BCSIR

Available online at www.banglajol.info

Bangladesh J. Sci. Ind. Res. **45(3)**, 243-248, 2010

BANGLADESH JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

E-mail: bjsir07@gmail.com

Secondary Metabolite Production in Callus Cultures of *Stevia rebaudiana* Bertoni

B. Janarthanam, M. Gopalakrishnan and T. Sekar*

Plant Tissue Culture Lab, P.G & Research Department of Botany, Pachaiyappa's College, University of Madras, Chennai - 600 030, Tamil Nadu, India

Abstract

Stevia rebaudiana is and an important non-caloric sweetening herb contains diterpene glycosides need to be explored for its commercialization. The evolving commercial importance of secondary metabolites in recent years has resulted in a great demand in the Pharma industry. Callus cultures were established from nodal and leaf explants. Leaf explants showed better callus initiation than nodal explants. Maximum callus biomass was observed in MS medium supplemented with 2, 4-D 1.0 mg/l. Further screening of callus culture was carried out on Murashige and Skoog (MS) medium with different concentration and combinations of 2, 4-D, NAA, IAA, IBA, BA and KN individually and in combinations. Remarkable callus biomass of 11.6 g/l dry weight (182.3 g/l fresh weight) was observed in MS media containing 0.5 mg/l 2, 4-D, 0.5 mg/l NAA and 1.0 mg/l KN. The harvested cell biomass was subjected to extraction of active principles. In this study, cell biomass extracts were compared with extracts from leaves of mother plants of *Stevia rebaudiana*. HPLC analysis of these extracts showed that the main components of the active principles namely Stevioside were present in sufficiently large amounts in the undifferentiated cultured cells.

Keywords: *In vitro* culture, Biomass, *Stevia rebaudiana*, Stevioside

Introduction

Stevia rebaudiana is a perennial herb belonging to the family Asteraceae. It is grown commercially in many parts of Brazil, Paraguay, Central America, Thailand, Korea and China (Soejarto *et al*., 1983). Leaves contain a large amount of stevioside (Geuns, 2004), which is formed by three molecules of glucose and one molecule of steviol, a diterpenic carboxylic alcohol. The leaves of Stevia are the source of the diterpene glycosides, stevioside and rebaudioside, which are estimated to be 100 - 300 times sweeter than sucrose (Ishima and Katayama, 1986; Tanaka, 1982). These Stevia extracts have long been used in Southern Africa to treat diabetes. Their ingestion causes a slight suppression of plasma glucose levels and significantly increased glucose tolerance in normal adult humans (Curi *et al.*, 1986). The use of stevioside results in a clinically significant hypotensive effect in spontaneously hypertensive rats, without adversely affecting their heart rates or serum catecholamine levels (Chan *et al*., 1998). The seeds of Stevia show a very low germination percentage (Goettemoeller and Ching, 1999) and propagation by seed does not allow the production of homogenous populations, resulting in great variability in important feature like sweetening level and composition (Tamura *et al.*, 1984). Vegetative propagation is also limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982). With the above mentioned difficulties, callus culture has been an alternative and efficient source for the production of secondary metabolites. Nevertheless, there are no reports available on *S. rebaudiana in vitro* cell mass production and screening of biosynthetic potential using modern techniques. Hence, the present investigation was carried out with the objective of providing new vistas on the cell and tissue culture and to elucidate the effect of plant growth regulators on cell growth and production of total Stevioside.

Materials and Methods

Healthy *S. rebaudiana* plants were obtained from RICH-SAAI, Solar nursery Chennai and were raised in pots containing Soil and Farm Yard Manure (1:1) under green house condition at P.G & Research Department of Botany, Pachaiyappa's college Chennai- 600 030, India. Juvenile leaf segment were cut from processed for aseptic culture. Explants were washed thoroughly under running tap water for 20 min, washed with a solution of Tween 20 (2 drops in

*Corresponding author: E-mail: tsekar_2005@yahoo.com

100 ml of water) for 1 min, and again washed with sterile distilled water. The cleaned explants were surface sterilized with mercuric chloride $HgCl_2$ (0.1%) for 4 minutes under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$.

