International Journal of Agricultural Research

ISSN 1816-4897

Academic Journals Inc.

www.academicjournals.com

International Journal of Agricultural Research 2 (12): 1006-1013, 2007
ISSN 1816-4897

In vitro Morphogenic Responses of Different Explants of Stevia (*Stevia rebaudiana* Bert.)

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Abstract: Leaf segments, internode and nodal segments of Stevia were cultured on MS medium supplemented with varying concentrations (1, 3, 5 and 7 mg L^{-1}) of growth regulators (2,4-D, BAP and NAA) along with coconut water for observe morphogenic responses (mainly formation of callus). Ninety five percent aseptic cultures were obtained when sterilized with 0.1% $HgCl_2$ for 10 min. Nodal segment initiated callus earlier than leaf segments and internode explant. Higher amount of callus were obtained from leaf segments than internode and nodal segment. Interaction effects of explant and growth regulators were significant for days of callus initiation, fresh weight and dry weight of the callus per culture. Among the twelve treatments the highest amount of callus was obtained in MS medium supplemented with 7 mg L^{-1} BAP followed by 3 and 5 mg L^{-1} NAA, respectively. On the other hand, 7 mg L^{-1} 2,4-D showed lowest performance followed by 5 mg L^{-1} 2,4-D and 5 mg L^{-1} BAP, respectively among the twelve treatments.

Key words: Explant, morphogenic, embryogenic, stevioside

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.) is a small, herbaceous, semi-bushy, perennial shrub of Compositae family originated from Paraguay. Centuries ago, natives of Paraguay used the leaves of this honey plant to sweeten their bitter drinks. It is one of 154 members of the genus Stevia, which produces sweet stevioside, a diterpenoid glycoside isolated from this plant leaves (Robinson, 1930; Soejarto *et al*., 1982). It grows well at the temperatures ranging between 15-30°C.

The first reports of commercial cultivation in Paraguay were in 1964 (Katayma *et al*., 1976; Lewis, 1992). Sumida (1968) began a large effort aimed at establishing Stevia as a crop in Japan. Since then, Stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Lee *et al*., 1979; Goenadi, 1983; Shock, 1982; Saxena and Ming, 1988; Brandle and Rosa, 1992; Fors, 1995). Currently Stevia production is centered in China and the major market in Japan (Kinghorn and Soejarto, 1985). In the Pacific Rim countries, China, Korea and Japan, Stevia is regularly used in preparation of food and pharmaceutical products. In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in the order of $220 million Canadian (Brandle and Rosa, 1992).

The property of the species that called attention to the plant was the intense sweet taste of the leave and aqueous extracts. From the leaves of Stevia (*S. rebaudiana*), stevioside, sweet crystalline diterpene glycosides are extracted. The dry leaves of this plant are 30 times sweeter than sugar, with zero calories. Whereas pure extract stevioside is non-caloric and 300 times sweeter than sugar

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(Bhosle, 2004). Other attributes of this natural, high-intensity sweetener include non-fermentable, non-discolouring, heat stability at 100°C and feature a lengthy shelf life. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages.

Stevioside is of special interest to diabetics, persons with hyperglycemia and the diet conscious. A sugar-free, no-calorie natural sweetener is especially helpful for people who are diabetic, prone to yeast infections, or trying to lose a few extra pounds by controlling calories. Additions of Stevia powder or liquid a pinch or drops at a time to tea, coffee, dairy products, or juices sweeten to taste. Some people detect a slight licorice aftertaste, depending on potency.

Though Stevia got its attention as sweetening agent other medicinal were reported. According to World Health Organization (WHO) findings it regulates blood pressure, fights cavities, induces pancreas to produce more insulin and act as bactericidal agent (Bhosle, 2004). No negative clinical reports have appeared in any of these countries where Stevia is readily available. For more than a decade stevioside has been approved and widely used in Japan.

Seed germination of Stevia is often poor (Miyazaki and Watenabe, 1974). Therefore, there are basically two options for multiplication. The first is the tissue culture and second the stem cutting. Callus culture is a good option because further embryogenesis or plant regeneration can be obtained. Callus induction from different explants has the following objectives:

- To identify the suitable sources of explants for callus induction.
- To find out suitable concentration of cytokinin (BAP) and auxins (NAA, 2,4-D) for callus induction.
- To produce powder stevioside from callus.

