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High-frequency induction of multiple shoots of *Stevia rebaudiana* bertoni through *in vitro* Micropropagation techniques

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ABSTRACT
An efficient, rapid and large scale propagation of the elite exotic, zero calorie, medicinal plant, *Stevia rebaudiana* Bert. through *in vitro* axillary bud multiplication was obtained on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of BAP, Kn and Ascorbic acid. However, the high frequency of morphogenetic response from axillary meristem was optimized on MS medium supplemented with 5.5µm BAP along with 50 mg/l Ascorbic acid. On average 90-91 shoots were induced per-explant of the initial material. Non-rooted shoots were sub-cultured to ½ MS medium containing IBA (0.2-1.0µm) for rooting. However, shoots readily formed roots (100%) when sub-cultured to ½ MS basal medium containing IBA 0.5 µm. The rooted micro-shoots were carefully transferred to soil rite ^{TC} mix: garden soil: compost (1:1:1 v/v/v) and subsequently shifted to the field with a survival rate of 70±7%.

Introduction

Stevia rebaudiana (Bert.) Bertoni is a perennial herb of Paraguay belonging to the family Asteraceae. Although there are more than 180 species of the Stevia plant, yet *Stevia rebaudiana* gives the sweetest essence due to the fact that its leaves accumulate zero-calorie ent-kaurene diterpene glycosides (stevioside and rebaudioside), a non-nutritive, high-potency sweetener, and substitute of sucrose which imparts 300 times sweetness than sucrose (Soejarto *et al*., 1982, 1983). Stevioside is one of the principal diterpene glycosides having a sweetness of 250-300 times that of sucrose. Stevioside is a white, crystalline and hygroscopic powder. Unlike many low calorie sweeteners, stevioside is stable at high temperature (100°C) and over a wide range of pH 3-9. It is also non- caloric and non fermentable and does not darken upon cooking. In addition to being a sweetener, its leaves are used in the treatment of diabetes, obesity, cavities, hypertension, fatigue, depression, sweet cravings and infections (Dyrskog *et al.*, 2005). Stevia has an ancient and venerable history in certain parts of the world. It is grown commercially in many parts of Brazil, Paraguay, Uruguay, Central America, Israel, Thailand, and China (Brandle and Rosa, 1992; Fors, 1995).

Reports on regeneration of Stevia are available through the culture of different explants such as from leaves (Ferreira and Handro, 1988; Sivaram and Mukundan, 2003), axillary shoots (Bespalhok *et al.*,1992), shoot tips (Tamura *et al*., 1984; Sivaram and Mukundan, 2003), callus culture (Patel and Shah, 2009), suspension culture (Ferreira and Handro, 1988) and anther culture (Flachsland *et al*., 1996). However, Pol *et al.*, (2007) characterized *Stevia rebaudiana* by comprehensive two-dimensional liquid chromatography. Whereas, Matsukubo and Takazoe, (2006) reported Stevia as sucrose substitutes and its role in caries prevention and Chang *et al*., (2005) studied increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats. Structural analysis of isosteviol and related compounds as DNA polymerase and DNA topoisomerase inhibitors has been done by Mizushina *et al.*, (2005).

Stevia plants can be propagated by seed or by cutting. Since, germination rate of seeds is poor and growth of seedling is very slow hence, it has become essential to develop an *in vitro* protocol for rapid and efficient multiplication of this imperative plant species. Therefore, to improve the quality and quantity, tissue culture techniques can be applied for its mass multiplication and regeneration. As far, no efficient and regenerative protocol has yet been standardized for large- scale multiplication. A new protocol has been developed and standardized in our lab for an efficient and rapid regeneration through nodal segments of *Stevia rebaudiana* Bert.

Material and methods

Explant selection: For inoculation, the explant was taken from actively growing shoots of 2-3 months old plants having axillary meristems (with one node each) ≈ 1-2cm were collected from field grown mature plants cultivated in Department of Botany, University of Rajasthan, Jaipur.

