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Optimization of the Protocols for Surface Sterilization, Regeneration and Acclimatization of *Stevia rebaudiana* Bertoni

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Abstract: The aim of the study was to investigate the factors needed for establishing an effective protocol for propagation of *Stevia rebaudiana.* Nodal explants from young branches were sterilized by using different concentration of bavistin and mercuric chloride ($HgCl_2$) and then cultured on BAP (0.5-2.0 mg/l) in combination with Kn or NAA (0.2-0.5 mg/l). The recorded data showed that the best results in sterilization of nodal explants were recorded with using Bavistin 0.2% + 8-HQC (200ppm) for 1 hour. High frequency bud break (80.5%) and multiple shoot formation (17.5) were induced from nodal segments explanted between June and August on MS medium supplemented with BAP (0.5 mg/l) + Kn (0.5 mg/l). Maximum shoot elongation (3.5) along with the highest number of leaves per shoot (8.7) was observed on the medium composed of MS salts and vitamins with GA_3 in the concentration of 0.5 mg/l. *In vitro* regenerated shoots were rooted on half strength MS medium supplemented with varying concentrations of auxins. Medium supplemented with 2.0 mg/l IBA showed highest rooting (69.76%) and early root initiation (7.1 days). Regenerated plantlets were successfully hardened and acclimatized in glass jar covered with poly propylene cap under net house conditions with 94.8 percent survival. The *in vitro* regenerated plants were uniform and identical in growth characteristics and morphology to the donor plants.

Abbreviations: BAP -6-Benzyl amino purine; 2,4-D - 2,4-Dichlorophenoxyacetic acid; MS - Murashige and Skoog (1962) basal medium; NAA - *a*-naphthalene acetic acid; IBA - Indole-3-butyric acid; IAA - Indole acetic acid; GA_3 - Gibberellic acid; 8-HQC-8-hydroxy quinnoline citrate

Key words: Multiple shoots · Nodal segments · *Stevia rebaudiana* · Acclimatization · Sterilization

INTRODUCTION

Stevia rebaudiana (Asteraceae), commonly known as natural sweetener is one of the most valuable tropical perennial herb. It grows wild in Paraguay and Brazil. Nowadays has found its way into markets in other parts of the world also.

Stevioside and redaudioside, sweet crystalline diterpene glycosides are extracted from the leaves of this important herb [1]. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste, non caloric and chemical stability [2].

The plant is used to cure many diseases like obesity, hypertension, heartburn, hypoglycemia and to lower the uric acid levels [3].

The seeds of *S. rebaudiana* show poor germination percentage and genetic variability [4]. Vegetative propagation is too slow and having the possibilities of pathogen accumulation in the tissues [5, 6].

Improved technology for vegetative propagation of mature elite plants of *Stevia rebaudiana* is highly desirable, as it will fulfill dual purposes, namely preservation and mass cultivation. Due to the above-mentioned difficulties, tissue culture is the only alternative method for rapid propagation of *S. rebaudiana* plants.

Although attempts have been made by several workers [3, 7-11] using various explants for the micropropagation of this magical sweetener, but considerable efforts are still required to make it more practical.

The aim of the study was to investigate various other factors such as sterilization treatments, explanting season, pre-acclimatization stage etc. influencing the different stages of micropropagation of *S. rebaudiana* from nodal explants.

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MATERIALS AND METHODS

Experiment 1. Effect of Different Sterilization Treatments on Explants Survival During the Establishment Stage: *S. rebaudiana* plants were procured from The Energy and Resources Institute (TERI), New Delhi for study and were maintained in glasshouse of Department of Botany, Kurukshetra University, Kurukshetra.

The explants (nodal segments) were excised from the healthy plants. All the collected explants were washed with liquid detergent. Thereafter, the detergent was completely drained out from the explants by washings it vigorously under running tap water (30 min.) to remove the microbial load and dust particles. For surface sterilization, the explants were agitated (1h) in 0.2% Bavistin alone or in a solution of 0.2% Bavistin + 200 ppm 8-hydroxy quinnoline citrate (8-HQC) followed by rinsing with autoclaved doubled-distilled water. Explants were also surface sterilized using 0.05 and 0.10 % $HgCl_2$ alone at different time scale (3-7 min.) in laminar airflow. After receiving the disinfection treatments, the explants were rinsed three times with sterile distilled water.

