Studii şi Cercetări	Biologie	12	37 - 40	Universitatea din Bacău	2007

ESTABLISHMENT AND MAINTENANCE OF CALLUS OF *STEVIA REBAUDIANA* UNDER ASEPTIC ENVIRONMENT

Raman Dang, Kuntal Das

Keywords: *Stevia, stevioside, phyto-hormones, tissue culture*

INTRODUCTION

The worldwide demand for high potency sweeteners increased in present years by introducing Stevia as a modern crop from wild plant. *Stevia rebaudiana* is commonly known as candy leaves or natural non-caloric sweet plant as was officially discovered by Dr. M.S. Bertoni in early 20^{th} century (1905) (Bertoni MS, 1905).

Stevioside and rebaudiosides are the active principal constituent of diterpene glycosides with differing sugar molecule attached as found in the leaves of Stevia plant and are responsible for high sweetness activity. Stevioside abundantly available in leaf of Stevia (13-20% in dry leaves) which shows 250-350 times sweeter and rebaudioside content in less quantities (1-3% in dry leaves) which shows 350-450 times sweeter while that of fresh healthy leaves only 30 times sweeter than table sugar. This sweetness property of Stevia focused multipurpose uses in human life. Due to sweetness and no side effect it can be used as an artificial sweeteners.

Other uses viz. hypoglycemic, cardiovascular, anti-microbial, digestive tonic, dental and skin care (Mowrey D, 1992).

These beneficial uses focused Stevia's importance and its availability throughout the year, but the main problem lies with Stevia seeds which show wide variation in the stevioside content probably due to gene segregation. To avoid segregation and to improve the yield of stevioside it is necessary to propagate a genetically homogeneous population from a selected elite plant with desirable characters. Micro-propagation techniques can serve as an alternative method to conventional techniques that can give disease free plant and assist in the preservation of germplasm by storing the *in vitro* developed propagules under low temperature.

Literature survey reveals initiation and establishment of callus from explant of Stevia by using various combinations of phyto-hormones but there is no such comparative tissue culture study on Stevia for initiation and maintenance in different strength of MS medium with different concentration of phyto-hormones so far been reported (Handro *et al*., 1977 and Ferreira and Handro, 1988). Geuns JMC (2003) and Nepovim *et al* (1998), reported stevioside content depends on biomass yield and method of cultivation, same thought followed in this present study for improving more amount of callus in suitable media. Therefore it is worthwhile to develop the callus in different strength of MS medium and medium selection with better callus initiation and their maintenance. HPTLC analysis study reveals that confirmation.

MATERIALS AND METHODS

Selection of Plant material: Stevia plants, grown in the green house of the herbal garden, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra, Bangalore, were selected as the source of mother plants. Plants parts like apical buds, axillary buds, and stem segments were the explants sources initially for shoot production .Leaf discs were used for the callus induction.

Chemicals: All the chemicals i.e. growth regulators, agar used for the culture media preparation were of analytical grade, obtained from the Hi-Media company.

Glass wares: Various glasswares like the Erlenmeyer flasks, test tubes, Beakers, pipettes, funnels, measuring cylinders and glass rods etc. were used. Glass bottles and jars with poly propylene caps were also used.

Preparation of Glass wares : All the glass ware were soaked in 5% Chromic acid, overnight and were washed in running tap water followed by rinsing with double distilled water. The cleaned glasswares were then dried in forced draft hot air oven at 100^0C temperature and were stored in dust proof cupboards until further use. Clean and dry glasswares were used for all kinds of tissue culture studies throughout the present research work.

Preparation of Stock solution: The stock solution of auxins- Indole acetic acid (IAA), Naphthalene Acetic Acid (NAA), Indole-3-butyric acid (IBA) and 2,4, Dichlorophenoxy acetic acid (2-4-D) were dissolved separately in minimal quantity of redistilled ethyl alcohol and volume was made up with double distilled water.

The stock solution of cytokinins-kinetin, Benzyl adenine or 6-benzyl amino purine (BAP) was dissolved in a few drops of 0.1 N HCl and volume was made up with double distilled water.

Stock solution of Poly vinyl pyrrolidone (PVP) was made (100 mg L^{-1}) by dissolving 0.1 N HCl or 0.1N NaOH.

All the above stock solutions were stored in a refrigerator at 5-6^{0}C then kept back to the ambient temperature before using them for the media preparation.

Preparatoin of Culture Medium: Various combinations of MS media were prepared with double distilled water with sugar concentration 3 g lt^{-1} along with different concentration of required growth hormones. 0.7% Agar was used as gelling agent. The media was then dispensed into culture tubes, 100ml EM flask, 200ml bottles with 15 ml, 40 ml and 60 ml media and PVP 5ml, 10ml and 15ml were added respectively to avoid browning of explants and were avoided using cotton plugs or Laxbro plastic caps and made air tight. They were then sterilized in an autoclave at a temperature of 121^{0}C and a pressure of 103.4 kPa for 20 minutes. The containers with cultured media were stored in a dust proof sterile chamber.