The surface sterilized leaf explants were cultured on MS (Murashige and Skoog 1962) basal media containing various concentrations of 2, 4-D (0.1, 0.5, 1.0, 2.5 and 5.0 mg/l); NAA (0.25, 0.5, 1.0, 2.5 and 5.0 mg/l) and BA (0.25, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/l) for callus induction.

Primary callus was established from leaf explant. For secondary callus production, a small portion of primary callus was excised using sterile knife holder and was subcultured periodically once in three weeks. The secondary callus was used for all experimental studies.

A standard approach of Latin square method (Collin and Edwards, 1998) was followed in screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2, 4-D, IAA, IBA and NAA) and Cytokinins (BA and KN) alone and in combinations. A range of 7 concentrations of auxins and Cytokinins (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 mg/l) were used in this study.

The growth measurement of callus was determined by following the method as described by Rao and Ravishankar (2000). Growth of the callus and its biomass was measured in terms of fresh (FW g/l) and dry weight (DW g/l). Fresh weight of callus was measured after removing the excess moisture and agar adhering to the callus surface using blotting paper. Dry weight of callus was determined by drying the callus in hot air oven at 60 ^oC for 24 h and was expressed in g/l DW culture.

The age of callus was determined by following the method as described by Gopi (2002). About 0.5gm of secondary callus culture from actively growing callus was inoculated in 250mL Erlenmeyer flask containing 50 ml of MS solid medium supplemented with 0.5 mg/l 2, 4-D, 0.5 mg/l NAA and 1.0 mg/l KN. The culture was incubated under 16/8h photoperiod at 25 ± 1^oC. Initial weight of the callus biomass was measured in terms of fresh (FW g/l) and dry weight (DW g/l). Observations were made from the 12th day after incubation up to 36th day with three days intervals and callus biomass yield data were recorded. At the 12th day the first Erlenmeyer flask containing callus was used and it was dried at 50 ^oC dark for 24 hrs. The same procedure was adopted for the callus harvested at different days of intervals 15th, 18th, 21st, 24th, 27th, 30th, 33rd and 36th days.

One gram of dried leaves of mother plant and standardized callus of *S. rebaudiana* were used. All samples were powdered, extracted with 25ml of water in Erlenmeyer flasks by shaking in a water bath at 70^oC for 30 min. After cooling, the extract was filtered through Sartorius RC membrane syringe filter (0.2mm) and 20ml of the sample used for the TLC and HPLC analysis. Preliminary identification of the Stevioside in the extracts was made by thin layer chromatography (TLC) using silica gel plates. The extracts were eluted with chloroform: methanol: water (30:20:4) and the chromatogram was developed by spraying with methanol: sulphuric acid (1:1) and heating to 110^oC (Pasquel *et al.*, 2002). High - performance liquid chromatography Shimadzu HPLC (Model SPD - 10A UV- VIS Detector) with a YMC- pack ODS - A, C18 column (250 × 4.6 mm; particle S - 5mm, 120 A). The linear gradient elution profile started with water: acetonitrile (75:25) and ended with water - acetonitrile (50:50) within 30min.

The detection was in the UV range at 205 nm and flow rate was adjusted to1ml/min (Vanek *et al.*, 2001). For both TLC and HPLC, Stevioside purchased from Wako Pure Chemical Industries Ltd., Japan was used as standard.

Results and Discussion

Development and growth of calli varied with explants. Leaf explants of *S. rebaudiana* were cultured on MS basal medium supplemented with individual concentrations of 2, 4-D, NAA and BA. The response percentage of calli development from leaf explants was significantly higher than that of nodal explant (Table I; Fig 1a, b). The moisture content of callus was also high as compared to other auxins supplemented media. The Callus was pale, yellowish green in colour. Callus grown on medium supplemented with BA was green in color more compact, hard granular. Callus was developed from leaf explants cultured on MS medium supplemented with 2, 4-D (1.0 mg/l) showed better callus induction and proliferation, it was 75% and the rate of proliferation was recorded from 12th day after inoculation up to 40 days with four day intervals. At 24th day the production of callus response was found to high compared to shorter duration. The above findings related to the nature of the callus was confirmed with the reports of Hildebrandt *et al.*, (1963), Davey *et al.*, (1971) and Kirkham and Holder (1981) in pea and tomato; *Atropha belladonna* respectively. These findings harmonized with Uddin *et al.*, (2006) in *Stevia rebaudiana* and also the results are accordance with the reports of

