Environment & Ecology 27 (1A) : 459—464, 2009
 ISSN 0970-0420

In vitro Propagation of *Stevia rebaudiana* Bertoni : An Important Medicinal Sweet Herb

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Abstract

A rapid propagation system was developed for medicinally important plant species *Stevia rebaudiana* Bertoni under *in vitro* conditions. Internodal explants from mature plant of *S. rebaudiana* were taken and cultured after surface sterilization. Calli was induced on Murashige and Skoog's medium supplemented with various concentration of auxins (2, 4-D, NAA, IAA) and cytokinins (BAP, Kn) individually and in combination. Calli was sub-cultured for indirect regeneration on MS medium fortified with BAP and 2,4-D. The *in vitro* raised shoots (>15—20 cm) were excised and transferred on half strength MS medium fortified with 1.0 mg/liter) IBA to produce roots. These rooted shoots were successfully acclimatized in pots containing sterilized soil : sand (1:1). After four weeks of implantation, these plantlets were finally transferred to field conditions. Seventy five percent of them survived.

Key words : *Stevia rebaudiana, In vitro* propagation, Multiple shoots, Internodal segment.

S. rebaudiana commonly known as sweet herb or honey leaf is an important medicinal plant belongs to family Asteraceae. It is a native to Paraguay, Brazil and northen section of South America where it grows near the ponds and streams in wild conditions (1). Various parts of this plant are of economic and medicinal value. About 200 species of Stevia are reported but the leaf of only *S. rebaudiana* is having sweetening properties. The leaves of *S. rebaudiana* contain glucosides i.e. stevioside, rebaudioside A and rebaudioside C (2). Steviosides are about 300 times sweeter than sucrose. The chemical, physical, pharmacological and toxiclogical characteristics of these compounds would suggest their use as natural acaloric supplement in human diet (3). The leaf extract is useful for treatment of diabetes. One acre of *S. rebaudiana* cultivation would produce sweetener equivalent to 36 acres of sugarcane crop. Conventionally the plant can be propagated through seeds. However, the viability of seeds is poor and highly variable. On the other hand, vegetative propagation through stem cutting is not efficient under various climatic conditions and erratic rooting behavior. The present investigation describe a method of raising plantlets from internodal segments under *in vitro* conditions which could be used for large scale multiplication of this important plant.

Methods

Internodal segments (1.0—1.5 cm) were taken from the mature plants growing in herbal garden, Department of Botany, Kurukshetra University, Kurukshetra. All the explants obtained from the mature plant were washed with teepol under running tap water, then surface sterilized by 0.1% (wt/vol) freshly prepared mercuric chloride solution for 3—5 minutes and subsequently given a dip in 70% ethanol. All the explants were washed with sterilized double distilled water to remove the traces of mercuric chloride. MS basal medium (4) containing 3% (wt/vol) sucrose and 0.8% (wt/vol) agar-agar was selected for the present investigations. The MS medium supplemented with various concentrations of auxins (0.5—2.0 mg/liter) and cytokinins (0.5—2.0 mg/liter) was used. The pH of the medium was adjusted to 5.8 before adding agar-agar and the medium was autoclaved at 121 C, 15 psi for 15 minutes. The surface sterilized explants were then inoculated on MS medium supplemented with 0.5—2.0 mg/liter of BAP, IAA, NAA, 2,4-D individually and in combination. The cultures were maintained under a continuous light (2000 lux) and temperature

Table 1. Response of internodal segment of *S. rebaudiana* on MS medium fertified with different concentrations of various growth regulators. + Poor callus, ++ Moderate callus, +++ Good callus, BAP-6-benzyl aminopurine; Kn-kinetin; IAA-indole-3-acetic acid; IBA-indole-3-butyric acid; NAA-α-naphthalene acetic acid.