Preparation of explants for Inoculation and Incubation: The selected explants were washed thoroughly under running tap water for 15 minutes. Explant was treated with Bavistin (0.1%) for 15 minutes (Patil *et al.*, 1996). The explants were washed with single distilled water and then it was taken to the laminar airflow chamber. The working bench of laminar airflow was sterilized using 70% alcohol. The treated plant materials were subjected to sterilization using 0.1% $HgCl_2$ for 5 minutes and repeatedly washed with sterile double distilled water. Then a known weight of tissue or a uniform plant material (leaf, axillary bud or meristem) were dissected and inoculated to the medium. The inoculated tubes, flasks and bottles were then incubated in the culture room at 25 ± 2^{0}C under fluorescent light with an intensity of 60 U E in m^{-2} Sec^{-1}. A photoperiod of 16 hours of light and 8 hours of darkness was maintained through an automatic timer, with a constant relative humidity of 65- 75% using an air-cooling system.

Growth measurement: In each replication of different treatments for an individual experiment, uniform explant tissues were inoculated and were incubated at 25 ± 2^{0}C. Each time a fixed number of replications were taken for the record. Weight of the callus and maintenance of the same were also done 30 days after inoculation and after every 16 days of subculturing respectively.

a) Effect of different combinations of auxin and cytokinin (kinetin) on callus induction of *Stevia rebaudiana* in different strengths of MS medium.

Treatments: Different strengths of MS Basal medium along with auxins (IAA, IBA, NAA, 2,4 D) 1 mg/ L, cytokinin (Kinetin) at 0.1, 0.2 and 0.3 mg/ L were used in combination.

Methodology: Leaf discs of 3 mm size were sterilized and inoculated in culture tubes containing 15 ml media.

Observations: 30 days after inoculation, observations were recorded. Results were tabulated in Table-1 with figures (Fig. 1 and 2).

b) Effect of different combination of auxin and cytokinins for callus maintenance of *Stevia rebaudiana* in half and single strength of MS medium.

Treatments: MS Basal medium along with auxins IAA, NAA, 2,4,D at 0.1 mg L^{-1} and cytokinins, Kinetin and BAP at 1 and 2 mg lt^{-1} were used in combination.

Methodology: As explained above.

Observations: After 30 days callus growth for different treatments were recorded that were tabulated in Table-2 with figures (Fig-3 and 4).

RESULTS AND DISCUSSIONS

a) Effect of different combination of auxins and Kinetin as cytokinins on callus induction of *Stevia rebaudiana* in different strengths of MS medium: The results (Table 1) show that the growth of callus was found independent due to an increased strength of MS media. Here, half strength of MS medium had given similar trend as like that of single strength of MS medium. Indole -3- butyric acid (IBA) and Kinetin combinations did not show any good response as like that of other combinations. 2,4-D 1.0 mg/L and Kinetin 0.2 mg /L combinations showed highest callus growth in both half (2.20 cm) and single strength (2.25 cm) MS medium. Whereas, IAA 1.0 mg/L + Kinetin 0.3 mg/L combination showed response to all strengths of MS media, but in terms of survival rate, 2,4- D and Kinetin showed good response among all. However, the overall growth response was better in single strength MS medium than others but maintenance of callus was better in half strength MS medium. Hence half strength MS medium was selected for callus growth.

Table 1. Effect of hormones on callus induction

Treatments	Callus response on MS media			Callus diameters (cm)			Maintenance of callus		
	½	1	2	½	1	2	½	1	2
NAA1.0+Kinetin0.1	No	No	No	--	--	--	--	--	--
NAA1.0+Kinetin0.2	Yes	Yes	No	1.00	1.08	--	++	+	--
NAA1.0+Kinetin0.3	Yes	Yes	No	1.12	1.13	--	+	+	--
IAA1.0+Kinetin 0.1	No	No	No	--	--	--	--	--	--
IAA1.0+Kinetin0.2	No	Yes	No	--	1.50	--	--	+	--
IAA1.0+Kinetin0.3	Yes	Yes	Yes	2.01	2.10	1.70	+	+	--

IBA1.0+Kinetin 0.1	No	No	No	--	--	--	--	--	--
IBA1.0+Kinetin0.2	No	No	No	--	--	--	--	--	--
IBA1.0+Kinetin0.3	No	Yes	Yes	--	1.70	1.60	--	+	--
2,4D1.0+Kinetin0.1	Yes	Yes	No	2.10	2.20	--	--	--	--
2,4D1.0+Kinetin0.2	Yes	Yes	No	2.20	2.25	--	++	++	--
2,4D1.0+Kinetin0.3	Yes	Yes	Yes	1.70	1.80	0.96	--	+	--

IBA= Indole butyric acid; IAA= Indole acetic acid; NAA=Naphthoxy acetic acid; 2,4 D = 2, 4 Dichloro acetic acid; (--) Dried; (+) Upto first subculture; (++) Upto second subculture;

b) Effect of different combination of auxin and cytokinins for callus maintenance of *Stevia rebaudiana* in half and single strength of MS medium.