Table I. Impact of auxin and cytokinin on callus induction from explants of *Stevia rebaudiana*

PGR Concentration (mg/l)			Juvenile leaf % response	Node % response
2,4-D	NAA	BA		
0.1	-	-	41.7 ± 2.8	25.0 ± 0.0
0.5	-	-	50.0 ± 5.0	25.0 ± 0.0
1.0	-	-	75.0 ± 5.0	43.3 ± 7.6
2.5	-	-	55.0 ± 5.0	20.0 ± 5.0
5.0	-	-	45.0 ± 5.0	20.0 ± 0.0
-	0.25	-	26.6 ± 5.0	18.3 ± 5.7
-	0.5	-	40.0 ± 5.0	25.0 ± 8.7
-	1.0	-	33.3 ± 7.6	16.6 ± 2.8
-	2.5	-	25.0 ± 5.0	15.0 ± 0.0
-	5.0	-	15.0 ± 5.0	10.0 ± 0.0
-	-	0.25	21.6 ± 5.7	15.0 ± 5.0
-	-	0.5	26.6 ± 2.9	26.6 ± 5.8
-	-	1.0	35.0 ± 5.0	16.6 ± 2.7
-	-	2.0	30.0 ± 0.0	8.3 ± 2.9
-	-	3.0	18.3 ± 2.8	6.7 ± 2.8

Data were recorded after 40 days of culture. Results represent mean ± SD of six replicated experiments.

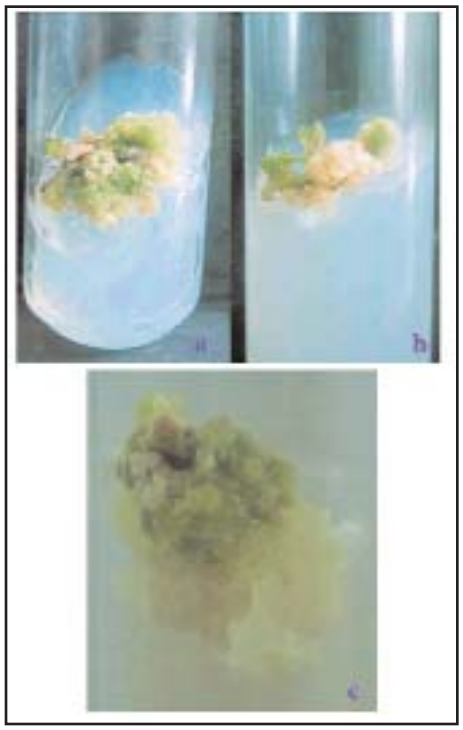

Fig. 1. Callus initiation from different explants of *Stevia rebaudiana*

(a) Callus initiation from nodal explants
(b) Callus initiation from leaf explants
(c) Secondary callus developed from primary callus of leaf explants

Thomas and Maseena (2006), in which callus induction was highest (90 %) in the leaf explants of *Cardiospermum halicacabum* on MS medium supplemented with 2, 4-D (5 mM). When the calli obtained from leaf explants were transferred to MS medium supplemented with different concentrations and combination of auxins and cytokinins, maximum growth of callus was obtained in MS medium amended with PGRs such as 2, 4-D at 0.5 mg/l followed by NAA at 0.5 mg/l, Indole-3- acetic acid (IAA) at 1.0 mg/l and Indole-3-butyric acid (IBA) 2.5 mg/l among the auxins. In the cytokinins, BA at 0.5 mg/l followed by kinetin 1.0 mg/l in MS medium was good in callus growth (Table II; Fig 2). The maximum callus growth was found with auxin such as 2, 4-D and NAA, and kinetin among cytokinins. Maximum callus growth using 2, 4-D, NAA and BA has been reported in *Gymnema sylvestre* (Reddy *et al.*, 1998; Gopi, 2002), *Eurycoma longifolia* (Siregar *et al.*, 2003); *Stevia rebaudiana* (Janarthanam and Kanimozhi, 2006) and *Rosa bourboniana* (Janarthanam and Seshadri, 2008). While the callus in 2,4-D supplemented medium was well developed, albino, spongy and loosely arranged (Fig 2a), it was pale, yellowish green in colour more friable, hard and granular in NAA supplemented media (Fig 2c). In IAA and IBA supplemented media, the callus was solid in nature and pale green in colour (Fig 2b, d). The nature of callus was green in colour and more compact, hard and granular in KN supplemented medium (Fig 2f), whereas in BA supplemented medium the callus was light green, less hard and compact (Fig 2e). The fresh and dry biomass yield was high on medium supplemented with KN followed by 2, 4-D (Table II).

