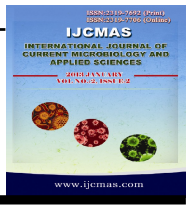
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7692 **Volume 2 Number 2 (2013) pp.56–61**
http://www.ijcmas.com



Original Research Article

Organogenic plant regeneration via callus induction in *Stevia rebaudiana* Bert

V. Guruchandran and C.Sasikumar*

Department of Biotechnology, Nehru Memorial College, Puthanampatti - 621 007, Tiruchirappalli, Tamilnadu, India
***Corresponding author:** drsasi_kumar5@yahoo.co.in*

Keywords

Stevia rebaudiana; Organogenesis; Callus; Growth regulators.

A B S T R A C T

Stevia rebaudiana is investigated as optional for artificial sweetening agents. In the present investigation an efficient and reproducible protocol for organogenesis using different explants from 3-4 months old plantlets of *S.rebaudiana* was studied. Maximum callus induction occurred in leaf explant cultured in the MS medium fortified with 2, 4 –D (1.5mg/L) in combination with BAP (0.5mg/L).Maximum number of multiple shoots regenerated on MS medium fortified with BAP (1.5mg/L) in combination with kinetin (0.5 mg/L). BAP (1.5 mg/L) in combination with GA_3 (1.0mg/ L) plays a vital role on the elongation of shoots. Half strength MS Medium in combination with NAA (1.0mg/ L) plays a vital role on rooting of shoots.

Introduction

Stevia rebaudiana Bert belongs to the family Asteraceae is one of the most valuable tropical medicinal plants. The genus *Stevia* consists of 154 species of which *S. rebaudiana* produces sweet steviol glycosides. (Soejarto *et al*., 1982). It is a small shrub by perennial growing up to 65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated above the middle. It is originally a South American wild plant, but could be now all found growing in semi-arid habitat ranging from grassland to shrub forest to mountain terrain all over the world. The first report of commercial cultivation in Paraguay was in 1964 began a large effort aimed at establishing *Stevia* as a crop in Japan (Katayma *et al*., 1976; Lewis, 1992; Sumida, 1968). Since then, it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada. The property of the species that called attention to the plant was the intense sweet taste of the leaves and aqueous extracts. From the leaves of *Stevia*, stevioside, sweet crystalline di terpene glycosides are extracted (Lee and Kang, 1979). Pure extract stevioside is non-caloric and 30 times sweeter than sugar (Bhosle, 2004). It is used as a table top sweetener, in soft drinks, baked goods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets. In the Pacific Rim countries like China, Korea and Japan *Stevia* is regularly

used in preparation of food and pharmaceutical products. In Japan alone, estimated 50 tons of stevioside is used annually with sales valued in order of $220 million Canadian Dollar (Brandle and Rosa, 1992). Seed germination of *Stevia* is often poor (Miyazaki and Wantenabe, 1974). Therefore, there are basically two options for multiplication; (i) tissue culture and (ii) stem cutting. The seeds of *Stevia* show a very low germination percentage. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura and Tamura, 1985). Vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982). Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid mass propagation of *Stevia* sp. Plant tissue culture techniques may help to conserve rare and endangered medicinal plants. Many important medicinal herbs have been successfully propagated *in vitro*, by organogenesis (Debnath *et al*., 2006).

Materials and Methods

Plant Material

Stevia rebaudiana plants were collected from Horticultural Research Station Yercaud, Tamilnadu, India. Leaf, node and shoot tip from 3-4 months old plantlets were used as an explant.

Surface sterilization

The explants were thoroughly washed with 0.1 % teepol followed by washing with sterilized distilled water thrice. The explants were then surface sterilized with different concentrations of mercuric chloride 0.1, 0.2 and 0.3 % for 3 to 5 minutes, followed by washing with sterile distilled water and transferred to culture tubes containing suitable medium under aseptic conditions. The observations on culture survival, extent of contamination and death of explants were recorded and expressed as percentage (Katayma *et al*., 1976).

Callus induction

Murashige and Skoog's medium (Murashige and Skoog, 1962) containing 0.8% agar (w/v) and 3% sucrose (w/v) was used. Growth regulators 2, 4 – Dichlorophenoxy acetic acid (2, 4-D, 0.5 – 2.5 mg/L) and in combination with BAP (0.5mg/L) were tested for the induction of organogenic callus from leaf, node and shoot tip explants. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15min. The cultures were maintained at 25 ± 2°C under 16 h photo periods with the light intensity of 3000 lux. Explants were subjected to two sub cultures at an interval of ten days each in the MS medium supplemented with the same concentration of growth regulators (Hossain *et al*., 2008).

Shoot regeneration

After 8 weeks of induction, the calluses were transferred to MS media containing 3% sucrose, 0.8% agar along with growth regulators with different concentration of BAP (0.5-2.5 mg/L) and incubated in culture conditions as mentioned above and subjected to two sub cultures at an interval of ten days each in the MS medium supplemented with the same concentration of growth regulators (Tamura *et al*., 1884).