The nodal segments (1.0- 1.5 cm), after trimming the ends, were finally inoculated on MS medium [12] with different concentrations and combinations of growth regulators containing 30 g/l sucrose and 8 g /l agar. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl and finally autoclaved at 1.05 kg/cm^2, 121°C for 20 min. Cultures were maintained at a temperature of 25±2ºC with a 16/8 h light/dark photoperiod under an illumination of 20 µmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool- white fluorescent light.

Experiment 2. Effects of Benzyladenine (BAP) During the Multiplication Stage: The nodal explant were excised and transferred on to semisolid MS medium supplemented with BAP (0.5-2.0 mg/l) in combination with Kn or NAA(0.2-0.5 mg/l) to study the effect of various PGRs on shoot regeneration.

Experiment 3. Effect of Explanting Season on Culture Establishment: Significant effect of season was noted on percent bud- induction in nodal explants on MS + 0.5 BAP + 0.5 Kn recorded after 4 weeks.

Experiment 4. Effects of Gibberellic Acid (GA_3) on Shoot Elongation of *In vitro* Regenerated Shoots: The regenerated shoots were then transferred to shoot elongation medium. Different concentrations of Gibberellic acid (GA_3) were used to study their effect on shoot elongation and leaf number.

Experiment 5. Effect of Various Auxins on *In vitro* Root Formation: The *in vitro* developed single/multiple shoots (2.5 - 3.0 cm long) were excised and implanted in culture tubes containing full and half strength MS medium fortified with IBA, IAA and NAA under aseptic conditions for *in vitro* rooting.

Experiment 6. Effect of Different Hardening Strategies and Growing Media During the Plantlet Acclimatization Stage: After development of sufficient roots, the plantlets were gradually pulled out from the medium and were subjected to hardening using two strategies, i.e. glass jars with polypropylene (PP) caps or plastic pots with polythene cover. Medium for both hardening methods was agro-peat supplemented with one-forth strength MS liquid medium (without organics) twice a week. Cultures were kept under the same controlled photoperiod and temperature condition as described earlier for nodal cultures. During 3rd week, the relative humidity inside the glass bottles was gradually reduced by loosening and finally removing the caps and the hardened plantlets were allowed to remain in the bottle for 3-4 days before being transferred to polybags containing a mixture of sand, soil and vermiculite (2:1:1) in a glasshouse during 6-8th week of hardening.

Statistical Analysis: Experiments were repeated thrice and data represents the mean of three experiments. Each treatment consisted of minimum twelve replicates. The percentage data was transformed using angular transformation (Arc Sinv %). Degree of variation was shown by Critical Difference (CD) at 5%.

RESULTS AND DISCUSSION

Sterilization Treatments: Plants growing in the external environments are invariably contaminated with microorganisms and dust generally confined to the outer surface of the plant. These external contaminants can easily be removed by washing in running water for 30-90 minutes. Dipping of explants into solutions (bavistin, streptomycin sulphate, teepol, 8-HQS, 8-HQC, $HgCl_2$) was found effective for the disinfection of explants obtained from field grown plants [13-15].

Table 1: Effect of different pre-sterilization treatments on surface sterilization and culture initiation on MS medium supplemented with BAP (0.5mg/l) + Kn (0.5mg/l)

Pre-treatment (min)		Survival (%) after 15 days	Contamination (%)	Bud Break (%)
0.05 (w/v) $HgCl_2$ for	3 min	3.5 (10.63)	82.8 (65.50)	2.0 (8.13)
	5 min	6.2 (14.30)	74.6 (59.74)	4.3 (11.83)
	7 min	15.8 (23.42)	60.8 (51.24)	12.7 (20.88)
0.10 (w/v) $HgCl_2$ for	3 min	9.1 (17.46)	59.8 (50.65)	4.7 (12.52)
	5 min	28.9 (32.52)	36.8 (37.35)	15.4 (23.03)
	7 min	34.3 (35.79)	42.5 (40.63)	31.5 (34.08)
C.D. at 5%		4.6	5.1	1.4
0.2% (w/v) Bavistin for	1 hr.	46.7 (43.11)	36.5 (37.17)	32.8 (34.94)
0.2% (w/v) Bavistin + 200 ppm 8-HQC for	1 hr.	65.8 (54.21)	30.0 (33.21)	40.0 (39.23)
C.D. at 5%		5.6	3.2	3.4