MATERIALS AND METHODS

Plant Materials

The experiment was conducted at October, 2005. The explants; nodes, internodes and leaf segments were used as experimental material. The explants were collected from 3-4 months old-field grown plants at BSRI (Bangladesh Sugarcane Research Institute) experimental field. These explants were treated with 1% savlon for 5-6 min with constant shaking and washed thoroughly with distilled water. Then the materials were taken under laminar air flow cabinet and surface sterilization was done with different concentrations (0.05, 0.1, 0.2 and 0.5%) of mercuric chloride ($HgCl_2$) for different durations (4, 6, 8, 10 and 12 min) followed by 4-5 times rinse with sterile distilled water to remove traces of $HgCl_2$ from the materials.

Preparation of Culture Media

Stock solutions of all components were prepared with appropriate amount of all the components. Appropriate amount of all the stock solutions were mixed following the standard media preparation procedure. The pH of the media was adjusted to 5.6-5.8 and growth regulators were added at different concentrations (1, 3, 5 and 7 mg L^{-1}). In each treatment three explants were inoculated on 36 test tubes, 12 test tubes each.

Inoculation Technique

Nodes and internodes were cut into 1 cm long pieces and the leaf segments into 1 cm^2 square pieces and then aseptically inoculated onto callus induction media. All inoculations and aseptic manipulations were carried out in a laminar airflow cabinet. Before use, the floor of the cabinet was cleaned with 90% ethyl alcohol to reduce the chances of contamination. The instruments like scalpels, forceps, needles etc. were sterilized by an alcoholic dip and flaming method inside the laminar airflow

chamber. Other requirements like Petri dish, bottles, conical flask, cotton, distilled water, etc. was sterilized by steam sterilization method. Before the onset of inoculation, hands were cleaned thoroughly by soap and then by spraying 70% ethyl alcohol. Surgical operations were carried out taking all possible care to ensure contamination free condition. Then the inoculated tubes were shelved in dark condition. All cultures were grown in an air-conditioned culture room in dark condition. The temperature of the culture room was maintained at 25±1°C.

All chemical compounds used in culture media as macronutrients and micronutrients in the present study were reagent grade (GPR) products of either Riedel-de-Haen, Germany; BDH, England or E. MERCK, Germany. The vitamins and growth regulators were mostly products of Sigma Chemical Company, USA and BDH, Germany.

Data Collection and Statistical Analysis

From the forth day of inoculation, regular visual observation was done upto five weeks to record the day of callus initiation and maturation. Then fresh weight of first five callus appearing tubes is selected and fresh weight of callus was recorded. After one day callus were dried in drier at 110°C and then again dry weight of callus were recorded and tabulated. The explants showing embryogenic callus was recorded after 5 weeks. Histological sections and microscopic observation was done under microphotograph. Data presented in the tables were analyzed using Duncan's Multiple Range Test (DMRT) by MSTAT-C.

RESULTS AND DISCUSSION

The surface sterilized leaf segment, internode and node explants of stevia were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 10% coconut water and different concentrations of 2,4-D, BAP and NAA. Days of callus initiation was observed after 4-21 days of culture and calli grew vigorously until nutrient of the medium became exhausted. Days of callus completion was observed within about 5 weeks. Number and percentage of callus producing explants were recorded after 5 weeks of culture.

From this investigation the unique findings could be presented by such a way that the leaf segments showed best callus formation among all explants and best callus was induced by BAP treatments followed by NAA and 2,4-D treatments. This results are well presented by the Table 1 showing the significant differences by DMRT at $p = 0.05$.

Irrespective to growth regulators, leaf segments showed best result in callus formation compared with other two explants. Table 3 shows a clear vision of that. Among all, the leaf explant shows the highest fresh callus weight on a average. Overall 89.58% explants showed callus formation. Some shoot and root along with callus were also found. Total shoot formation was 10.19% and total root formation was 14.12%. BAP promoted shoot formation, whereas NAA facilitated root formation (Table 2). At the time of callus initiation it was colored as light creamy and at the time of completion as deep creamy.

Effect of 2,4-D on Morphogenetic Responses of Different Explants of Stevia

Best callus induction was observed in MS medium containing 1 mg L^{-1} 2,4-D irrespective to all explants of stevia (Fig. 4). Increasing concentration of 2, 4-D shows decreased callus induction. In case of 2,4-D average days of callus initiation (DCI) was about 11.87, fresh weight 148.30 mg and dry weight 18.54 mg (Table 3). About 80.56% explants showed response (Fig. 1). Callus formed predominantly from different explants of stevia using different concentration of 2,4-D. But in case of 1 mg L^{-1} 2,4-D out of 12 replication only one replication showed shoot with callus (about 8.33%) from nodal segments. Root was not observed at all. Leaf segments expressed better results from that of the internode and nodal segments (Table 2).

