researcharticle | International Journal of Plant Sciences, (July to December, 2009) Vol. 4 Issue 2 : 559-563

In vitro regeneration through callus culture of medicinally important plant *Stevia rebaudiana* (Bert.) Bertoni

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Accepted : June, 2009

SUMMARY

Stevia is versatile herb with incredible sweetness that can be safely used in herbal medicines; tonics for diabetic patients and also in the daily usage products. *Stevia rebaudiana* (Bert.) Bertoni is a small perennial herb of the family Asteraceae and it's a single sweetener has antidiabetic properties. Its sweetening power ranges from 100-300 times more than sucrose. The sweet compounds found in Stevia leaves are diterpene glycosides (Steviol glycosides), which are synthesized in the initial stages. Tissue Culture Technique has been adopted as an alternative method for rapid and large scale multiplication of *Stevia rebaudiana* for attaining market demand. Multiple shoots were obtained from callus obtained through leaves of Stevia when cultured on Murashige and Skoog's (1962) (MS) medium supplemented with 1.0-4.0 mg/l BAP and 0.5-1.0 mg/l NAA. These regenerated shoots when cultured on MS medium Supplemented with 2.0 mg/l BAP gave maximum shoot multiplication rate (10-12 folds). Rooting of these *in vitro* regenerated shoots were obtained on MS medium supplemented with 0.5-2.0 mg/l IBA.

Key words : MS medium, Tissue culture, Shoot multiplication, Rooting

The herb *Stevia rebaudiana* (Bert.) Bertoni (family: Asteraceae), commonly known as sweet leaf, sweet herb or honey herb is a native of highlands of Paraguay, where it has been used by aboriginals as a sweetener for centuries. It has been recently introduced in India and its name has been coined as "Madhu-patra". Due to its huge applications in food, drug and pharmaceutical industries, it is now commercial cultivated in many countries of the world *viz.*, Brazil, Paraguay, Uruguay, Central America, Israel, Thailand, China and Japan. Stevia is a natural alternative to artificial sweeteners (Chalapathi *et al.*, 1999). Dry Stevia leaf is up to 100-300 times sweeter than sucrose due to the presence of two compound stevioside and rebaudioside A (Kinghorn and Soejarto, 1984; Liu and Liu, 1995; Striedner *et al.*, 1991; Sekihasti *et al*., 2002; Brandle *et al*., 1998; Nepovim and Vanek, 1998; Debnath *et al.*, 2008; Brandle and Rosa, 1992; Brandle, 1999). Stevia has been used for cavities, depression, diabetes, fatigue, heart support, hypertension, hyperglycemic, infections, obesity, sweet cravings, tonic, urinary insufficiencies and as a sweetener. In the US, Stevia is mostly employed as a sugar substitute. It is recommended for diabetes and has been used by humans with no side effects (Megeji *et al.*, 2005). There is growing international market for Stevia. There are offers to buy container loads of Stevia leaves at a price of two-five dollars per kilo.

Stevia leaves can be used because of its anti-fungal and anti-bacterial property. Mild Stevia leaf tea offers excellent relief for an upset stomach. A wet Stevia leaf bag provides a cooling effect on eyes (similar to using cucumber). The leaves effectively tighten the skin and are good for wrinkles. Stevia has proved to give exceptional benefits when used regularly in skin care. It also has a healing effect on blemishes, wounds, cuts and scratches. Stevia is helpful in weight and blood pressure management. It has also been reported that stevia lowers incident of colds and flu. Medicinal uses of stevia include regulation of blood sugar, preventing hypertension, treatment of skin disorders and prevention of tooth decay (Planas and Kuc, 1968; Chen *et al*., 2005).

Vegetative propagation of this plant through stem cuttings is also limited by the low rate of multiplication and the production of plants from seeds that otherwise have very low chances of germinating and growing. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in sweetening levels and composition (Sakaguchi and Kan, 1982). Tissue Culture Technique has been adopted as an alternative method for rapid and large scale multiplication of *Stevia rebaudiana* for attaining market demand (Nakamura and Tamura, 1985).

Tamura *et al*. (1984) established clonal propagation of *Stevia rebaudiana* by culturing stem tips with an increasing demand of stevioside in food industry. Ferreira

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and Handro (1988) described a method for production maintenance and plant regeneration from cell suspension cultures of *S. rebaudiana*. Bespalhok *et al*. (1993) establish somatic embryogenesis from leaf explant of *S. rebaudiana*. Bespalhok and Hattori (1997) obtain embrogenic callus formation and histological studies from *S. rebaudiana*.

MATERIALS AND METHODS

Plant material/explant source:

Fully expanded *in vitro* leaves taken from *in vitro* multiplied shoots were used as an explant material for organogenesis in *Stevia rebaudiana*. For this, nodal segments measuring 2-4 cm in length and 0.4-0.8 cm in diameter were selected from *Stevia rebaudiana*. Nodal segments were cleaned with 70% alcohol; these were sterilized with 0.1% $HgCl_2$ for 5 min and washed thoroughly with sterilized distilled water. The explants were then cultured on MS medium supplemented with BAP. Shoots proliferated were excised and multiplied on MS medium supplemented with BAP. The *in vitro* leaves excised from *in vitro* shoots were used for initiation of callus.