Figures in parentheses are arcsine transformed value

Table 2: Effect of growth regulators on culture initiation in *S. rebaudiana*

Growth regulators				
BAP	NAA	Culture initiation (%)	Days required for bud break	No. of shoots per explant
0.5	0.2	31.2(33.89)	8.9	2.1
1.0	0.2	34.2(37.52)	9.6	3.2
2.0	0.2	50.1(45.00)	8.4	3.6
0.5	0.5	40.8(39.70)	7.2	1.9
1.0	0.5	41.7(40.22)	6.1	3.2
2.0	0.5	54.8(47.75)	5.7	4.3
1.0	0.2	21.6(27.69)	9.4	3.1
2.0	0.2	35.8(36.75)	8.7	3.2
1.0	0.5	28.1(31.95)	10.1	3.1
2.0	0.5	34.1(35.67)	10.4	3.4
0.0	0.0	16.1(23.58)	14.8	1.3
C.D. at 5%		5.1	1.5	0.51

Figures in parentheses are arcsine transformed value

Here a total of 8 disinfection treatments using Bavistin (0.2%), 0.2% Bavistin + 200 ppm 8-HQC, $HgCl_2$ (0.05%) and $HgCl_2$ (1.0%) were tested in this study. After receiving the disinfection treatments, the explants were rinsed three times with sterile distilled water. The explants receiving the different disinfection treatments were cultured on a basal MS medium. The explant survival percentage was recorded after four weeks of culturing. Significant differences were observed among the treatments tried. The recorded data showed that the best results in sterilization of nodal explants were recorded with using Bavistin 0.2% + 8-HQC (200ppm) for 1 hour followed by 0.2% Bavistin alone for 1 hour. The treatments comprising $HgCl_2$ or Bavistin alone gave statically poor response (Table 1). The Bavistin is a common systemic fungicide, which effectively controls different latent fungal contaminations under field conditions. Similarly, 8-hydroxy quinnoline citrate has bactericidal action and was also effective, that is why in present study in combination they have shown good result. The result is also in conformity with Prasad [16] who had used these two chemicals in combination to control the microbial contamination in axillary bud explant in rose.

Furthermore, it was observed that *Stevia* was generally more susceptible to damage caused by the $HgCl_2$ as compared to Bavistin and 8-HQC disinfectants. The obtained result explained the sensitivity of plant tissues to different surface sterlizants. Arafa *et al.* [17] and Hussein [18] reported that surface sterilization with $HgCl_2$ followed by Chlorox resulted in the highest decontamination and survival percentage of *Dieffenbachia exotica* cv. Tropic-Snow and *Aglaonema spp.*, respectively.

Growth Regulators: Effect of different plant growth regulators supplemented to MS medium on culture establishment and time to bud break is shown in Table 2 & 3. Among the different combinations tried, 0.5 mg/l BAP + 0.5 mg/l Kn showed better response with highest (17.5)

Table 3: Effect of growth regulators on shoot multiplication in *S. rebaudiana*

Growth regulator (mg/l)			
BAP + Kinetin		No. of shoots per explant	Length of shoots (cm)
0.2	0.0	8.70	0.21
0.5	0.0	13.70	0.94
0.0	0.2	7.90	1.11
0.0	0.5	10.20	1.16
0.2	0.2	15.20	2.41
0.5	0.5	17.50	2.51
C.D. at 5%		2.3	0.02

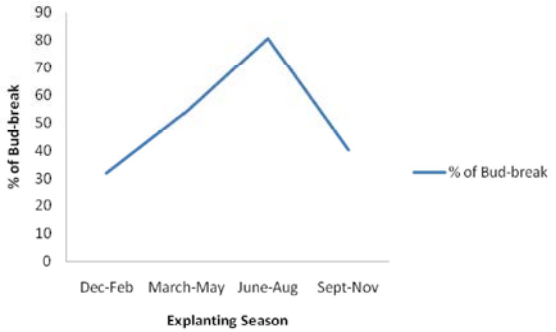


Fig. 1: Effect of Season on percent bud induction in nodal explants of *S. rebaudiana* on MS medium supplemented with BAP (0.5mg/l) + Kn (0.5mg/l)