Table 1: Interaction effect between explants, growth regulator (hormone) and hormone concentration on callus production in stevia (values are means from 5 replications)

Interaction between explants, hormone and hormone concentration (mg L^{-1})		Days of callus initiation (DCI)	Fresh weight (mg)	Dry weight (mg)
Leaf				
2,4-D	1	11.00c-g*	256.20c-f	35.62c-j
	3	14.00abc	324.20cd	46.64d-h
	5	12.60a-g	260.60cde	34.88c-j
	7	12.80a-f	243.80c-g	34.72e-j
BAP	1	12.00b-g	572.00b	90.34ab
	3	13.00a-e	537.20b	66.02cd
	5	14.00abc	224.20c-h	29.60f-k
	7	13.40a-d	882.40a	97.10ab
NAA	1	11.00c-g	292.80cde	52.16def
	3	10.20d-i	664.20b	100.54a
	5	10.80c-g	525.40b	79.36bc
	7	12.20b-g	302.00cd	47.60d-h
Internode				
2,4-D	1	9.60e-j	164.40c-i	13.96j-m
	3	10.40d-h	79.20ghi	10.04klm
	5	12.80a-f	33.60i	4.36m
	7	13.40a-d	37.40i	4.38m
BAP	1	10.60c-g	142.20d-i	25.20h-m
	3	12.00b-g	168.60c-i	21.26i-m
	5	15.60a	129.40c-i	16.68i-m
	7	9.20g-l	234.00c-h	29.88f-k
NAA	1	6.00l	314.80c	48.46d-g
	3	7.20h-l	244.40c-g	27.76g-l
	5	6.00l	313.00cd	39.74c-i
	7	6.80jkl	166.80c-i	18.12i-m
Node				
2,4-D	1	9.40f-k	232.60c-h	21.86i-m
	3	9.40f-k	89.60f-i	8.64klm
	5	12.20b-g	37.00i	5.10lm
	7	14.80ab	21.00i	2.28m
BAP	1	11.80b-g	328.20c	54.40de
	3	10.60c-g	284.20cde	35.98e-j
	5	11.20c-g	70.60hi	8.12klm
	7	9.20g-l	513.40b	55.28de
NAA	1	6.60jkl	317.20c	48.50d-g
	3	6.20kl	248.20c-f	29.56f-k
	5	6.60jkl	288.80cde	38.22e-i
	7	7.00i-l	210.20c-h	22.06i-m

*: Values within column followed by different letter(s) are significantly different by DMRT at p = 0.05

Table 2: Morphological responses of stevia explants cultured in MS media supplemented with different growth regulators

	Explants		
Hormone (mg L^{-1})	Leaf	Internode	Node
2,4-D			
1	C	C	C,C+S
3	C	C	C
5	C	C	C
7	C	C	C
BAP			
1	C	C, C+S	C, C+S
3	C	C, C+S	C, C+S
5	C	C, C+S	C, C+S
7	C	C, C+S	C, C+S
NAA			
1	C, C+R	C, C+R	C, C+R, C+S
3	C, C+R	C, C+R	C, C+R, C+R+S
5	C, C+R	C, C+R	C, C+R, C+S
7	C, C+R	C, C+R	C, C+R, C+R+S

C: Callus, S: Shoot, R: Root, C+S: Both callus and shoot, C+R: Both callus and root, C+R+S: Both callus, root and shoot

Table 3: Summary of morphogenic responses of different explants using different growth regulators (2,4-D , BAP and NAA)