Medium and culture conditions:

MS medium containing 3% sucrose was used in all the experiments. The pH of medium was adjusted to 5.8 before adding 0.7% agar-agar (w/v) and sterilized by autoclaving at 15 Ibs (1.8 kg/cm^2) pressure at 121^{0}C for 15 minutes. Cultures were incubated at 25 ± 2^{o}C for 16 hours in light (illuminated by 40 watt fluorescent tubes, 1200 lux) and for 8 hours in dark cycle irradiance by cool fluorescent tubes. The cultures were regularly transferred on fresh medium to check the browning of cultures.

Induction and development of callus:

Various combinations of phytohormones i.e BAP, Kn, NAA, IAA and 2,4-D were tested for their effect on callus formation on leaf explant. MS medium lacking growth regulators served as control. *In vitro* leaves were inoculated on medium and data on callus was recorded as the per cent of the leaf explant forming callus.

Multiplication and differentiation of callus (shoot organogenesis):

The pale green compact callus was transferred on MS medium supplemented with BAP (1.0-4.0 mg/l) and NAA (0.5-1.0 mg/l). Data on organogenic response percent, multiplication rate of callus, number of shoots induced per callus clump and length of differentiated shoots were recorded after 4-5 weeks of incubation. Organogenic callus were transferred and cultured on maintenance medium.

Organogenic response % (callus) = Number of organogenic cultures x 100/Total cultures inoculated

Multiplication rate = Final fresh weight of callus/ Initial weight of callus inoculated

In vitro shoot multiplication and in vitro rooting:

Shoots of size upto 1-2 cm were multiplied on MS medium supplemented with BAP and multiplication rate was calculated. Experiments on *in vitro* rooting of *in vitro* shoots were attempted with 2-3 cm long shoots produced during shoot multiplication. Shoots were cultured on MS medium supplemented with auxins. Single shoot was cultured for initiation of roots and observations were recorded after 5 weeks interval.

Statistical analysis:

All experiments were repeated thrice. Each treatment consisted of 12 replicates. The data representing means of three experiments were analyzed with the help of statistical packages *viz.,* Excel ver 2.0 for data of a completely randomized design. The data recorded for various parameters during the study were subjected to one and two way analysis of variance (ANOVA). The significance of the data were ascertained by F-test and the critical difference (C.D.) values at 5% computed, for comparing differences in means of various treatments.

RESULTS AND DISCUSSION

Nodal segments surface sterilized for 5 minutes with 0.1% mercuric chloride ($HgCl_2$) gave 90-95% bud break response (Table 1). Excised *in vitro* leaves were cultured

Table 1 : Effect of cytokinin (BAP) in MS medium on axillary bud induction using nodal segments of *Stevia rebaudiana* (Data was recorded after 3 weeks)

BAP (mg/l)	Response %	Mean shoot number	Mean shoot length (cm)
Control	8.33 ± 0.012	0.30 ± 0.02	0.23 ± 0.17
0.5	22.50 ± 0.006	0.80 ± 0.30	0.43 ± 0.16
1.0	55.83 ± 0.006	1.80 ± 0.30	0.85 ± 0.03
2.0	85.00 ± 0.029	4.30 ± 0.50	0.88 ± 0.06
3.0	55.00 ± 0.029	2.70 ± 0.30	0.68 ± 0.03
4.0	44.66 ± 0.006	1.80 ± 0.30	0.76 ± 0.05
5.0	35.00 ± 0.029	1.50 ± 0.02	0.78 ± 0.07
Significance	***	***	***
C.D. (P=0.05)	0.05	0.90	0.29

on MS medium supplemented with different combinations of BAP (1.0-4.0 mg/l) and NAA (0.5-1.0 mg/l) for callus induction and its further growth. Callus was initiated on MS medium supplemented with BAP and NAA. MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA gave maximum 50% callus formation (Fig.1). A good amount of pale green compact callus was obtained on this medium. 50 mg of callus was inoculated for callus multiplication and regeneration of more number of shoots. Best callus multiplication rate of 3-4 folds was obtained on MS + 3.0 mg/l BAP + 0.5 mg/l NAA with maximum number of 4-5 shoots produced per callus clump in 75%

Table 2 : Effect of hormonal interactions on callus induction in MS medium using *in vitro* leaves in *Stevia rebaudiana* (Data recorded after 5 weeks)

Hormonal concentration (mg/l)	Response %	Degree of callusing	Remarks
1.0 BAP + 0.5 NAA	10.00 ± 0.03	+	White compact callus
2.0 BAP + 0.5 NAA	25.33 ± 0.10	++	Yellow white compact callus
3.0 BAP + 0.5 NAA	50.50 ± 0.08	+++	Pale green compact callus
4.0 BAP + 0.5 NAA	42.23 ± 0.04	++	Pale green compact callus
1.0 BAP + 1.0 NAA	40.34 ± 0.12	++	Pale green compact callus
2.0 BAP + 1.0 NAA	35.34 ± 0.12	++	Pale green compact callus
3.0 BAP + 1.0 NAA	29.33 ± 0.09	++	Pale green compact callus
4.0 BAP + 1.0 NAA	20.34 ± 0.12	++	Green compact callus
Significance	***		
C.D. (P=0.05)	3.12		

