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Cyanobacterial extract and MS media as a novel tool for *In-vitro* regeneration of *Stevia rebaudiana* Bertoni

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ABSTRACT

It is now evident that plant tissue culture is an essential component of plant Biotechnology which offers novel approach to the production, propagation conservation and manipulation of plants. In *Stevia rebaudiana* Bertoni the use of *in- vitro* techniques as alternative or complement to conventional procedures of propagation and genetic improvement could facilitate obtaining disease free homogenous populations. The purpose of the present study was to show the effect of Cyanobacterial Media and Murashige and Skoog media for *in-vitro* propagation of *Stevia rebaudiana* Bertoni by taking different explants and by taking different concentrations of hormones. Using cyanobacterial media might be useful to obtain *in-vitro* callusing from the explants of *Stevia rebaudiana* Bertoni.

Key words: Cyanobacterial Media (CM), Murashige and Skoog's Medium (MS), *Stevia rebaudiana* Bertoni, *in-vitro* propagation.

INTRODUCTION

Stevia rebaudiana Bertoni is a small perennial herb with green leaves that belongs to the Asteraceae family. It grows primarily in the mountain range of Paraguay but over 150 various species of stevia have been identified around the world (Antonie, 2000). The plant has gained wide access to Pacific Rom countries, where in recent decades it is being cultivated domestically, used in its raw leaf form and is now commercially

processed into sweetener. After first report of commercial cultivation in Paraguay in 1964 (Lewis, 1992), it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Brandle and Rosa 1992). It is a natural, non-caloric sweet-tasting plant used around the world for its intense sweet taste. The sweet herb of Paraguay, stevia produces sweeteners in its leaves are natural plant products (Starratt and Gizen, 2004). Leaves of this plant produce zero-calorie diterpene glycosides (stevioside and rebaudioside), a non-nutritive, high-potency sweetener and substitute to sucrose, being sweetener than sucrose (Nakamura and Tamura, 1985).

Stevia plants grow better having temperature range of 0-40^{0}C. So these conditions allow stevia, growing annually in areas having diverse environment. However, it is cultivated on commercial basis by seed, cutting or division of mother plants in green house during winter every year. The seeds of *Stevia* show a very low germination percentage also. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition. Vegetative Propagation too, is limited by lowering number of individuals that can be obtained from single plant. Propagation through stem cutting requires enough stocks of stem cutting and high lebour inputs (Carneiro *et. al.*1997). Due to above mentioned difficulties tissue culture is the only alternative method to prepare sufficient amount of plants within short time duration for mass propagation of Stevia plants. Plant tissue culture of plant through shoot tip or axillary bud culture

may help to rare medicinal plants by recovery of genetically stable and true to type progeny. It is now evident that plant tissue culture is an essential component of Plant Biotechnology which offers novel approaches to the production, propagation, conservation and manipulation of plants(Sakaguchi and Kan, 1982). The success of *in vitro* culture depends mainly on the growth conditions of the source material medium composition and culture conditions and on the genotypes of donor plants (Thorpe, 1993).

Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation, conservation of germplasm, study and production of bioactive compounds, and for genetic improvement. Medicinal plants have vast genetic diversity, which is a valuable source of agronomic genes of interest for the future. Large-scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemicals independent of plant availability and more consistent product quality (Caswell *et. al*. 2004). Minimal growth of tissue in culture and cryopreservation has been used to store plant materials from a wide variety of species (Delporte *et. al.* 2001). Various compounds of cyanobacteria could be useful sources to enhance or substitute the influenced of synthetic plant growth regulators on tissue cultures of different plants *in-vitro*. The approach of using cyanobacterial cultures would overcome many barriers of micropropagation were costly synthetic chemicals involved (Banerjee and Sarkar, 2008). Cyanobacteria or Blue green algae are prokaryotic

photosynthetic microorganism that produces a wide array of substances, including plant growth regulators (Metting and Pyne, 1996).

The present paper deals with the use of MS media and cyanobacterial extract as a liquid culture media for the induction and multiplication of adventitious shoot bud to ensure high yield of biomass of this medicinal herb. This protocol is very cost effective and totally novel where liquid culture of cyanobacterial extract have been used for the efficient regeneration of *Stevia rebaudiana* Bertoni.