Growth regulators	Days of callus initiation			Fresh weight (mg)			Dry weight (mg)		
	Average	Higher	Lower	Average	Higher	Lower	Average	Higher	Lower
2,4-D									
Leaf	12.60	14.00	11.00	271.20	324.20	243.80	37.97	46.64	34.72
Internode	11.55	13.40	9.60	78.65	164.40	33.60	8.19	13.96	4.36
Node	11.45	14.80	9.40	95.05	232.60	21.00	9.47	21.86	2.28
BAP									
Leaf	13.10	14.00	12.00	553.95	882.40	224.20	70.76	97.10	29.60
Internode	11.85	15.60	9.20	168.55	234.00	129.40	23.25	29.88	16.68
Node	10.70	11.80	9.20	299.10	513.40	70.60	38.44	55.28	8.12
NAA									
Leaf	11.05	12.20	10.20	446.10	664.20	292.80	69.92	100.54	47.60
Internode	6.50	7.20	6.00	259.75	314.80	166.80	33.52	48.46	18.12
Node	6.60	7.00	6.20	266.10	317.20	210.20	34.58	48.50	22.06

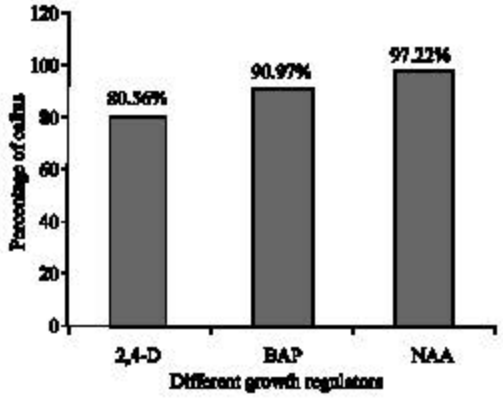

Fig. 1: Percentage of callus formation using different growth regulators irrespective to explants

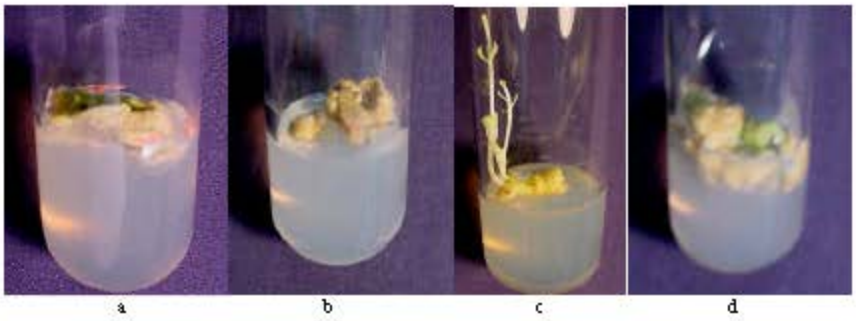

Fig. 2: Callus production from BAP using different concentration. (a) = 1 mg L^{-1} BAP, (b) = 3 mg L^{-1} BAP, (c) = 5 mg L^{-1} BAP and (d) = 7 mg L^{-1} BAP

Effect of BAP on Morphogenetic Responses of Different Explants of Stevia

Best callus induction was observed in MS medium containing 7 mg L^{-1} BAP irrespective to all explants of stevia. Then 1 mg L^{-1} BAP, 3 mg L^{-1} BAP and lastly 5 mg L^{-1} BAP showed better callus formation (Fig. 2). In case of BAP days of callus initiation was about 11.88; fresh weight 340.53 mg and dry weight 44.15 mg (Table 3). About 90.97% explants showed response (Fig. 1). Callus formed predominantly from different explants of stevia using different concentration of BAP. But shoot formed with callus from internode and nodal segments (about 27.08%). Root was not observed at all.

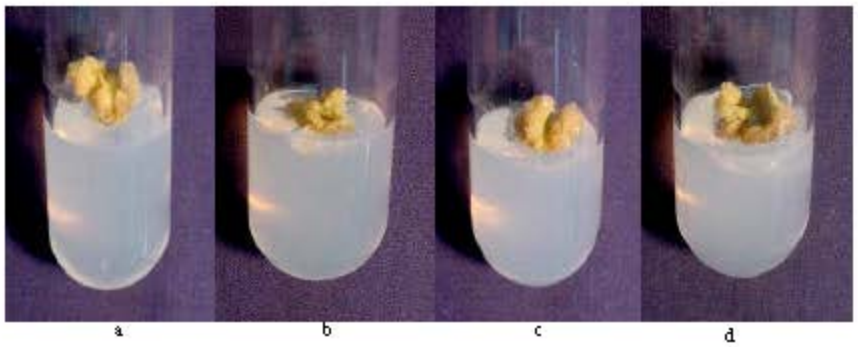

Fig. 3: Callus production from NAA using different concentration. (a) = 1 mg L^{-1} NAA, (b) = 3 mg L^{-1} NAA, (c) = 5 mg L^{-1} NAA and (d) = 7 mg L^{-1} NAA

