

Plant Regeneration from Leaf Derived Callus of *Stevia rebaudiana* Bertoni

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Abstract

Juvenile leaf explants of *Stevia rebaudiana* Bertoni produced maximum callus than the nodal explants cultured on MS containing 11.31 μM 2, 4-D and 2.22 μM BAP. Callus transferred to MS supplemented with 4.44 μM BA and 1.34 μM NAA showed better growth response and produced 14.0 ± 1.0 shoots with an average length of 5.6 ± 0.1 cm after 28 days. All plantlets produced profuse rooting within 25 days after transfer to half strength of MS basal medium supplemented with 2.46 μM IBA. Rooted plantlets were transferred for hardening, with 90% of plantlets successfully established in the field.

Introduction

Stevia rebaudiana Bertoni a perennial shrub belongs to Asteraceae. It is grown commercially in many parts of Brazil, Paraguay, Central America, Israel, Thailand, Korea and China (Soejarto et al. 1983). Its leaves and extracts are widely used as health food in tea bags, tablets, soft drinks, sea food, dessert items and confectionaries in recent years in Japan and South America (Fujita and Edahiro 1979, Nabors and Gelardi 1991). The leaves of *Stevia* are the source of the diterpene glycosides, stevioside and rebaudioside, which are reported to be 100 - 300 times sweeter than sucrose (Ishima and Katayama 1986, Tanaka 1982). Use of stevioside as a natural sweetener has been reported to be safe (Geuns 2004). The seeds of *Stevia* show a very low germination percentage (Goettemoeller and Ching 1999) and propagation by seed does not produce homogenous populations, resulting in great variability in important feature like sweetening level and composition (Tamura et al. 1984). Vegetative propagation is also limited by small number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan 1982). With the above mentioned difficulties, tissue culture has been the alternative for rapid mass propagation of *Stevia*.

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Earlier *in vitro* propagation of *Stevia* has been attempted through different explants: *viz.* leaf, shoot tips and axillary buds (Ferreira and Handro 1988, Ferreira and Handro 1987, Patil et al. 1996, Janarthanam and Kanimozhi 2005). However, callus regeneration from leaf explants has not been reported so far. Leaf tissues are reported to be more organogenic in various plants, such as *Coleus forskohlii*, *Cajanus cajan*, *Gloriosa superba*. The present study aims at developing a simple, rapid, economical, and high frequency regeneration protocol from leaf explants of *S. rebaudiana* so as to give rise to true-to-type clones for potential application in large-scale propagation.

Materials and Methods

Healthy *Stevia rebaudiana* Bertoni plants were obtained from RICHSAAI, Solar nursery Chennai and were raised in pots containing soil and farm yard manure (1 : 1) under greenhouse condition at P.G. & Research Department of Botany, Pachaiyappa's College, Chennai-600 030, India. Leaf explant was collected from potted plants and processed for aseptic culture. Explants were surface sterilized by cleaning thoroughly under running tap water for ten min, washed with a solution of Tween 20 (two drops in 100 ml of water) for one min, and again washed with sterile distilled water. The cleaned explants were finally treated with HgCl₂ (0.1%) for three - four min under aseptic conditions and washed five times with sterile distilled water to remove traces of HgCl₂. After surface sterilization, the explants were cut into small pieces and inoculated in MS supplemented with 2, 4-D (0.45, 2.26, 4.52, 11.31 and 22.62 µM); NAA (1.34, 2.69, 5.37, 13.43 and 26.85 µM) and BAP (1.11, 2.22, 4.44, 8.88 13.32 and 22.20 µM) in different concentrations and combinations for the production of callus. The primary callus obtained from the leaf explant was subcultured in the same medium. For shoot multiplication, 0.5 g of 24 to 28 - days old callus was transferred to MS supplemented with different concentrations and combinations of BAP (0.56 - 22.20 µM) and NAA (0.54 - 5.36 µM). After 35 days, the fully developed shootlets were transferred to half strength of MS basal medium supplemented with IBA (0.49 - 2.46 µM) for root development. After a further 25 days, culture vessels containing the rooted plantlets were transferred to greenhouse under 60 - 70% humidity. The plantlets were fertigated with dilute MS basal media. After 15 days, the fully acclimatized plantlets were transferred to 6 cm pots containing red soil, vermiculite and farmyard manure (1 : 1 : 1).