MATERIAL AND METHODS

Plant and media preparations

Different parts of the plant were used as explants for the *in-vitro* regeneration of *Stevia rebaudiana* Bertoni. The seeds of the plant were obtained from J.N.K.V.V. Jabalpur, M.P. and algal stock cultures of *Scytonema sp, Mastigocladus laminosus and Aulosira fertilissima* were obtained from the Department of Bioscience, Barkatullah University, Bhopal and grown in BG-11 free nitrogen medium (Rippka, 1979). For *in-vitro* regeneration of *Stevia rebaudiana* Bertoni Murashige and Skoog's medium was used with various concentrations of plant growth regulators (Murashige and Skoog., 1962).

Explant preparation

Leaf, nodal and inter-nodal segments, shoot tips etc were collected as explants from several days (2-4 month) old plant of *Stevia rebaudiana* Bertoni. Shoot apex of *Stevia rebaudiana* Bertoni can also regenerate shoots when culture on Murashige and Skoog medium supplemented with different growth regulators. The explants were cut into small pieces (about 0.1 m long) and then were treated with 1%

savlon for 5-6 min with constant shaking and washed thoroughly with distilled water. Then the explants were taken under laminar airflow cabinet and surface sterilized with a 0.1% mercuric chloride solution containing two-three drops of tween-80 for 5 min under aseptic condition and then washed four times with sterilized distilled water. The explants were then inoculated aseptically into culture medium with different concentrations and combinations of growth regulators.

Surface sterilization of explant

Surface sterilization is necessary in order to disinfect the explant before it was placed over media. For this following steps are performed:

- The explant were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the dust particles and microbes from the surface.
- In the next step explants were soaked in an aqueous soap solution containing 1% Labolene (Qualigens) for 5-7 minutes and then washed with distilled water.
- Explants were treated with 2% Bavistin (antifungal) for 10 minutes and antibiotic for 5 minutes.
- This was followed by gentle wash in double distilled water for 5 minutes for two cycles.

Sterilization procedure under aseptic condition

After the surface sterilization explants were taken inside the laminar flow hood. Here 2-3 washings were given with sterile double distilled water. Further explants were surface disinfected with freshly prepared 0.1% (w/v)

aqueous solution of mercuric chloride for 5 minutes. They were then thoroughly washed for 3-4 times with sterile double distilled water to remove any traces of mercuric chloride for 5 minutes (two cycles). The explants were immersed in 70% ethanol for 5 min. This was followed by at least three rinses in double distilled water. Rinsed explants were inoculated in prepared Murashige and Skoog's medium (MSM) (Murashige and Skoog., 1962).

Glasswares

The glassware used for culture work comprised of 6"Õ 1" borosil test tubes, 250ml, 500ml and 1000ml borosil flasks. In addition other glassware includes graduated measuring cylinder, Petri dishes, beakers and a range of pipettes. Before use, glasswares were thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in2 (121^0C) for 15 to 20 minutes.

Culture conditions

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25±2ºC, relative humidity (RH) of 60-70% and a light intensity of approx. 2500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 hr (light/dark).

Subcultures

Cultures were maintained through regular monthly subcultures. The cultured tissues were aseptically transferred on to fresh media without being subjected to chemical sterilization. To achieve shoot elongation, the multiple shoot clusters were dissected into finer units. The adhering agar and necrotic tissues were removed without damaging the shoot primordial/buds. These units were finally recultured on to fresh media having appropriate plant growth regulators (PGRs). Subcultures were also performed as and when necessary after evaluation of growth changes.