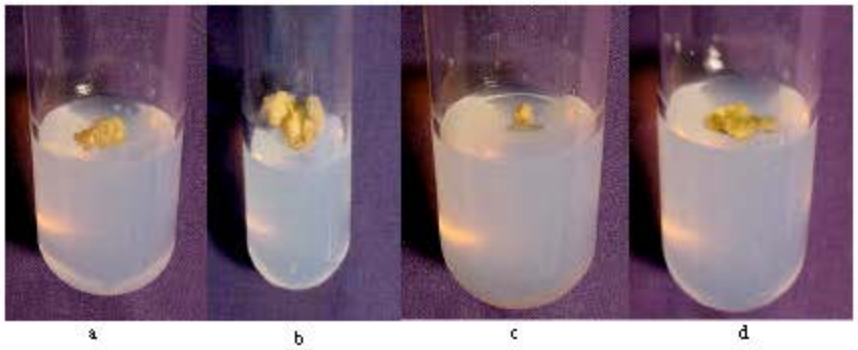

Fig. 4: Callus production from 2,4-D using different concentration. (a) = 1 mg L^{-1} 2,4-D, (b) = 3 mg L^{-1} 2,4-D, (c) = 5 mg L^{-1} 2,4-D and (d) = 7 mg L^{-1} 2,4-D

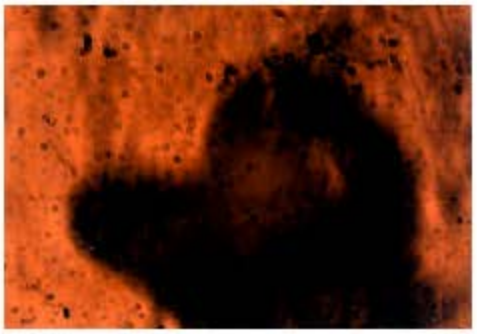

Fig. 5: Heart shaped embryogenic callus cell under microphotograph

Leaf segments expressed better results from that of the internode and nodal segments (Table 2). In case of MS medium supplemented with different concentration of BAP red color shown on medium with callus formation. About 14.58% experiments were found as red color.

Effect of NAA on Morphogenetic Responses of Different Explants of Stevia

Best callus induction was observed in MS medium containing 3 mg L^{-1} NAA irrespective to all explants of stevia. Then 5 mg L^{-1} NAA, 1 mg L^{-1} NAA and lastly 7 mg L^{-1} NAA showed

better callus formation (Fig. 3). In case of NAA days of callus initiation was about 8.05; fresh weight 332.98 mg and dry weight 46.01 mg (Table 3). About 92.22% explants showed response (Fig. 1). Callus formed predominantly from different explants of stevia using different concentration of NAA. But root formed with callus from leaf, internode and nodal segments (about 42.36%) (Fig. 4). Shoot also occurred in case of different concentration of NAA in addition of callus and root along with callus formation. Leaf segments expressed better results from that of the internode and nodal segments (Table 2). In case of MS medium supplemented with different concentration of NAA no extra color shown on medium with callus formation as red on BAP.

Leaf segments showed better response compared with node and internode because of its tendency of having undifferentiated cells which produces callus. As BAP promotes shoot development along with callus and NAA promotes rooting, research findings also suggest that along with callus these growth regulators may act as shooting and rooting media, respectively. Microphotograph shows that fragile callus is heart or torpedo shaped i.e., embryogenic (Fig. 5), whereas few organogenic callus which is physically compact were also found. According to Bespalhok *et al.* (1993), Wada *et al.* (1981) and Miyagawa *et al.* (1986) somatic embryogenesis can be done from leaves and stems.

Length of day under which Stevia is cultivated has an effect on stevioside concentration, which is supported by Kinghorn and Soejarto (1985). The authors observed that in subtropical regions of the world, stevioside concentration is high in Stevia plant when it is cultivated under long days.

Further investigation about the sweetening strength of stevioside from callus is recommended. If dry callus can be a suitable source of stevioside, it can be commercially marketable as alternative sugar, in large amount, but in short space.

ACKNOWLEDGMENTS

The author is extremely grateful to his honorable research co-supervisor, Dr. Md. Amzad Hossain, Associate Cane Nutritionist, Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh for his constant and constructive guidance, encouragement, valuable criticism and for providing necessary Laboratory facilities to accomplish research.

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