For all the above studies, MS basal medium containing 3% w/v sucrose was used. All the plant growth regulators were filter-sterilized using a 0.2 µm filter (Minisart®, Sartorius, VivaScience AG, Hannover, Germany) before addition to culture media. The pH of the medium was adjusted to 5.6 ± 0.2, gelled with 0.9 % agar (Hi media, India), and autoclaved at 121°C at 15 psi for 15 min. Cultures

were maintained at $25 \pm 1^\circ\text{C}$ under 16h light and 8h dark cycle. Lighting was provided using white cool fluorescent tubes of $40 \mu\text{mol}/\text{m}^2/\text{s}$ light intensity. Each experiment was repeated three times and each treatment had six replicates. The data were subjected to ANOVA and means were performed by DMRT using SPSS (SPSS ver. 16.0).

Results and Discussion

Leaf and nodes explants of *Stevia rebaudiana* were cultured on MS supplemented with 2, 4-D or NAA in combination with $2.22 \mu\text{M}$ BAP. Both 2, 4-D and NAA were effective at inducing callus formation when used in combination with BAP. While 2, 4-D generated higher frequencies of callus formation than NAA, the percentage response was not significantly different between BAP and NAA. Callus response varied between nodal and leaf explants with 60 and 80%, respectively (Table 1, Fig. 1A). MS supplemented with BAP and 2, 4-D was more effective for inducing callus from leaf explants than supplementation with NAA and BAP. MS supplemented with $11.31 \mu\text{M}$ of 2, 4-D and $2.22 \mu\text{M}$ of BAP yielded two times more callus than the other media. Both friable and green compact calluses were observed where the callus was white and friable initially and later turned into compact nodular callus after prolonged incubation. Early callus formation from internode explants of *S. rebaudiana* was reported using MS containing $13.56 \mu\text{M}$ 2, 4-D which gave maximum callus production (Uddin et al. 2006), whereas in the present study leaf explants produced maximum callus. The formation of early callus from leaf explant of *Stevia rebaudiana* in MS containing 2, 4-D and BAP supported the present finding (Ferreira and Handro 1988). The morphogenic callus generated from leaf segments after 24 - 28 days was transferred to MS supplemented with different concentrations and combinations of BAP ($2.22 - 22.20 \mu\text{M}$) and NAA ($2.69 - 26.85 \mu\text{M}$). Callus demonstrated differential responses according to the hormonal concentration (Table 2), with shoot initiation observed three weeks after subculture. BAP has been considered to be most effective for the induction of shoot in plant tissue culture (Asamenew and Narayanaswamy 2000, Baskaran and Jayabalan 2005, Russell 1979). In the present study, BAP and NAA were not effective in inducing shoots when applied alone. Indeed, shoots were formed only when the medium was supplemented with BAP at $4.44 \mu\text{M}$ in addition to NAA. Concentrations of this BAP above and below this level were ineffective for inducing *de novo* shoot regeneration (Table 2). When BAP was present at $4.44 \mu\text{M}$, addition of all levels of NAA tested was effective for stimulating regeneration of shoots. However, $1.34 \mu\text{M}$ of NAA was most effective for inducing 80% of the callus to form shoots and induced greatest number of shoots (14.0 ± 1.0) per explant. Addition of higher amounts of NAA was supraoptimal causing a reduction in both

percentage of shoot regeneration and their number per explant. (Table 2, Fig. 1B,C). Addition of 4.44 μM BA and 2.68 μM NAA the culture produced green

Table 1. Influence of various concentrations and combinations of 2, 4-D, NAA, and BAP on callus induction from node and leaf explants of *S. rebaudiana*.