A total of 105 combinations of auxins and cytokinins were tried for optimum callus biomass production. The hormone combination for optimum callus biomass production was standardized and the callus biomass was 182.3 g/l fresh weight and 11.6 g/l dry weight MS medium supplemented with 0.5 mg/l 2, 4-D, 0.5 mg/l NAA and 1.0 mg/l KN after 24 d of culture (Fig 3). When the above combination was used for suspension culture, optimum biomass production was achieved with 112.2 g/l fresh weight and 8.75 g/l dry weight after 21 d of culture (Fig 3). The 24th day callus (Solid medium) extract was used for TLC and HPLC analysis. The thin layer chromatography analysis (Pasquel, 2002) showed a similar banding with an R_f value of 0.692, 0.682 and 0.686 in standard, standardised callus and leaves of mother plant, thus confirming the presence of stevioside in leaf and callus tissues (Fig. 4). Earlier studies indicate the use of water and methanol for Stevioside extraction with comparable efficiency (Vanek *et al*., 2001). The stevioside content was higher in leaf (19.25 g/kg) than callus (17.63 g/kg) respectively (Fig. 5). The presence of stevioside to be higher

Table II: Effect of auxins and cytokinins on *Stevia rebaudiana* callus culture

PGR	2,4-D		IAA		IBA		NAA		BA		KN	
(mg/l)	FW(g/l)	DW(g/l)	FW(g/l)	DW(g/l)	FW(g/l)	DW(g/l)	FW(g/l)	DW(g/l)	FW(g/l)	DW(g/l)	FW(g/l)	DW(g/l)
0.10	43.0	2.48	8.60	0.62	15.16	1.76	12.25	1.15	53.34	6.94	86.64	8.43
0.25	55.3	3.10	18.8	1.23	12.18	0.96	17.16	1.06	59.50	6.83	95.85	9.35
0.50	97.4	4.50	46.34	3.08	24.15	1.70	66.12	3.45	66.07	7.66	97.59	9.69
1.00	92.3	4.16	66.46	3.56	45.10	2.92	59.46	3.32	61.08	6.97	132.6	12.43
2.50	80.5	3.87	48.64	3.12	56.40	3.26	42.32	2.02	52.92	5.65	123.6	12.05
5.00	57.6	3.15	34.38	2.98	18.12	1.26	33.40	2.61	45.00	5.01	94.28	9.29

Data were recorded after 40 day of culture.

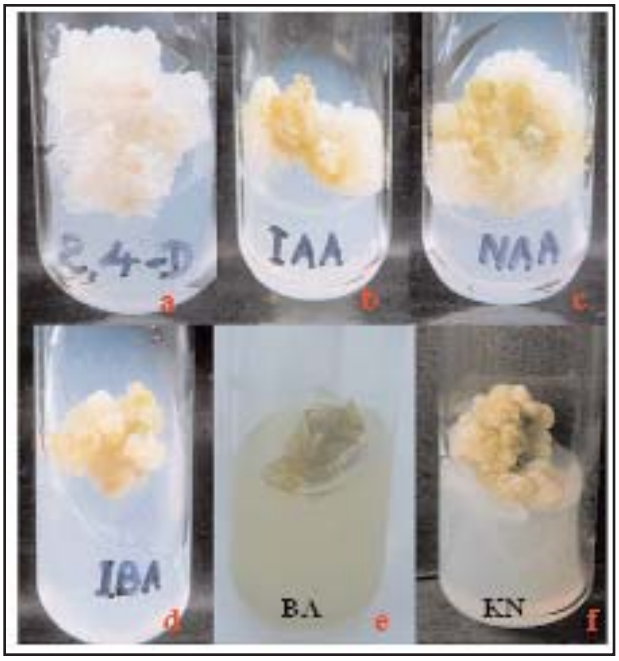

Fig. 2. Callus culture on MS medium supplemented with auxins and cytokinins (a) 2,4 -D (0.5 mg/l); (b) IAA (1.0 mg/l); (c) NAA (0.5mg/l); (d) IBA (2.5 mg/l); (e) BA (0.5 mg/l) and (f) KN (1.0 mg/l)