The results (Table 2) show that 2,4-D 0.1 mg L^{-1} and Kinetin 2 mg L^{-1} gave higher callus growth of 5.10 cm and 4.90 cm respectively in half and single strength of MS medium respectively and least one was IAA 0.1 mg L^{-1} and BAP 1.0 mg L^{-1} combinations for half strength (1.00 cm) and NAA 0.1 mg L^{-1} and Kinetin 2.0 mg L^{-1} combination for single strength (1.03 cm). Callus culture in combinations of 2,4-D, IAA with Kinetin and BAP were dried after 2 weeks of inoculation, while combination of NAA with BAP and Kinetin were of viable in nature and green in color, but more stable was recorded with NAA and BAP combinations in both half and single strength MS media. Therefore, it may be concluded that half strength MS medium was proved superior for callus maintenance in hormone combinations of NAA and BAP.

Table 2. Effect of hormones on maintenance of callus

Treatments	Callus initiation		Callus growth (cm)		Maintenance of callus	
	½MS	1 MS	½MS	1 MS	½MS	1 MS
IAA0.1+Kinetin1.0	No	No	--	--	--	--
IAA0.1+Kinetin2.0	Yes	Yes	1.90	1.95	--	+
IAA0.1+BAP1.0	Yes	Yes	1.00	1.10	--	--
IAA0.1+BAP2.0	No	Yes	--	1.05	--	--
NAA0.1+Kinetin1.0	No	No	--	--	--	--
NAA0.1+Kinetin2.0	No	No	--	--	--	--
NAA0.1+BAP1.0	Yes	Yes	3.10	2.81	++	+
NAA0.1+BAP2.0	Yes	Yes	4.30	4.25	++	++
2,4D0.1+Kinetin1.0	Yes	Yes	1.80	1.80	+	--
2,4D0.1+Kinetin2.0	Yes	Yes	5.10	4.90	+	+
2,4D 0.1+BAP1.0	Yes	Yes	2.10	2.15	--	--
2,4D 0.1+BAP2.0	Yes	Yes	2.03	3.00	+	--

Table 3. HPTLC analysis data for micro propagated callus study

Treatments	Fresh weight (g)	Dry weight (g)	% stevioside
Half MS medium	2.72	0.512	5.60
Full MS medium	2.31	0.331	4.45

The production of Stevioside in callus cultures has been positively reported by Komatsu *et al* (1976) while Handro *et al* (1977) reported that their culture were not sweet but in this present study effect of MS medium at half and full strength was evaluated on sweet stevioside content in Stevia.

From the Table 3, it was found that half MS medium was better (5.60 %) than full strength (4.45 %) in respect of stevioside content. The results of the present investigation has confirmed the earlier results reported by Nepovim *et al*., (1998) and Geuns (2003).

REZUMAT

The callus culture of leaves of Stevia rebaudiana was initiated and maintained on different strengths of Murashiage and Skoog (MS) medium supplemented with various phytohormones. Kinetin in combination with Naphthalene Acetic Acid (NAA) and 2,4-D showed better results in callus initiation whereas combined application of Benzyl adenine or 6-benzyl amino purine (BAP) and NAA showed most satisfactory results of callus maintenance in half strength of MS medium.

CONCLUSIONS

The NAA 0.1 + BAP 2.0 treatment combination has been proved superior in callus initiation, growth and maintenance. The use of half MS medium was found better with respect to fresh weight, dry weight and percentage of stevioside content compared to full MS medium irrespective of different treatments.

REFERENCES

1. BERTONI M.S., LEE KAA HE-E.SA., 1905 - Nature et ses properties. Ancient. Paraguayos. 1(5): 1-14.
2. MOWREY D., 1992 - Life with Stevia: How sweet it is. 1-14.

3. HANDRO W., HELL K.G., KERBAUY G.B., 1977 - Tissue culture of *Stevia rebaudiana*, A sweetening plant, Planta medica, 32, 115-117.
4. FERREIRA C.M., HANDRO W., 1988 - Micropropagation of *Stevia rebaudiana* through leaf explants from adult plants, Planta medica, 157-160.
5. GEUNS JMC. 2003 - Molecules of interest: Stevioside. Phytochem, 64, 913-21.
6. NEPOVIM A., DRAHOSOVA H., VALICEK P., VANEK T., 1998 - The effect of cultivation conditions on the content of stevioside in *Stevia rebaudiana* Bertoni plants cultivated in the Czech Republic. Pharmaceutical Pharmacological Letters, 8, 19-21.
7. PATIL V., ASHWINI K.S., REDDY P.C., PURUSHOTHAM M.G., PRASAD T.G., UDAYAKUMAR M., 1996 - *In vitro* multiplication of *Stevia rebaudiana*, Current Science, 70 (11), 960.
8. KOMATSU K., NOZAKI W., TAKEMURA M., NAKAMINAMI M., 1976 – Production of natural sweetener, Chemical abstract, 84, 169.

AUTHORS' ADDRESS

DANG RAMAN - Al-Ameen College of Pharmacy, Opp.Lalbagh Main Gate, Hosur Road, Bangalore-27

DAS KUNTAL - St. John's College of Pharmacy, #6, R.P.C Lay out, Vijaya Nagar, Bangalore-40. India