Growth regulators (μM)			% explants forming callus from explant type	
2,4 D	NAA	BA	Node	Leaf
0.0	0.0	0.0	Nil	Nil
2.26	0.0	2.22	40.0 \pm 0.0 ^a	46.7 \pm 2.8 ^a
4.52	0.0	2.22	61.6 \pm 5.7 ^c	50.0 \pm 5.0 ^{ab}
11.31	0.0	2.22	45.0 \pm 5.0 ^{ab}	80.0 \pm 5.0 ^c
22.62	0.0	2.22	40.0 \pm 0.0 ^a	63.3 \pm 7.6 ^b
0.0	2.69	2.22	46.7 \pm 2.8 ^{ab}	55.0 \pm 5.0 ^{ab}
0.0	5.37	2.22	46.7 \pm 2.8 ^{ab}	60.0 \pm 5.0 ^{ab}
0.0	13.43	2.22	48.3 \pm 2.8 ^b	55.0 \pm 5.0 ^{ab}
0.0	26.85	2.22	55.0 \pm 5.0 ^{bc}	50.0 \pm 5.0 ^{ab}

Explants were cultured on MS supplemented with 2, 4-D, NAA, or BAP. Data were recorded after 40 days of culture. Results represent mean \pm SD of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$ level.

callus and in other concentration of BA along with NAA swelled, turned brown and failed to grow later. Callus transferred to MS containing BA alone (1.11 - 8.88 μM) grew vigorously and developed into green compact structures (Table 2) which ultimately turned brown and failed to develop in to normal shoots. Callus transferred to NAA did not show any development or growth but the callus showed light green in colour. The regenerated shoots attained a height of about 5.6 cm in about 28 days. Though addition of NAA in lower concentrations (1.34 and 2.67 μM) along with BA (4.44 μM) increased the shoot length, whereas higher concentrations do not have positive influence on shoot length. An earlier report on the callus initiation from foliar discs and its regeneration of plant from cell suspension culture of *Stevia rebaudiana* also reported the production of shoot buds on (Linsmaier and Skoog 1965) macro-, micro, nutrients, vitamins (Nitsch 1969) medium supplemented with NAA and Kn. However, the results reported the development of plantlets through cell suspension culture and also using hormone intervention during two stages (Ferreira and Handro 1988). On the other hand, use of BAP and NAA for shoot development from callus observed in this study is in agreement with earlier reports on organogenesis in dicotyledon plants (Asamenew and Narayanaswamy 2000, Dhar and Joshi 2005, Hee et al.

