0	1.0	0.0	0.0	0.0
4	C4	2.0	2.0	0.0	0.0	0.0
5	C5	2.0	3.0	0.0	0.0	0.0
6	C6	0.0	0.0	2.0	0.0	0.0
7	C7	0.0	0.0	3.0	0.0	0.0
8	C8	0.0	0.0	4.0	0.0	0.0
9	C9	0.0	0.0	5.0	0.0	0.0
10	C10	0.0	0.0	6.0	0.0	0.0
11	C11	0.0	0.0	0.0	1.0	0.0
12	C12	0.0	0.0	0.0	2.0	0.0
13	C13	0.0	0.0	0.0	3.0	0.0
14	C14	0.0	0.0	0.0	4.0	0.0
15	C15	0.0	0.0	0.0	5.0	0.0
16	C16	0.0	0.0	0.0	6.0	0.0
17	C17	0.0	1.0	0.0	1.0	0.0
18	C18	0.0	1.5	0.0	1.0	0.0
19	C19	0.0	1.0	0.0	2.0	0.0
20	C20	0.0	1.5	0.0	2.0	0.0
21	C21	0.0	0.0	0.0	1.0	0.5
22	C22	0.0	0.0	0.0	1.0	1.0
23	C23	0.0	0.0	0.0	1.0	1.5
24	C24	0.0	0.0	0.0	0.0	2.0
25	C25	0.0	0.0	0.0	0.0	2.5

BAP: Benzyl amino purine; NAA: Naphthalene acetic acid; KN: Kinetin; 2,4-D: 2,4-Dichlorophenoxyacetic acid; IBA: Indole-3-butyric acid.

3.2 Callus Induction

Leaf was used as a primary explant. The experiments were repeated many times, each with three replicates and callus were recorded after four weeks of culture initiation. The most frequently used auxins were 2,4-D, NAA, IBA, BAP, Kinetin (Table 2). Callus initiation was observed from leaf after two weeks of culture initiation. Best response (100%) of callus regeneration was seen in C17 (2,4-D 1.0mg/l + NAA 1.0mg/l) medium. Callus produced from this concentration were soft, globular/irregular, fragile and whitish green in color as shown in Fig. 1. The other media, C20 showed 84% callus while C19 and C13 showed 81% callus induction. The Callus appeared slightly whitish and yellowish green, amorphous and moderately fragile. The lowest callus induction (33%) was recorded in C6 (KN 2.0mg/l) medium after four weeks of culture initiation (Table 3). Many authors developed the protocol for the establishment of callus from the leaf and nodal segments of stevia (Hsing *et al*., 1983; Abou-Arab *et al*., 2010; Swanson *et al*., 1992; Das *et al*., 2006; Banerjee and Sarkar, 2008; Janarthanam *et al*., 2009; Gupta *et al*., 2010; Pande & Khetmalas, 2012 and Guruchandran and Sasikumar, 2013). Callus formation from nodal segments of *S. rebaudiana* was reported using MS basal medium supplemented with 2,4-D gave maximum callus which showed less conflict than our findings (Uddin *et al*., 2006). On the other hand callus establishment from leaf segments in MS basal media fortified with 2,4-D and BAP supported our findings (Ferreira and Handro, 1988).

Table 2. Effect of disinfectants on explants survival and establishment.

S. No.	Ethanol (70%) Exposure Time	$HgCl_2$ (0.1%) Exposure Time	Response of Explants	Contamination
1.	1 minute	6 minutes	Brown (dead)	-
2.	1 minute	5 minutes	Brown (dead)	-
3.	1 minute	4 minutes	Brown (dead)	-
4.	1 minute	3 minutes	Green (live)	-
5.	1minute	2 minutes	Green (live)	+
6.		6 minutes	Brown (dead)	-
7.		5 minutes	Brown (dead)	-
8.		4 minutes	Brown (dead)	-
9.		3 minutes	Green (live)	+
10.		2 minutes	Green (live)	+

Table 3. Effect of different concentrations of phytohormones on callus induction and growth.