number of shoots per explant (Table 3; Fig. 2b). Therefore, it was the best treatment for shoot regeneration. Supplementation of NAA in place of Kn also enhanced the number of shoots per explants culture establishment and minimized the time to bud break (Table 2; Fig. 2a). BAP has been the most commonly used cytokinin in the plant tissue culture media [20, 21-23]. The effectiveness of BAP treatments either singly or in combination with a low level of NAA/Kn than individual or combined kinetin treatments has also been noted in *S. rebaudiana* by earlier workers also [4, 24, 25].

Seasonal Variation: The explants surviving from the establishment stage were used as a source for the plant material used for the following *in vitro* experiments, which were conducted during the multiplication stage.

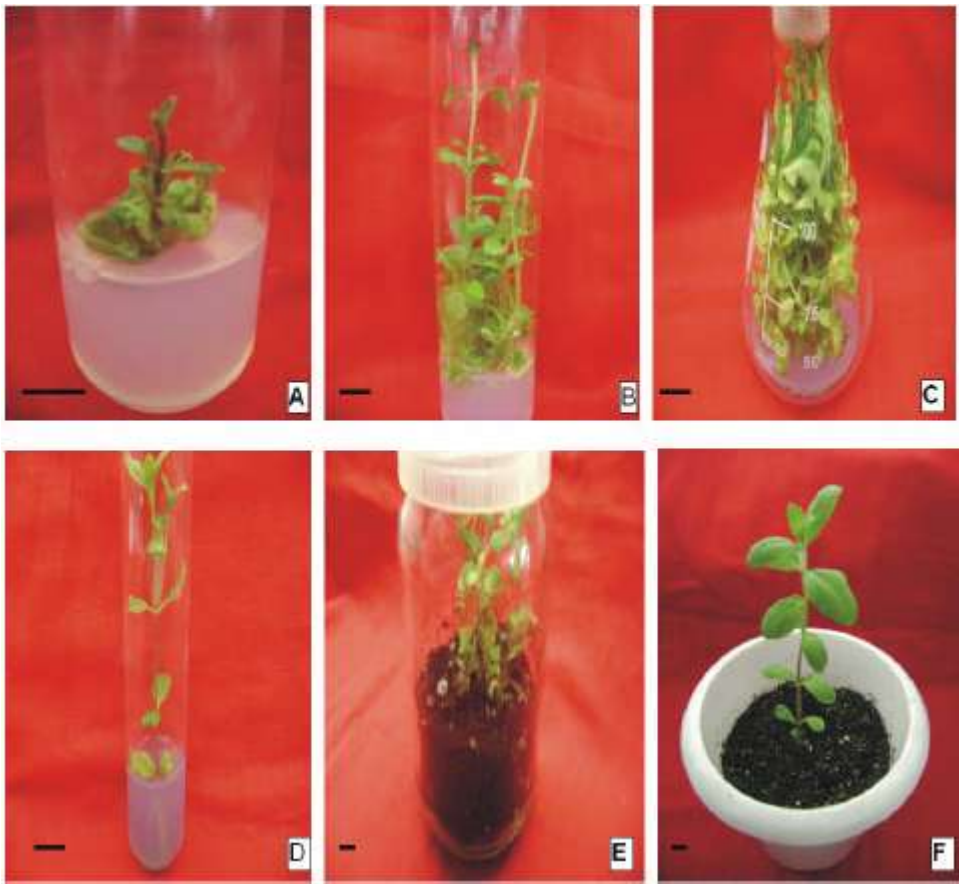
The shoot proliferation was greatly influenced by the month of the year during which the explants had been collected [19]. Best results (80.5 % bud induction) occurred with nodal explants excised between June-August, followed by 54.8 % bud-break in the months of March-May. Other explanting periods (December-February and September-November) were comparatively less suitable (Fig. 1), for obtaining optimal bud-induction, giving only 31.9 % and 40.22 % bud break, respectively.