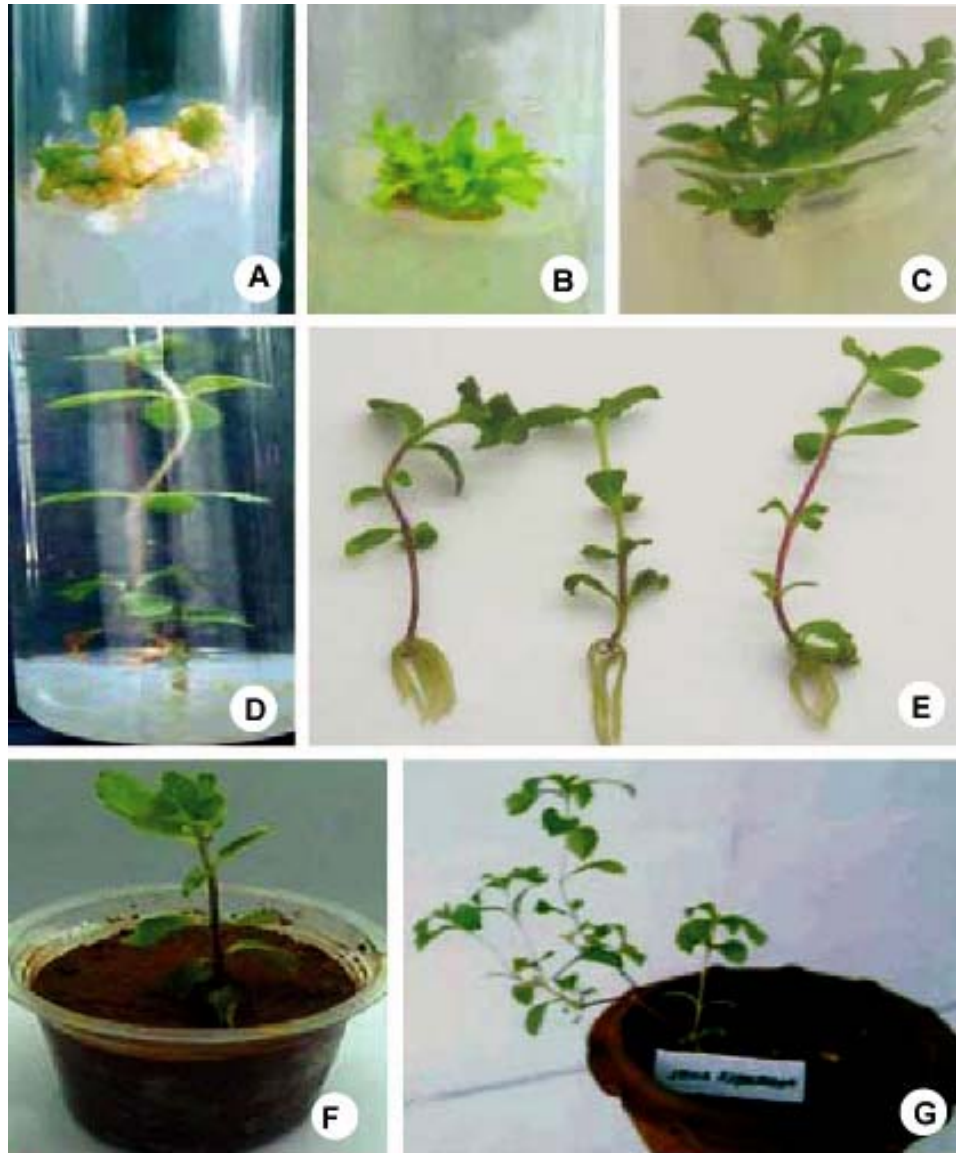


Fig. 1. Regeneration of multiple shoots from leaf derived callus of *Stevia rebaudiana*. (A) Callus initiation of leaf explant. (B & C) Proliferation of multiple shoots from leaf derived callus at day 28. (D) A rooted *Stevia rebaudiana* plant after 25 days. (E) Three well established rooted plants. (F) Well established plants successfully transplanted to the paper cups. (G) Plants transferred to pots.

2006, Kumari and Pardhasaradhi 1992, Rev and Mroginski 1996, Roche et al. 1986). Hence, the synergistic combination of auxin and cytokinin on organogenic differentiation has been well established in several systems (Koroch 2002, Pereira et al. 2000, Pretto and Santarém 2000, Thomas and Puthur 2004, Xie and Hong 2001).

Table 2. Morphogenic effect of BAP and NAA on leaf derived callus of *Stevia rebaudiana*.