Medium composition (mg/l)	Percent explant callusing	No. of days for callus induction	Color & texture of callus	Visual growth of callus
Control	–	–	–	–
BAP (0.5)	–	–	–	–
(1.0)	–	–	–	–
(1.5)	47.23	18.7	Creamish yellow, soft	+
(2.0)	49.91	19.1	Creamish yellow, soft	+
Kn (0.5)	–	–	–	–
(1.0)	42.56	18.5	Creamish yellow, soft	+
(1.5)	49.08	19.7	Creamish yellow, soft	+
(2.0)	50.24	20.2	Creamish yellow, soft	++
IAA (0.5)	–	–	–	–
(1.0)	67.21	16.4	Light green, soft	+
(1.5)	70.43	16.9	Light green, soft	++
(2.0)	74.74	16.2	Light green, soft	++
NAA (0.5)	90.21	16.1	Brownish, compact	+
(1.0)	96.11	15.2	Brownish, compact	+
(1.5)	94.32	15.6	Brownish, compact	+++
(2.0)	92.01	16.4	Brownish, compact	++
2,4-D (0.5)	86.34	15.3	Light green, fragile	+
(1.0)	90.11	15.0	Light green, fragile	++
(1.5)	94.21	14.0	Light green, fragile	++
(2.0)	100	13.9	Light green, fragile	+++
BAP (0.5) + 2,4-D (0.5)	90.2	15.3	Light green, fragile	+
BAP (0.5) + 2,4-D (1.0)	93.8	15.2	Light green, fragile	++
BAP (0.5) + 2,4-D (2.0)	100	15.2	Light green, fragile	++
BAP (1.0) + 2,4-D (0.5)	96.1	14.8	Light green, fragile	++
BAP (1.0) + 2,4-D (1.0)	98.2	14.2	Light green, fragile	++
BAP (1.0) + 2,4-D (2.0)	98.5	14.7	Light green, fragile	++
BAP (2.0) + 2,4-D (0.5)	93.0	14.0	Light green, fragile	+
BAP (2.0) + 2,4-D (1.0)	94.1	14.1	Light green, fragile	++
BAP (2.0) + 2,4-D (2.0)	100	14.0	Light green, fragile	+++

of 25 ± 2 C and 50—60% humidity. The calli were maintained by sub-culturing on fresh medium with same concentrations after a period of four weeks. The calli derived from various explants were transferred to regeneration medium having various growth regulators i.e. 2,4-D, IAA, NAA, BAP and Kn in different concentrations. The elongated shoots (15—20 cm) were aseptically transferred to rooting medium. Healthy rooted plantlets were taken out from the rooting medium and washed with sterile distilled water to remove the traces of agar-agar. The *in vitro* regenerated plantlets were transferred to sterilized sand : soil (1:1). The plantlets were irrigated with half strength MS salts solutions on alternate days. These potted plants were placed in culture room in continuous light and under high humidity. After about two weeks, the regenerants were exposed to natural conditions periodically for 3—4 hours for gradual hardening. After four weeks, the plantlets were transferred to the field conditions.

Results and Discussion

Internodal explant excised from the young branches of the mature plant for callus induction failed to induce callus on MS medium devoid of growth regulators (Table 1). Among the auxins, 2,4-D showed 100% callus induction after 13.9 days of inoculation (Table 1). The medium supplemented with IAA resulted in lesser percent of callus induction. Beside low response to callus induction, the time taken for callus induction was also more in IAA. IAA at a con-

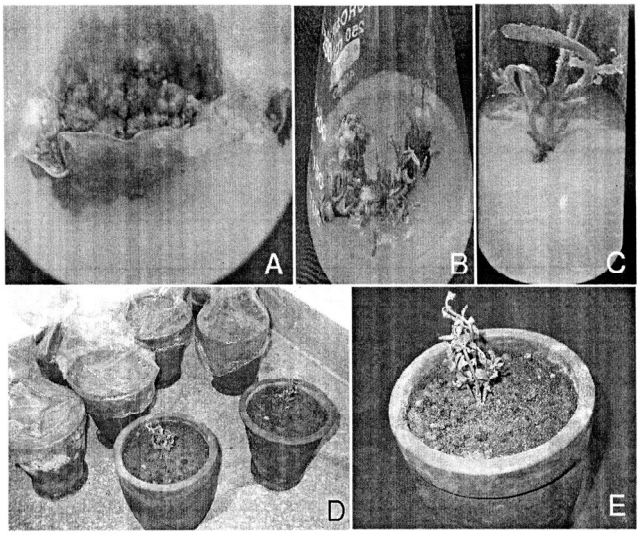

Figure 1. *In vitro* propagation of *Stevia rebaudiana* Bertoni. A. Callus formation from internodal explant in NS medium supplemented with BAP (2.0 mg/l) + 2,4-D (2.0 mg/l). B. Regeneration of plantlets from callus in medium supplemented with BAP (2.0 mg/l) + 2,4-D (1.0 mg/l). C. Rooting of plantlets in MS half strength medium fortified with IBA (1.0 mg/l). D. Hardening of plantlets. E. Plantlets successfully transfered to pot.

centration of 0.5 mg/liter was unable to induce callus in internodal explants upto 28 days of inoculation (Table 1).