+ Sign indicates Degree of callus formation + poor
++Moderate +++High

Table 3 : Effect of cytokinin (BAP) in MS medium on *in vitro* shoot multiplication of *Stevia rebaudiana* (Data recorded after 3 weeks)

BAP (mg/l)	Mean shoot number	Mean shoot length (cm)	Multiplication rate
Control	12.25 ± 0.84	0.73 ± 0.02	2.04 ± 0.14
0.5	40.42 ± 1.48	1.15 ± 0.01	6.37 ± 0.25
1.0	40.58 ± 0.98	0.92 ± 0.05	8.76 ± 0.16
2.0	62.67 ± 1.08	1.30 ± 0.12	10.54 ± 0.18
3.0	30.92 ± 0.81	1.01 ± 0.09	6.15 ± 0.14
4.0	26.75 ± 1.75	0.88 ± 0.10	5.56 ± 0.29
5.0	25.08 ± 1.52	0.79 ± 0.05	4.18 ± 0.25
Significance	***	***	***
C.D. (P=0.05)	3.38	0.21	0.58

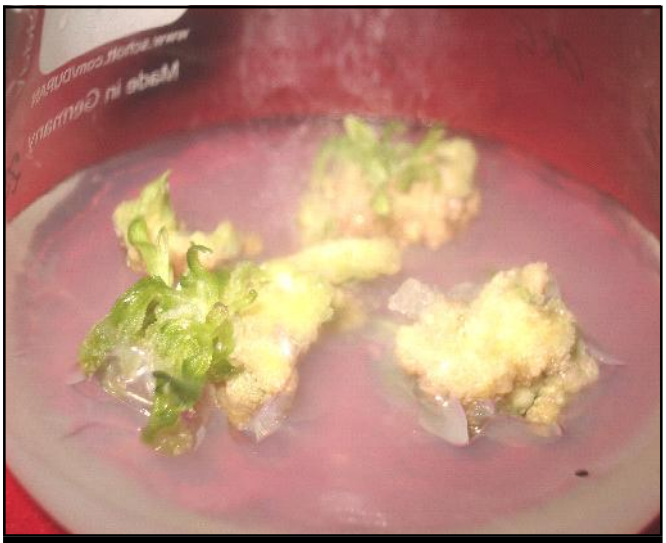

Fig. 1 : Induction of shoots from callus on MS medium supplemented with 3.0 mg/l BAP + 0.5 mg/l NAA

of the callus cultured (Table 2). Organogenic calli multiplied on other combinations of BAP and NAA in MS medium gave varied percentage of shoot organogenesis (20-60%). Therefore, all cultures were maintained on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA and regular subculturing was carried out every 5 weeks to multiply the callus and to maintain the organogenic potential of callus. Multiplication rate of 10-12 folds was obtained on MS medium supplemented with 2.0 mg/l BAP after repeated subculturing (Table 3, Fig. 2). Similar results on BAP supplemented media have been reported in a number of medicinal plants (Sudha and Seeni, 1996; Fracaro and Echeverrigaray, 1991; Purohit *et al.*, 1994; Wawrosch *et al.*, 1999).

Fig. 2 : *In vitro* shoot multiplication on MS medium supplemented with 2.0 mg/l BAP

Table 4 : Effect of auxin (IBA) in ½X MS medium on ½X MS medium on *in vitro* rooting of *in vitro* shoots in *Stevia rebaudiana* (Data recorded after 5 weeks)

IBA (mg/l)	Rooting %	Mean root number	Mean root length (cm)
0.0	12.50 ± 2.42	1.60 ± 0.24	0.82 ± 0.11
0.1	20.83 ± 2.38	9.00 ± 0.70	0.83 ± 0.12
0.5	62.50 ± 2.40	10.40 ± 1.03	1.05 ± 0.14
1.0	87.00 ± 2.42	10.60 ± 0.86	2.28 ± 0.14
1.5	79.17 ± 2.39	9.40 ± 1.03	2.20 ± 0.18
2.0	54.83 ± 2.40	8.00 ± 0.70	1.72 ± 0.16
Significance	***	***	***
C.D. (P=0.05)	7.28	3.04	0.41

*** indicates significant of value at P=0.1
± Values represent the Standard Error

In vitro roots were induced from *in vitro* raised shoots on ½X MS medium supplemented with auxins (NAA or IBA) in concentration from 0.1-2.0 mg/l. It was found that 1.0 mg/l IBA supplemented in half strength MS medium gave maximum rooting response of 87% with 10-12 roots produced per shoot after 5 weeks, on reducing the level of IBA to 0.1 mg/l in ½X MS medium rooting percentage decreased up to 12-15% (Table 4).

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