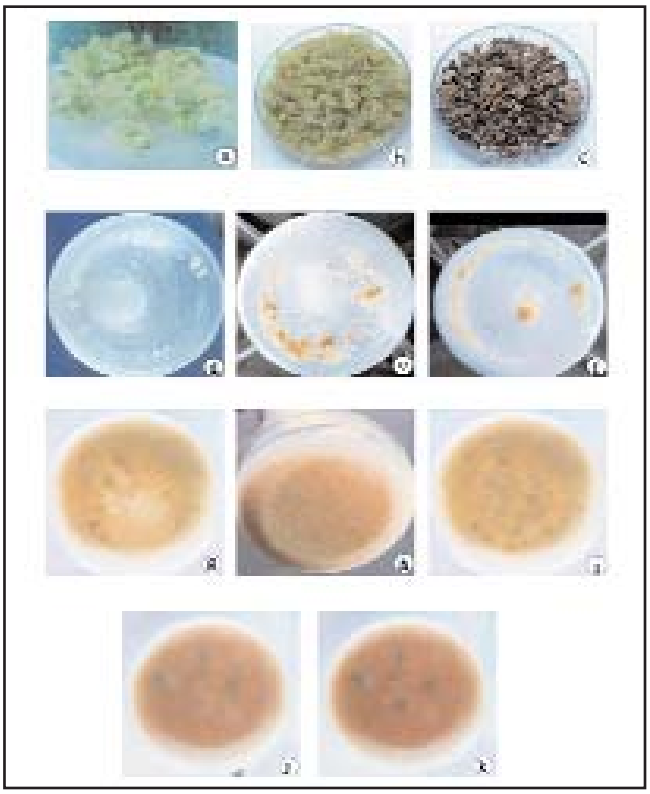

Fig. 3. Solid and liquid *suspension cultures* of *Stevia rebaudiana* UP: Growth of cells in solid culture using MS medium supplemented with 0.5 mg/l 2, 4-D, 0.5 mg/l NAA and 1.0 mg/l KN; (a) 24th day; (b) & (c) Fresh and Dry weight callus biomass. Bottom: Growth of cells in liquid culture using MS medium supplemented with; (d) 9th day, (e) 12th day, (f) 15th day, (g) 18th day, (h) 21st day, (i) 24th day, (j) 27th day and (k) 30th day

in leaf than callus observed in this study is in agreement with earlier report (Sivaram and Mukundan, 2003). The accumulation of active principles in cultured cells at a higher level than those in native plants through optimization of cultural conditions has been observed in *Panax ginseng* (Ushiyama, 1991). Rosmarinic acid by *Colleus blumei* (Ulbrich *et al.*, 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), diosgenin by *Dioscorea* (Rokem *et al.*, 1984), ubiquinone-10 by *Nicotiana tabacum* (Matsumoto, 1980) were accumulated in much higher levels in cultured cells than in the intact plants. Sometimes cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant (Whitaker *et al.*, 1986). Since the demand in pharmaceutical industries for plant based raw materials is ever increasing. The present study is a stepping stone for *in vitro* production of required active principles *of Stevia rebaudiana*. So far,

Table III. Age of calli and production of *Stevia rebaudiana* on Solid and liquid medium

Age of callus (Days)	Callus biomass on MS solid medium		Callus biomass on MS liquid medium	
	FW (g /l)	DW (g /l)	FW (g /l)	DW (g /l)
9	93.43	6.43	61.50	5.06
12	103.5	6.97	83.80	6.55
15	146.9	8.53	95.80	7.36
18	168.4	9.97	110.9	8.27
21	172.3	10.1	112.2	8.75
24	182.3	11.6	102.2	8.12
27	168.2	9.63	98.37	7.01
30	146.8	8.57	94.40	6.52
33	143.3	7.33	92.13	6.50
36	140.2	7.03	90.18	6.12

Data were recorded after 40 day of culture.