Shoot elongation

Proliferated multiple shoots with an average height of 2 cm were carefully excised from the explant, segregated in to small clusters

containing 2-3 shoots. The shoot clusters were transferred to shoot elongation medium containing BAP (1.5mg/L) in combination with different concentrations of GA_3 (0.5-2.5 mg/L). The cultures were maintained at 25 ± 2°C under 16h photoperiod (Uddin *et al*., 2006).

Root formation

After 3 weeks, elongated shoots (1-1.3 cm in height) were transferred to half strength MS basal medium and full strength MS basal medium containing NAA 1.0 mg/L. The cultures were maintained as described above (Hossain *et al.*, 2008).

Hardening

After two weeks of culture in the root induction medium, the rooted plants were removed from the culture tubes and were washed in running tap water to remove agar from the root surface. Then the rooted plants were transplanted to plastic pots containing sterile soil: sand: vermiculite (1:1:1 v/v/v) (Sairkar *et al*., 2009).

Results

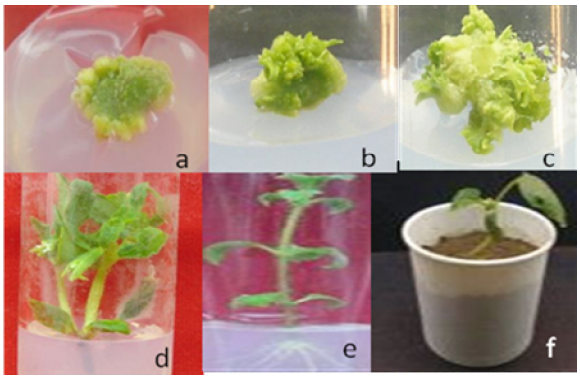
Surface sterilization

The shoot tips were found to be less contaminable when compared to the nodal and leaf segments. The degree of contamination reduced with the increase in the concentration of mercuric chloride and higher period of exposure. The contamination percentage was lowest when shoot tips were treated with 0.3 % mercuric chloride for 5 minutes. Highest contamination was recorded in the nodal segments, when treated with 0.1% mercuric chloride for 3minutes. Surface sterilization is essential to obtain successful tissue culture plantlets in *in vitro* systems. The efficiency of the sterilization varies, which depends upon the chemical concentration used, time of exposure etc. In the present study it was observed that treatment of explants with 0.3% mercuric chloride for 5 minutes duration was found to be the best for obtaining contamination free cultures and higher survival percentage.

Callus induction

Callus producing ability of shoot tip, leaf, node explants derived from 30days old plantlets of *S. rebaudiana* was evaluated on MS medium supplemented with different concentrations of 2, 4-D in combination with BAP. Callus occurred at the cut end of the explants after 20 days of culture initiation. Maximum callus induction (Figure.1a) occurred in the MS medium fortified with BAP (0.5mg/l) and 2, 4 –D (1.5mg/l) as shown in Table 1.

Figure.1 Organogenic plant regeneration via callus induction in *S.rebaudiana* Bert



a. Callus induction occurred in leaf explant cultured in the MS medium fortified with 2,4 - D (1.5mg/L) in combination with BAP (0.5mg/L)
b. Prolifertion of multiple shoots from callus
c. Regenerated shoots on MS medium fortified with BAP (1.5mg/L) in combination with kinetin (0.5 mg/L).
d. Elongation of shoots in MS medium containing BAP (1.5mg/l) and GA_3 (1.0mg/l)
e. Rooting of shoots in half strength MS medium containing NAA(1.0mg/L).
f. Hardened plantlets.

Table.1 Effect of 2, 4-D and BAP on callus Induction

Plant growth regulator (mg/L)		Explants		
2,4-D	**BAP**	**Leaf**	**Node**	**Shoot tip**
0.5	0.5	--	--	--
1.0	0.5	+	--	--
1.5	0.5	++	+	+
2.0	0.5	+	--	--
2.5	0.5	--	--	--

(--) No response
(+) Response up to first subculture
(++) Better response up to second subculture

Shoot regeneration

Various growth regulators at different combinations and concentrations were examined for regeneration of shoots. Maximum number of multiple shoots (Figure.1b, c) produced from the callus cultured on MS medium fortified with BAP (1.5mg/l) in combination with Kinetin (0.5mg/l) (Table 2). It is interesting to note that there was no response when the explants were cultured in the MS medium without any supplementation, suggesting that it is essential to add growth regulators exogenously to obtain desirable results.