The medium supplemented with cytokinins (BAP and Kn) failed to initiate high percent of callus even after 28 days of inoculated explant at higher concentration (2.0 mg/liter) of BAP and Kn. However, the percent callus induction was low in these media. The colors of calli in cytokinins were creamish yellow and soft in texture (Table 1). The calli initiated from the cut ends of the explants and later on, it covers the whole explant (Fig. 1 A). The calli were fragile and light green in colour in 2,4-D fortified media where as it was compact in texture and whitish green to light green in colour in other media with NAA and IAA.

To obtain a good amount of callus for further study the explants were inoculated on various combinations of auxins and cytokinins. A combination of BAP with 2,4-D showed 90.2—100 percent calli induction in internodal explant (Table 1, Fig. 1 A). A combination of BAP (2.0 mg/liter) + 2,4-D (2.0 mg/liter) showed maximum percent of plantlets regeneration from callus with maximum number of shoots formation (Table 2, Fig 1 B). The color of calli was light green and fragile in texture. Callus growth in terms of fresh and dry weight was recorded after 28 days on MS medium supplemented with various concentration of BAP in combination with 2,4-D.

Supplementation of auxins to the MS medium resulted in the formation of roots after 20.9—24.8 days of inoculation. The maximum number of roots was formed at 1.0 mg/liter of IBA in MS half strength medium (Fig. 1 C), which took 24.6 days for rooting and most of roots were healthy and long (Table 3). The MS media fortified with NAA and IAA (1.0 and 2.0 mg/liter) and IBA (2.0 mg/liter) were failed to develop roots. The regenerated plantlets were successfully acclimatized and transferred to pot (Fig. 1 D, E). Seventy five percent of the regenerants survived well.

To achieve micropropagation through callus, it requires the induction and formation of the callus as an initial step. It was found that MS medium without growth regulator failed to induce callus from tested explants which probably due to the insufficient level of endogenous growth regulators in explants to induced callus and it required an exogenous supply. However, when the medium was supplemented with auxins and cytokinins alone the results were not encouraging. The cytokinins showed a moderate percent callus induction from internodal segments

Table 2. Formation of shoots from callus of *S. rebaudiana* on MS medium fortified with different concentration of BAP+2,4-D. + Poor callus, ++ Moderate callus, +++ Good callus.

Medium composition (mg/l)	Visual growth of callus	Number of shoots	Length of shoots (cm)
BAP (0.5)+2,4-D (0.5)	+	13.0±0.012	11.6±0.013
BAP (0.5)+2,4-D (1.0)	++	12.8±0.011	12.8±0.016
BAP (0.5)+2,4-D (2.0)	++	15.1±0.014	14.9±0.022
BAP (1.0)+2,4-D (0.5)	++	14.6±0.015	12.4±0.011
BAP (1.0)+2,4-D (1.0)	++	16.1±0.014	14.9±0.014
BAP (1.0)+2,4-D (2.0)	++	17.8±0.016	15.7±0.011
BAP (2.0)+2,4-D (0.5)	+	13.1±0.012	10.9±0.012
BAP (2.0)+2,4-D (1.0)	++	18.7±0.017	14.9±0.014
BAP (2.0)+2,4-D (2.0)	+++	22.0±0.011	16.4±0.012