Fig. 4. Analysis of stevioside content in the leaf and callus culture of *Stevia rebaudiana* (a) Stevioside standard (b) Callus sample (c) Leaf sample

there is no known report on the production of active principles of *Stevia rebaudiana* by callus. This is the first successful attempt for of production of secondary metabolites of *Stevia rebaudiana* where, production levels can be manipulated with appropriate PGRs. Further studies will be directed towards large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

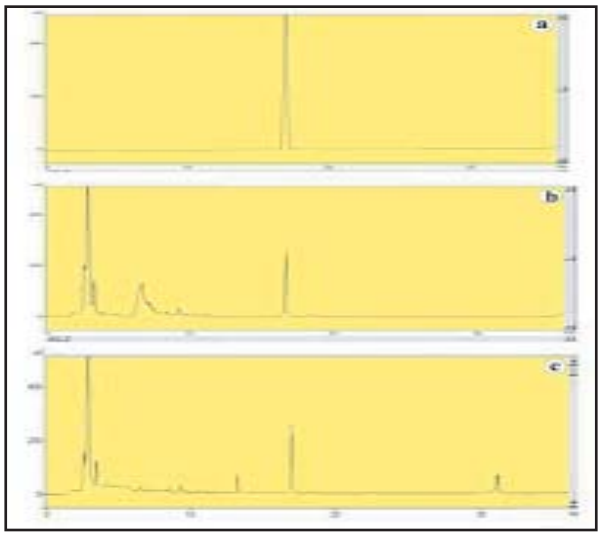

Fig. 5. HPLC analysis of stevioside content in the leaf and callus culture of *Stevia rebaudiana* (a) Stevioside standard (1 mg/ 1ml) (Wako Pure Chemicals, Japan); (b) Leaf; (c) Callus.

20 µl of sample was injected for every analysis (One gram of dried leaves and callus extracted with 25ml of water and concentrated residue dissolved in 1mg / ml of water). Leaf and Callus sample extracts were filtered using Sartorius Millipore filter and injected for further analysis.

Acknowledgement

The author Dr. B. Janarthanam thanks the principal and Dr. C. R Bojan, H.O.D of Botany Pachaiyappa's college for providing lab facilities and encouragement for this work.

References

Chan P., Xu D. Y., Liu J. C., Chen Y. J., Tomlinson B., Huang W. P. and Cheng J. T. (1998) The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats. *Life Sci.* **63**: 1679-1684.

Collin H. A. and Edwards S. (1998) The plant Cell Tissue Culture. In: D. Rickwood and C. Howe (ed.). (Bios scientific publishers Limited, Oxford, UK), P. 32.

Curi R., Alvarez M., Bazotte R. B., Botion L. M., Godoy J. l. and Bracht A. (1986) Effect of *Stevia rebaudiana* on glucose tolerance in normal adult human. *Brazil J. Med Bioi Res.,* **19**: 771-774

Davey M. R., Flower M. W. and Street H. E. (1971) Cell clones contrasted in growth, morphology and pigmentation isolated from a callus culture of Atropha belladonna Var. *Letea Phytochem.,* **10**: 2559 - 2575.

Geuns J. M. C. (2004) Review: The safety of stevioside used

as a sweetener. pp. 85-127 in "Proceedings of the first symposium on the Safety of Stevioside". Geuns, J.M.C. and Buyse, J., Eds. Kuleuven, 2004. Euprint Editions ISBN 9074253024.

Goettemoeller J. and Ching, A. (1999) Seed germination in Stevia rebaudiana. In: J. Janick (ed.), Perspectives on new crops and new uses. (ASHS Press, Alexandria, VA) pp. 510-511.

Gopi C. (2002) *In vitro* production of cell biomass and major metabolites of medicinal importance of *Gymnema sylvestre* R.Br. Ph.D., thesis, University of Madras, Chennai, India.

Hildebrandt A. C., Wilme J. C., Johns H. and Riker J. A. J. (1963) Growth of chlorophyllous plant tissue *in vitro. Am. J. Botany.,* **50**: 248 - 254.

Ishima N. and Katayama O. (1986) Sensory evaluation of stevioside as a sweetener. *Rep. Natl. Food Resp. Inst*. **31**: 80 - 85.