Table.2 Effect of BAP and Kinetin on shoot induction

Plant growth regulator (mg/L)		Callus responded %	Mean Number of Shoots produced	Basal callus
BAP	Kinetin			
0.5	0.5	6%	2	-
1.0	0.5	42%	7	-
1.5	0.5	72%	12	-
2.0	0.5	65%	8	+
2.5	0.5	59%	6	+

+ Basal Callus Present; - Basal Callus Absent

Table.3 Effect of GA_3 along with BAP on elongation of shoots

Plant growth regulator(mg/L)		Shoot responded	Mean Shoot length (cm)
GA_3	BAP		
0.5	1.5	10%	0.5
1.0	1.5	48%	5.5
1.5	1.5	38%	4.5
2.0	1.5	25%	3.2
2.5	1.5	23%	2.8

Shoot elongation

Shoots (1.0cm height) harvested and transferred to shoot elongation medium containing GA_3 (0.5-2.5mg/l) in combination with BAP (1.5mg/l). After two weeks of culture, maximum shoot elongation (Figure.1d) occurred in the MS medium containing BAP (1.5mg/l) and GA_3 (1.0mg/l) as shown in the Table 3.

Root formation

The rooting of elongated shoots varied significantly with regard to type and concentration of nutrients present in the rooting medium. Maximum frequency of rooting as well as production of normal roots was observed in half strength MS basal medium (Figure1e).

Discussion

The concentration of mercuric chloride prevented the growth of the microbial contaminants. The cultured explants showed more than 80% contamination free cultures when treated with 0.1% mercuric chloride for 2 minutes. Higher concentration of mercuric chloride and longer time of

exposure produced cultures with less contamination, but percentage of survival of explants decreased. Among the three explants best callus induction was occurred in leaf explant. Earlier (Sairkar *et al* ., 2009) reported that 2.0 mg/L of 2, 4-D with 1.0mg/L kinetin showed 88% callus induction after 30 days. Hossain *et al*. (2008) reported that shoot tips showed better response for shoot proliferation than nodal segments and BAP (1.0 mg/L) was superior to all other hormonal treatments for shoot proliferation. Higher percentage of rooting were obtained in half strength MS basal medium supplemented with NAA (1.0 mg/L), were as in the early report full strength MS medium supplemented with NAA (1.5 mg/L) was the best medium for rooting (Hossain *et al.*, 2008). The above study reveals that an efficient plant regeneration via callus and shoot organogenesis was established.

ACKNOWLEDGEMENT

We place on record our deep sense of gratitude to the Secretary, the Principal and the management of Nehru Memorial College (Autonomous), Puthanampatti, and Tiruchirappalli for providing necessary equipments and constant encouragement throughout the work.

References

Bhosle, S. 2004. Commercial Cultivation of *Stevia rebaudiana.* Agrobios Newsletter.3 (2): 43-45.

Brandle, J.E., and Rosa, N. 1992. Heritability for yield, leaf- stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. *Canadian Journal of Plant Science*. 72: 1263-1266.

Debnath, M., Malik, C.P., and Bisen, P. S. 2006. Micropropagation: A Tool for the Production of High Quality Plant-based Medicines, Curr *Pharm Biotechnol*, 7(1): 33-49.

Hossain, M. A., Kabir A. H. M., Jahan, T. A., and Hasan M. N. 2008. Micropropagation of Stevia, *Int. J sustain crop prod.*, 3(4):1-9.

Katayma, O., Sumida, T., Hayashi, H., and Mitsuhashi, H. 1976.The Practical application of *Stevia* and R&D data (English translation) ISU Company. Japan. 747.

Lee, J. I., and Kang, K. K. 1979. Studies on new sweetening resource plant *Stevia rebaudiana* in Korea, *Plant Sci* 40: 112-117.

Lewis, W. H. 1992. Early uses of *Stevia rebaudiana* leaves as sweetener in Paraguay. *Econ Bot*. 46: 336-337.

Miyazaki, Y., and Wantenabe, H. 1974. Studies on the cultivation of *Stevia* on the propagation of plant (Eng Abstr). *Jap J Trop Agric*. 17: 154-157.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant*. 15 :473-479.

Nakamura, S., and Tamura, Y. 1985. Variation in the main glycosides of *Stevia rebaudiana* Bertoni. *Jpn J Trop Agric*. 29:109-116.

Sairkar, P., Chandravanshi, M. K., Shukla, N. P., and Mehrotra, N. 2009. Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. *Journal of Medicinal Plants Research*. 3(4): 266-270.

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

Soejarto, D., Kinghorn, A.D., and Fransworth, N.R. 1982. Potential

sweetening agents of plant origin. *J Nat Prod*. 45: 590-599.

Sumida, T. 1968. Reports on stevia introduced from Brazil as a new sweetness resource in Japan (English summary). J Cent Agric Exp Stn. 31: 1-71.

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

Uddin, M. S., Chowdhury, M. S. H., Khan, M. M. M. H., Uddin, M. B., Ahmed, R., and Baten, M. A. (2006). In vitro propagation of *Stevia rebaudiana* Bert in Bangladesh, *African J. Biotechnol*., 5(13): 1238-1240.