Shoot proliferation medium

MS media in two strengths i.e., full MS and ½ MS (all the ingredients 0f MS medium were reduced to half of its original concentration) supplemented with either 6- benzyladenopurine (BAP) or kinetin (kn) at varying concentrations (0.1, 0.5, 1.0 and 1.5 mg/l) were prepared for shoot proliferation. After mixing all stock solutions 3% and 1.5% sugar was added for full MS and half MS respectively then the pH of the media was adjusted to 5.7 and 5.8. Nodal segments with a single axillary bud) about 0.5-0.8 cm were prepared aseptically and were implanted vertically on MS medium fortified with specific concentrations of growth regulators (BA, KIN and NAA) singly or in combination adding30 g lG1 sugar (market sugar) and 0.7% Difco Bacto-agar. The pH of the medium was adjusted to 5.7 with 0.1 NaOH before autoclaving at 1.06 kg cmG2 and 121°C for 20 min. The cultures were incubated at a constant temperature of 25±2°C with 16 h photoperiod (2000 lux).Subcultures were done every 21 days interval. Nodal segments from the proliferated shoots were subcultured again for further

multiple shoot induction. Regenerated multiple shoots were cut and individual shoots were placed in MS medium containing different concentrations of IBA, NAA and IAA for root induction.

Callus induction medium

A number of treatments with different concentrations and combinations of auxins (NAA, 2, 4-D) and cytokinin (BA) were employed for callus induction. Data were recorded after 24 days or culture. Twelve test tubes were selected randomly from 36 tubes of each treatment. Then calli were washed and remove the water molecule using tissue paper and fresh weight data was recorded.

Root induction media

Shoots that developed in liquid multiplication medium were transferred individually into half strength MS agar (0.7%) medium supplemented with IAA (1.0 mg/l).The shoots were maintained for 4 weeks under the same culture condition as for development of roots. After this time, the percentage of rooted shoots was recorded. Rooted plantlets were transplanted to pots filled with sterilized mixture of sand, vermicompost and soil (1:1:1) and grown for 5 weeks in greenhouse conditions to determine the percentage of plants that survived.

Cyanobacterial culture media

MS supplemented with CaCl2 (0.44 gm/l), 3% (w/v) sucrose, cyanobacterial media was used in this experiment. The pH of the medium was adjusted to 5.8. The culture vials containing the media were autoclaved at 121ºC at 15 lbs pressure for 20 min. All the cultures were maintained at 25 ± 2°C under 16 hr photoperiods and 8 hr dark periods with a photosynthetic photon flux density (PPFD) of 50 µ mol m^2/s provided by cool white fluorescent lamps (Phillips, India) and with 50-55%

relative humidity. Effect of hot extract of cyanobacterial species (10- 90 ml) + MS (10- 90 ml) + sucrose 3% (W/v) + CaCl2 (0.45 gm) and hot extract of cyanobacterial Species (10-90 ml) + MS (10- 90 ml) + Kn (1.0 mg/l) + sucrose3% (w/v) + CaCl2 (0.45gm) was studied (Banerjee and Sarkar, 2010).

Transplantation

The rooted plantlets were first transferred to plastic pots containing garden soil, sand and peat moss (1:1:1), covered with transparent polythene bags and placed in acclimatization room at 28±2°C with 70-90% relative humidity, after five days temperature was increased from 28 to 32°C. After two weeks, transparent bags were removed from pots for proper hardening. After four weeks, the plants were then shifted in greenhouse and in field under low light intensity. The data for various growth attributes were recorded such as % explants regeneration, multiple shoot formation, shoot length, % plantlets rooted, number of roots per plantlet, and survival of plants during acclimatization and in the field was recorded.

Effect of Cytokinin (BAP) on initiation of explant of *Stevia rebaudiana* Bertoni

The experiment was performed to study the effect of interaction of BAP and explant on various parameters viz. the frequency of bud break and bud elongation and mean number of shoots and shoot length. This experiment consisted of explants viz. nodal segments. A total of 5 concentrations of BAP (0.5.1.0, 2.0, 3.0, 4.0, 5.0 mg/l) were used. There were three replicates for each treatment and a total of 12 treatments were performed for each parameter.

Effect of Cytokinin (Kn) on initiation of explant of Stevia *rebaudiana* Bertoni

This experiment was performed to study the effect of interaction of KN and explant on bud break and bud elongation frequencies and mean shoot number and shoot length. This experiment was similar to Experiment 1 and used a different plant growth regulator cytokinin (Kn) at various concentrations (0.5, 1.0, 2.0, 3.0, 4.0.5.0 mg/L).