Gibberellic Acid: The proliferated microshoots were thin and small. To make them strong, longer with more and bigger size of leaves and suitable for induction of rhizogenesis, they were transferred to elongation medium. Gibberellic acid (GA_3) is involved in several important biochemical and morphogenetic responses which include the promotion of elongation in axial organs, such as stems and flower pedicels, along with the stimulation of root growth [26]. The effect of GA_3 on shoot elongation of *in vitro* grown *Stevia* was noted in this study. Medium containing GA_3 in the concentration of 0.5 mg/l showed maximum elongation (3.5) and leaves number (8.7) within four weeks (Table 4; Fig. 2c).

Root Induction: The *in vitro* regenerated shoots were excised aseptically and implanted on full and half strength MS medium without or with growth regulators (0.5 - 2.0 mg/l NAA, IAA and IBA) for rhizogenesis (Table 6). Excised shoots failed to develop roots on both full and half strength MS medium without growth regulators. MS half strength medium supplemented with 2.0 mg/l IBA

Table 4: Effect of Gibberellic acid (GA_3) on shoot elongation in *S. rebaudiana*

Gibberellic acid (mg/l)	Avg. shoot length (cm)	No. of leaves per shoot
0.1	2.1	6.1
0.5	3.5	8.7
1.0	3.2	7.3
C.D. at 5%	0.57	1.21



*Bar= 1.0cm and it represents the length of the plant

Fig. 2: *In vitro* propagation of *Stevia rebaudiana* (A) Shoot induction on MS medium + BAP (2.0 mg/l) + NAA(0.5 mg/l); (B) Multiple shoot formation on MS + 0.5 mg/l BAP + 0.5 mg/l Kn; (C) Shoot proliferation on MS + 0.5 mg/l GA3; (D) *In vitro* rooting on ½ MS + IBA (2.0 mg/l) (E) A regenerated *Stevia* plant hardened on agro-peat in Glass jar (F) Initial stage of acclimatization

Table 5: Effect of auxins on rooting of *S. rebaudiana* micro-shoots

Treatment (mg/l)	Rooting (%)	Days to rooting	No. of roots/ shoot	Mean length of shoot (cm) after 15 days
IAA 0.5	65.21 (53.79)	9.2	3.8	3.4
1.0	74.4 (59.54)	8.5	4.3	3.9
2.0	76.8 (61.21)	8.4	4.7	3.8
IBA 0.5	81.0 (64.16)	8.6	4.2	4.1
1.0	84.5 (66.74)	8.0	4.3	4.8
2.0	87.8 (69.76)	7.1	5.6	4.6
NAA 0.5	60.1 (50.77)	8.0	7.4	2.3
1.0	74.7 (59.80)	8.4	7.6	2.1
2.0	81.4 (64.38)	9.1	8.9	2.7
C.D. at 5%	4.3	1.07	0.12	0.13

Table 6: Effect of different strategies on hardening of *S. rebaudiana* plantlets

Strategy	Plantlet survival (%)	Days to hardening
Plastic pot with polythene cover	82.71 (65.42)	24.8
Glass jar with PP cap	94.8 (76.82)	19.4
C.D. at 5%	5.7	3.1

proved best with 87.8 % rooting and early root initiation (7.1 days) (Fig. 2d). IBA has been observed to induce strong rooting response and has been extensively used to promote rooting in a wide range of plant species by Rani and Rana; Steephen *et al.*; Yadav and Singh [19, 27, 23].

Hardening: *In vitro* raised plantlets transferred to glass jars with PP caps were clearly better strategy as compared to plastic pots with polythene cover (Fig. 2e). As it recorded highest plantlet survival (94.8%), obtaining high success during acclimatization as compared to plastic pots with polythene cover. Plantlets of glass jar received less open space but appropriate relative humidity and could be transferred to glasshouse in a shorter duration (19.4 days) (Table 6; Fig. 2f). Similar strategy has earlier been reported by Singh *et al*. [28] and Alizadeh *et al*. [29]. The *in vitro* derived plantlets have a characteristic feature is poorly developed epicuticular waxy layer. This leads to uncontrolled foliar water loss when the plants are taken out from the culture vessels. However, when the plants are kept at high humidity conditions, they synthesize more epicuticular wax, which enhances the survival success during acclimatization [30]. Besides, the *in vitro* raised plants have poorly developed epicuticular waxes, more stomata per unit area and raised guard cells with wide opening, which result in more transpiration losses and less survival of plantlets [16].

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