S. No.	Explants	Medium code	Callus induction (%)	Callus morphology
1	Leaf	C1	60.0 ± 4.71	Slight whitish green
2	Leaf	C2	52.0 ± 6.32	Light green
3	Leaf	C3	56.0 ± 6.99	Light green
4	Leaf	C4	42.0 ± 6.32	Light yellowish green
5	Leaf	C5	52.0 ± 6.32	Light green
6	Leaf	C6	33.0 ± 4.83	Light green
7	Leaf	C7	40.0 ± 4.71	Yellowish Green
8	Leaf	C8	42.0 ± 7.89	Light yellowish green
9	Leaf	C9	50.0 ± 9.43	Yellowish Green
10	Leaf	C10	43.0 ± 8.23	Light brown
11	Leaf	C11	63.0 ± 6.75	Slight whitish Green
12	Leaf	C12	70.0 ± 4.71	Light green
13	Leaf	C13	81.0 ± 3.16	Light yellowish green
14	Leaf	C14	62.0 ± 4.22	Light green
15	Leaf	C15	51.0 ± 8.76	Light green
16	Leaf	C16	52.0 ± 7.98	Light yellowish green
17	Leaf	C17	100.0 ± 00	Slight whitish green
18	Leaf	C18	70.0 ± 4.71	Light green
19	Leaf	C19	81.0 ± 3.16	Light yellowish green
20	Leaf	C20	84.0 ± 5.16	Slight whitish green
21	Leaf	C21	51.0 ± 3.16	Yellowish green
22	Leaf	C22	43.0 ± 6.75	Light green
23	Leaf	C23	51.0 ± 3.16	Yellowish green
24	Leaf	C24	41.0 ± 3.16	Light green
25	Leaf	C25	43.0 ± 6.75	Light brown

(Results are mean ± SD of three replicates)

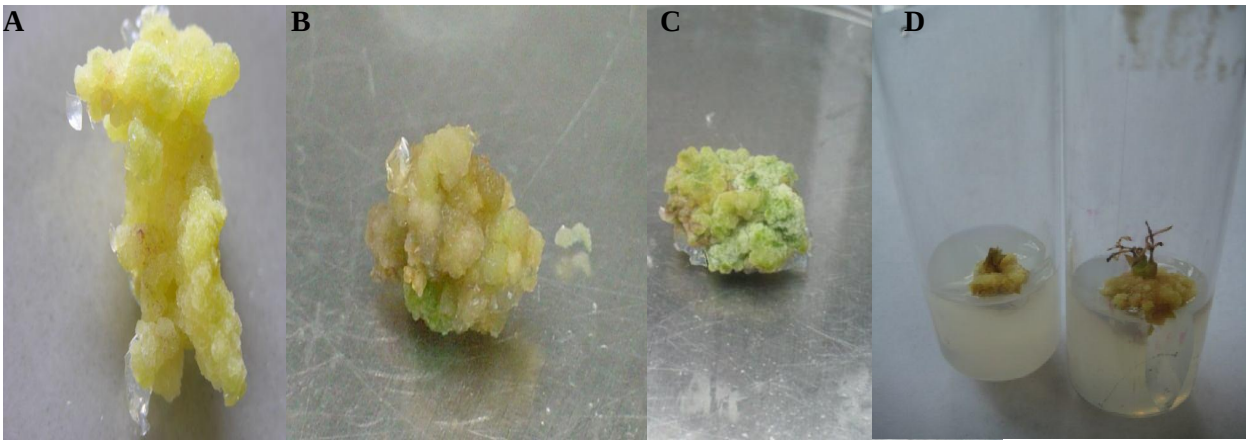

Fig. 1. Proliferation of callus in media supplemented with different phytohormones in different concentration (A: Light yellowish green callus; B: Light greenish yellow callus; C: Greenish yellow callus; D: Brownish yellow callus).