Effect of BAP, Kn and NAA on MS medium for regeneration of Shoot from nodal explants of *Stevia rebaudiana* Bertoni

Leaf explants were cut from petiolar end and placed on sterile medium with dorsal surface in contact with the medium supplemented with BAP and NAA. All the cultures were incubated in culture room, at a temperature of 25±2°C, 16 h photoperiod and light intensity of 25 μmol/(m^{-2} s^{-1}) provided by white fluorescent tubes. After 4 weeks the shoot buds induced on leaf explants were excised from base along with some portion of mother explants and placed on proliferation medium supplemented with BAP (3.8μM) and Kn (2.0 μM). Cultures were established in different culture vessels and it was observed that shoots regenerated in flask shows normal morphology and well elongated. After 2–4 weeks there was significant increase in shoot number.

RESULTS AND DISCUSSION

BAP is a plant growth regulator that play important role in initiation of explant. The effect of the different concentration of BAP was found to be significant for all parameters after 3-4 weeks of inoculation. Maximum frequencies of bud break and bud elongation and mean number of

regenerated shoots and shoot length were observed at 5mg/l BAP i.e. maximum frequencies of bud break is 72% and bud elongation is 85% and mean shoot number is (14.58) and mean shoot length is (3.46). While at concentration lower then 5.0 mg/l treatment resulted decline in all parameters (Table1).

Table 1- Effect of Cytokinin (BAP) on initiation of explant of *Stevia rebaudiana* Bertoni

BAP(mg/L)	FBB	FBE	MNS	MSL
0.00	28.73	38.42	1.82±0.5774	1.86±0.071
0.5	36.78	46.56	2.26±0.4923	2.08±0.066
1.0	42.22	68.62	4.25±0.6216	2.12±0.052
2.0	56.56	78.56	5.17±0.5774	2.24±0.065
3.0	62.44	80.12	6.0±0.603	2.88±0.079
4.0	64.82	82.02	9.5±0.6742	3.02±0.075
5.0	72.52	85.92	14.58±0.6686	3.46±0.065

In the experiment of effect of different conc. of Kn on initiation of explant were studied on different parameters. Explants stayed fresh in Kn supplemented medium till the next subculture and Kn supported the elongation of the explants. Explants were inoculated on MS medium supplemented with 3% vitamins, 3% sucrose, 0.8% agar and different concentration of Kn (cytokinin) on various parameters were studied (Table 2).

Table 2- Effect of cytokinin (Kn) on initiation of explant of *Stevia rebaudiana* Bertoni

Kn (mg/L)	FBB	FBE	MNS	MSL
0.00	22.56	36.44	1.80±0.5149	2.12±0.079
0.5	24.26	42.48	2.22±0.452	2.26±0.022
1.0	36.42	52.88	3.58±0.5146	2.42±0.0462
2.0	44.58	68.56	4.16±0.3892	2.54±0.0667
3.0	52.22	78.68	4.75±0.4523	3.02±0.0522
4.0	58.92	80.02	5.58±0.5149	3.52±0.0574
5.0	62.56	86.48	5.75±0.4523	4.46±0.0792

Experiment that show the effect of BAP, Kn and NAA on MS medium for regeneration of shoot from nodal explants of *Stevia rebaudiana* Bertoni, it was observed that best result obtained from (2.5 μM) of BAP with (2.9 μM) of NAA which produced 88% shoots formation and showed highest number of shoots (8.5±0.5) and highest average length of the shoot (6.9±0.3) per culture. During induction synergistic effect of BAP (2.5 μM) and NAA (2.9 μM) at lower concentration was found to be optimum for regenerating maximum number, i.e. four to eight shoot buds from nodal and leaf explants. The initial BAP and Kn combination at BAP (3.8 μM) and Kn (2.0 μM) level worked well for the purpose of shoot proliferation and elongation from the explants (Table 3).