whereas auxins supported better callus formation. The initiation of the callus is perhaps because of exogenous supply of growth regulators that disturbed the established polarity and induced the callus formation. Callus initiated from the cut ends of the explants and finally whole surface of the explant was involved. Similar observations have been made in hypocotyls and cotyledon explants of *Sesbania grandiflora* (5) and *Leucaena leucocephala* (6). This may be due to the production of endogenous auxins from the damaged cell of cut surface which trigerred the cell division as found in *Ornithogallum* (7) where active division observed at cut ends of the tissue. But in the present studies the auxins released by the damaged cells were not sufficient to trigger the cell division and it required the exogenous supply of auxins. Among the auxins, 2,4-D was found to be better in terms of percent callus induction and number of days required for induction. The combination of auxins and cytokinins were used to induce calli from various explants. The fortification of auxins and cytokinins together in the medium in variable concentration produced better results in terms of both callus induction and growth. The medium supplemented with 2.0 mg/liter BAP + 2.0 mg/liter 2,4-D was proved better for induction and callus growth. However, the extent of callus growth differed with the concentration of BAP and 2,4-D and with the nature of the explants. It has been noticed that with the increase in the concentration of auxins and cytokinins, percent callus induction increased. This indicates that to achieve maximum callus induction, a defined auxin-cytokinin ratio

Table 3. Formation of roots in excised shoots regenerated *in vitro* on MS media fortified with different concentrations of IBA. –No response.

Medium composition (mg/l)	Avg no. of days required for root induction	Avg no. of roots shoot	Nature of roots
MS basal medium	–	–	–
MS half basal medium	–	–	–
MS + IBA (0.5)	24.4	3.9±0.015	Healthy, small
(1.0)	24.8	5.8±0.011	Healthy, long, stout
(1.5)	23.9	4.9±0.016	Healthy, long
(2.0)	–	–	–
MS (1/2)+IBA (0.5)	24.3	4.3±0.013	Healthy, long
(1.0)	24.6	5.0±0.022	Mostly Healthy, long
(1.5)	23.4	4.4±0.024	Long, stout
(2.0)	24.2	1.9±0.013	Small

was required. The role of auxins and cytokinins in callus induction was also advocated by Rao and De (8) in *Albizzia,* Yasmeen and Rao (9) in *Vigna radiate*, Latto et al. (10) in *Chlorophytum arundinacem,* Singh et al. (11) in *Chlorophytum borivilianum*. In many other herbs and tree species like *Thevetia peruviana* (12), 2,4-D alone was used for callus induction while in *Fraxinus angustifolia* (13) BAP was found to be superior than 2,4-D. Young explants exhibited better response as compared to mature explants as these younger tissues are physiologically and biochemically more active, and they have less rigid cell wall. Therefore, they showed better morphogenetic potential(14). This is also evident from the present study. The calli obtained in this experiment were sub-cultured after 28 days on the same medium with the purpose to observe the change in callus growth and to maintain the calli for further investigations. No change in the growth pattern was observed in any of calli produced by different explants even after 2—3 subcultures. The present study has revealed that the differentiation was found to be dependent on appropriate concentration of auxins and cytokinins in the medium. Similar observations were also made in *Sesbania grandiflora* (15), *Delonix regia* (16), *Melia azedarach* (17). Low concentration of auxins are required for root induction whereas an increase in auxins concentration suppressed the root induction because of its supra optimal concentration. Such obser-

vations have also been reported in *Eucalyptus tereticornis* (18), *Dalbergia latifolia* (19), *Prosopis cineraria* (20), *Melia azedarach* (17), *Cardispermum halicacabum* (21), *Rotula aquatica* (22), *Gloriosa superba* (23), *C. borivilianum* (24). The most crucial step in the micropropagation is the hardening and acclimatization as it is the process, which makes the plantlet capable of tolerating the external environmental conditions and survives. In present investigation, the rooted plantles after sufficient root length were acclimatized by gradual shifting to sterilized soil and sand mixture (3:1) under high humidity and subsequently to the field conditions.

Similar acclimatization procedure for establishment of *in vitro* regenerated plants have been successfully established by Das and Mitra (25), Raghva Swamy et al. (19), Nagalkshini and Pullaiah (26), Gupta et al. (27), Shahzad and Siddiqui (17), Rajore et al. (28). Hardening and acclimatization of plants were done because the plants raised under *in vitro* on synthetic carbohydrate supplemented medium under artificial light fail to acclimatize abruptly to rigor of natural environments. Therefore, a careful transfer of plants in the soil after hardening and acclimatization is required. In the present study, 75% of the plants survived well. Similar observations were also made by Nuehaimi and Toruan (29), Sivaram and Mukundan (30), Joshi and Dhar (31), Saini et al. (32), Lattoo et al. (10) and Vadodaria et al. (33).

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