Janarthanam B. and Kanimozhi J. (2006) *In Vitro* study on *Stevia rebaudiana* Bertoni and its active compounds in "National Conference on Path to Health Biotechnology Revolution in India", November 24th - 26th, Anna University, Chennai, Tamil Nadu, India.

Janarthanam B. and Seshadri S. (2008) *In vitro* manipulations of *Rosa bourboniana*. *Acta Horticulturae,* **769**: 357-370.

Kirkham M. S. and Holder P. L. (1981) Water osmotic and turgor potentas of Kinetic treated callus. *Hort. Sci.* **1**: 306 - 307.

Matsumoto T. (1980). *Agr. Biol. Chem.* **84**: 967.

Murashigue T. and Skoog F. (1962) A revised medium for rapid growth and biossays with tobacco tissue cuItures. *Physiologia Plantarum*. **15**: 473-497.

Pasquel A., Meireles M. A. A., Marques M. O. M. and Petenate A. J. (2002) Extraction of Stevia glycosides with co_2 + water, co_2 + ethanol, and co_2 + water + ethanol. *Braz. J. Chem. Eng.,* 17 SP .

Rao R. and Ravishankar G. A. (2000) Biotransformation of Protocatechuic Aldehyde and caffeic acid to vanillin and capsaicin in freely suspended and immobilized cell culture of *Capsicum frutescens*. *J. Biotechnol.,* **76**: 1371-146.

Reddy P. S., Gopal G. R. and Sita G. L. (1998) *In vitro* multiplication of *Gymnema sylvestre* R.Br. -An important medicinal plant. *Curr. Sci.* **75**: 843-845.

Rokem J. S. (1984) *Plant Cell Rep.*, **3**: 159-160.

Sakaguchi M. and Kan T. (1982) Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. *Ci. Cult.,* **34**: 235-248.

Siregar L. A. M., Keng L. C. and Lim P. B. (2003) Selection of Cell Source and the effect of pH and MS Macronutrients on Biomass Production in cell cultures of Tongkat Ali (Euryocoma Longifoli Jack). *J. Plant Biotechnology,* **5**:131-135.

Sivaram L. and Mukundan, U. (2003) *In vitro* culture studies on *Stevia rebaudiana. In vitro* Cell Development *Biol. - Plant.,* **39**: 520-523.

Soejarto D. D., Compardre C. M., Medon P. J., Kamath S. K. and Kinghorn A. D. (1983) Potential sweetening agents of plant origin II Field search for sweet tasting *Stevia species. Econ. Bot.,* **37**: 71-75.

Takahashi S., Fujita Y. and ibid, (1991) CMC Co. Ltd. Tokyo., pp.72-78.

Tamura Y., Nakamura S., Fukui H. and Tabata M. (1984). Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. *Plant Cell Reports*., **3**: 183-185.

Tanaka O. (1982) Steviol Glycosides; new natural sweeteners. *Trends in Analytical Chemistry.,* **1**: 246 - 248.

Thomas T. D. and Maseena E. A. (2006). Callus and plant regeneration in *Cardiospermum halicacabum* Linn. An important medicinal plant, *Sci. Hortic*., **108**: 332 -336.

Uddin M. S., Chowdharry M. S. H., Haque Khan M. M. M., Uddin M. B., Ahmed R. and Md. Azizul Baten. (2006). *In vitro* propagation of *Stevia rebaudiana* Bert in Bangladesh. *African Journal of Biotechnology.* **5**: 1238-1241.

Ulbrich B., Weisner W. and Arens H. (1985) In Primary and Secondary Metabolism of Plant Cell Cultures, Eds. Neumann, KH, Reinhard E Springer-Verlag, Berlin. pp. 293-303.

Ushiyama, Takahashi, Fujita (1991) Rokem *et al.*, 1984; Matsumoto *et al.*, 1980 Ushiyama, K., In "Plant Cell Culture in Japan", Eds. Komamine A, Misawa M, DiCosmo F. CMC Co. Ltd., Tokyo, pp.92-98.

Vanek T., Nepovim A. and Valicek P. (2001) Determination of Stevioside in plant material and fruit Teas. *J. Food Composit. Anal.,* **14:** 383 - 388.

Whitaker R. J. *Am* (1986) *Chem. Soc. Symp. Ser,* **317**: 347-362.

Received : May 13, 2009;
Accepted : January 12, 2010