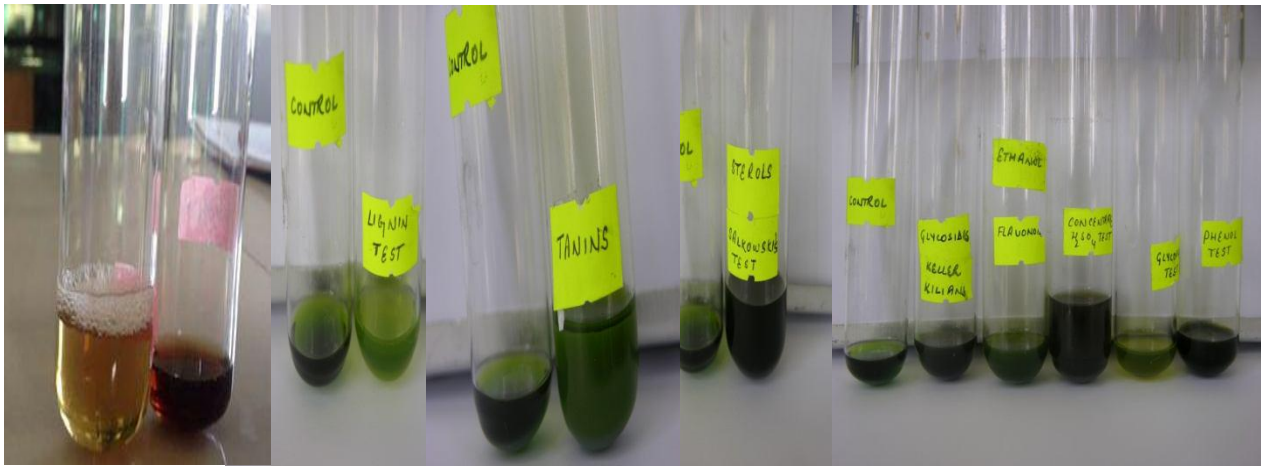

Fig. 2. Phytochemical Screening.

3.3 Biochemical Analysis

Different phytochemical tests have been done for the detection of presence of bioactive compounds (Harborne, 1973; Trease and Evans, 1987). The present study conducted on the *Stevia* plant revealed the presence of bioactive compounds. Table 4, showed the result of phytochemical screening of *S. rebaudiana* in green leaf and callus. Green leaf showed the maximum presence of flavonoids, glycosides, phenol, tannins, sterols and saponins, whereas callus showed minimum presence. A similar study was carried by Upadhyay *et al*., 2013, also showed the presence of flavonoids, glycosides, phenol, saponins, reducing compounds, alkaloids and tannins in the aqueous extract of leaves.

Table 4. Phytochemical Screening.

S. No.	Sample	Flavonoids	Glycosides	Phenol	Lignin	Sterols	Tannins	Saponins
1	Green leaf	+++	+	+++	_	++	+++	+++
2	Callus	++	+	++	_	+	++	++

+ Positive notification of the presence of tested group, +++ Distinct notification, ++ Clear notification, - Absence

4. Conclusion

The current study was done to develop a rapid and simple protocol for the establishment of regenerative callus for *Stevia*. *In vitro* analysis was conducted using various combinations and concentration of phytohormones on which the best callusing was obtained with the combination of MS basal medium fortified with 2,4-D (1mg/l) and NAA (1mg/l). Thus, this could be a suitable medium to produce the maximum amount of callus from a leaf. The leaf and callus were further screened for the presence of various phytochemicals. The series of qualitative tests carried out showed that the leaves of Stevia contained relatively more amount of flavonoids, glycosides, phenol, tannins, sterols and saponins as compared to callus; thereby making both active sources of secondary metabolites. Since tissue culture technology is the only process for mass propagation; this study provides a rapid and efficient protocol to generate the maximum number of platelets within a limited time. This study is first of its kind in Nepal and describes a strong way for the rapid and reliable multiplication and commercial production of regenerative Calli. The callus so produced can be used for the multiple shoot regeneration and establishment of somaclonal variants for better yield and productivity.

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