BA (μM)	NAA (μM)	Response (%)	Av. No. of shoots	Av. shoot length (cm)	Morphogenic response
2.22	0.0	0.0	0.0	0.0	Green nodular structures
4.44	0.0	0.0	0.0	0.0	"
6.66	0.0	0.0	0.0	0.0	Calli turned pale brown
8.88	0.0	0.0	0.0	0.0	"
0.0	0.54	0.0	0.0	0.0	No growth
0.0	1.34	0.0	0.0	0.0	"
0.0	2.69	0.0	0.0	0.0	"
0.0	5.37	0.0	0.0	0.0	"
2.22	0.54	0.0	0.0	0.0	Tissue enlarged but failed to grow further
2.22	1.34	0.0	0.0	0.0	"
2.22	2.69	0.0	0.0	0.0	"
2.22	5.37	0.0	0.0	0.0	"
4.44	0.54	$50.0 \pm 5.0^{\text{ab}}$	$6.3 \pm 1.1^{\text{a}}$	$2.6 \pm 0.1^{\text{b}}$	Small green shoots
4.44	1.34	$80.0 \pm 5.0^{\text{c}}$	$14.0 \pm 1.0^{\text{b}}$	$5.6 \pm 0.1^{\text{d}}$	Well developed green shoots
4.44	2.69	$55.0 \pm 5.0^{\text{b}}$	$7.0 \pm 1.0^{\text{a}}$	$3.0 \pm 0.1^{\text{c}}$	Small green shoots
4.44	5.37	$40.0 \pm 5.0^{\text{a}}$	$5.3 \pm 1.1^{\text{a}}$	$1.8 \pm 0.1^{\text{a}}$	"
6.66	0.54	0.0	0.0	0.0	Calli turned brown
6.66	1.34	0.0	0.0	0.0	"
6.66	2.69	0.0	0.0	0.0	"
6.66	5.37	0.0	0.0	0.0	"
8.88	0.54	0.0	0.0	0.0	"
8.88	1.34	0.0	0.0	0.0	"
8.88	2.69	0.0	0.0	0.0	Growth inhibition
8.88	5.37	0.0	0.0	0.0	"

Data were recorded after 28 days of culture. Results represent mean \pm SD of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$ level.

Individual shoots from a multiple shoot mass were separated after 28 days transferred to half strength of MS supplemented with $2.46 \mu\text{M}$ IBA (Table 3). In all media, roots appeared after one to two weeks and after 25 days, roots were well developed (Fig. 1 D,E). The maximum rooting response achieved on medium supplemented only with $2.46 \mu\text{M}$ IBA was 100%, with an average of 7.3 ± 0.25 roots per shoot (Table 3). Though lower concentration of IBA produced

significantly higher rooting and root length, higher concentration of IBA did not produce results as expected. The observation on the reduction of MS salts strength to one half to enhance the rooting frequency is in agreement with earlier finding in *S. rebaudiana* (Sivaram and Mukundan 2003).

Table 3. Rooting response of *in vitro* derived *S. rebaudiana* shoots.

IBA (μM)	% response	Roots/shoot	Root length (cm)
0.49	30.0 \pm 5.0 ^a	4.2 \pm 0.20 ^a	3.2 \pm 0.2 ^a
0.98	41.7 \pm 7.6 ^a	4.3 \pm 0.17 ^a	3.3 \pm 0.3 ^{ab}
2.46	100 \pm 0.0 ^c	7.3 \pm 0.25 ^c	5.5 \pm 0.2 ^c
4.92	55.0 \pm 5.0 ^b	5.3 \pm 0.12 ^b	3.8 \pm 0.1 ^b
12.30	36.6 \pm 2.8 ^a	5.0 \pm 0.10 ^b	3.1 \pm 0.1 ^a

Data were recorded after 25 days. Results represent mean \pm Sd of six replicated experiments. Values denoted by different letters differ significantly at $p < 0.05$ level.

Cent per cent plantlet survival was seen after hardening of the regenerated *Stevia rebaudiana* in red soil, vermiculite, and farmyard manure (1 : 1 : 1) for three weeks. However, the rate decreased as some of the plants died over the next four to six weeks on transfer to soil. It was observed that gradual acclimatization of *in vitro* grown plants to the external environment is most essential for *Stevia*. Ninety per cent of the plants transferred to pots survived and resumed growth (Fig. 1F, G). There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics compared to each other and to the control plants of *Stevia rebaudiana*. In conclusion, the results reported here demonstrated the ability to regenerate whole plants of *Stevia rebaudiana* from leaf explants. Significant numbers of shootlets were produced in the present study through an intervening callus phase with all regenerates showing normal phenotypes. This is the first report describing callus-mediated regeneration of *Stevia rebaudiana* from leaf explants and describes a rapid and simple protocol to obtain large numbers of plantlets within a short period. The authors estimate that more than 50,000 plantlets could be produced within five subcultures from the leaf callus explants. Further work to study the genetic fidelity and phytochemical availability is underway.

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