Table 3- Effect of BAP, Kn and NAA on MS medium for regeneration of shoot from nodal explants of *Stevia rebaudiana* Bertoni

Plant growth Regulators (µM)			**Shoot Form-ation (%)**	**No. of shoot bud induced per explants Mean ± S.E.**	**Average length of shoot (cm) Mean ± S.E.**
BAP	Kn	NAA			
0.3	0	0	25	3.5±0.5	4.5±0.5
2.1	0	0	69	5.5±0.5	5.0±0.5
4.3	0	0	76	7.0±0.3	6.8±0.5
8.7	0	0	30	4.7±0.6	5.8±0.2
0	0.5	0	23	1.4±0.2	4.5±0.5
0	2.4	0	31	3.9±0.5	3.9±0.5
0	4.5	0	66	5.5±0.5	4.7±0.4
0	9.3	0	41	3.2±0.5	4.1±0.2
0.3	4.5	0	44	3.5±0.5	4.5±0.3
1.6	3.8	0	55	4.1±0.5	4.1±0.5
3.8	2.0	0	81	6.5±0.5	5.5±0.3
4.3	0.5	0	64	4.8±0.2	4.9±0.4
2.5	0	2.9	88	8.5±0.5	6.9±0.3
2.3	0	4.7	73	5.2±0.5	5.6±0.3
2.3	0	9.3	45	2.7±0.5	3.4±0.3

Studies also show the effect of combination of cyanobacterial media and MS on shoot induction of *Stevia rebaudiana* Bertoni for rapid and large-scale multiplication. The combined effect of cyanobacterial media and MS

(70 ml + 30 ml) was found optimal for maximum shoot proliferation showing 20 shoots per explant with 9.5 shoot length (Table 4). Shoot multiplication was observed when cytokinin (Kn and BAP) were added in the shoot initiation media (cyanobacterial media and MS). Effect of combination of different plant growth regulators on root formation on MS media was also observed (Table 5).

Table 4- Effect of different ratio of cyanobacterial media (CM) and MS on *in- vitro* shoot proliferation from nodal segment of *Stevia rebaudiana Bertoni*

MS + CM (Conc.)	**% of Shoot Number Formation**	**% of Shoot length formation**	**% of Survi -val rate**
0.000 (Control)	----------	*0.9* ± 0.9	0
MS and CM (10ml + 90 ml)	15 ± 2.25	8.5 ± 2.5	98
MS and CM (20ml + 80 ml)	12 ± 1.75	4.8 ± 3.1	77
MS and CM (30ml + 70 ml)	21 ± 2.79	9.5 ± 2.12	100
MS and CM (40ml + 60 ml)	17 ± 2.90	8.8 ± 2.21	99
MS and CM (50ml + 50 ml)	14 ± 2.90	8.0 ± 2.01	96
MS and CM (60ml + 40 ml)	09 ± 1.54	3.2 ± 1.50	72
MS and CM (70ml + 300 ml)	12 ± 1.88	4.0 ± 1.72	82
MS and CM (80ml + 20 ml)	8 ± 1.29	2.9 ± 1.70	52
MS and CM (90ml + 10 ml)	13 ± 2.0	6.2 ± 1.85	79

Table 5- Effect of IAA, IBA and NAA in MS medium for root formation

Growth Regulators (mg/l)	**Root Formation (%)**			**No. of total roots/culture mean ± S.E.**			**Average length of root (cm) Mean ± S.E**		
IAA+IBA+NAA	IAA	IBA	NAA	IAA	ABA	NAA	IAA	IBA	NAA
0.5	35	28	31	2.8±0.5	2.2±0.5	2.5±0.5	3.1±0.5	2.5±0.4	2.5±0.5
1.0	48	45	48	3.5±0.4	2.4±0.8	3.3±0.4	4.6±0.3	3.1±0.5	3.4±0.8
1.5	65	51	38	7.6±0.5	4.6±0.5	5.0±0.4	5.5±0.4	4.8±0.4	4.6±0.3
2.0	41	22	26	4.5±0.5	3.9±0.3	3.5±0.5	4.1±0.3	3.2±0.3	3.2±0.4

CONCLUSION

In the present course of investigation, we have optimized *in-vitro* regeneration of Stevia rebaudiana Bertoni by using Cyanobacterial media and MS media. The investigation was carried out with the aim to provide a protocol for iv-vitro callusing in Stevia plant. By using the method described above, hundreds of clonal copies of plant can be produced from one explant by performing continuous subculturing of shoot propagules.

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