Surface sterilization: For surface sterilization, they were immersed in $HgCl_2$ (0.1%) for 2-3 min. Stem segments were surface-disinfested with Tween 20 and Bavistin (0.2 %) for ½ an hour before treating with sterilants, showed good results in controlling the contamination. To remove any trace of the sterilant, the materials were washed with sterile deionized water at least 4-5 times.

Shoot multiplication:

Explants consisting of shoot segments were individually placed basal end down in Jam bottles containing shoot induction medium (SIM) for micropropagation. SIM consisted of MS salts (Murashige and Skoog, 1962) supplemented with 30g•L^{-} sucrose, a carbohydrate source and 8.0 g•L^{-} Agar (Qualigens) and various levels of cytokinins (BAP and Kn) alone and in combination then BAP and Ascorbic acid in different ratios and concentrations as shown in table 1 & 2 . The pH of the medium was adjusted to 5.7-5.8 using 0.1N NaOH or 0.1N HCl, before sterilization and solidified by 8.0 g•L^{-} agar and autoclaved at 121^0 C for 15 min. All the cultures were placed in plant growth

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room at 26±2^{0}C with 16/8hr light/dark cycle. Light (2000 lux) was provided by cool white fluorescent tubes (Crompton India Ltd.). The newly formed shoots were sub-cultured after every 30 days on SIM for their further growth and elongation. This protocol eliminates use of separate shoot elongation medium as SIM provoke both shoot induction and elongation.

Rooting and acclimatization: For rooting, the regenerated shoots obtained from the explants were cultured on ½ MS basal medium containing IBA 0.5 µm. After 4 weeks of culture, plantlets with well developed roots were washed with tap water and transferred to earthen pots containing soil rite ^{TC} mix: garden soil: compost (1:1:1 v/v/v) and irrigated with tap water regularly. To maintain high humidity, the plantlets were covered with polythene bags and after 15 days, the polythene bags were removed and established plantlets were transferred to net house.

Results and Discussion

In the primary set of present investigation, effects of cytokinins (BAP and Kn) at different concentrations (3.0-7.0 µm) were evaluated on micropropagation of *Stevia rebaudiana* Bert. The results obtained during culture establishment and shoot multiplication suggests the involvement of cytokinins, particularly BAP stimulated the production of more shoots per-explant regardless of concentration than kinetin and the highest number of shoots per explant was observed on MS medium containing 5.5 µm BAP after 4 weeks of culture. The caulogenic response of BAP has also been shown in taxa of the asteraceae (Emmanuel *et al.*, 2000; Korach *et al.*, 2002; Dhaka and Kothari, 2005; Trejgell, 2009).

In the second set of experiment effect of various concentrations of Ascorbic acid in combination with low levels of BAP to the culture medium were tested. It has been found when 50 mg/l Ascorbic acid was supplemented with 5.5 µm BAP significantly increased the number of shoots and their proliferation. However, increased levels of Ascorbic acid (>50 µm) and BAP (>5.5 µm) were not accompanied by an increase in shoot multiplication and their elongation. The maximum number of shoots (90.6±0.5) per explant and the elongated plantlets (9.56±0.5) were obtained when Ascorbic acid (50 mg/l) was supplemented in culture medium containing BAP (5.5µm). (Figure 1a)

Latha and Usha (2003) also reported in Stevia that BAP was found to be more effective than other cytokines for shoot induction from nodal explants, shoot apex and leaf explants. However, in the present investigation, though BAP alone could record more initial establishment (73.2±1.9), but addition of Ascorbic Acid improved the initial establishment to (90.6±0.5) per explant. A similar observation was made with capsicum in shoot tip explant (Agarwal *et al.*, 1988) and in cotyledon (Gunay and Rao, 1978). Similarly, Christopher and Rajam (1994) and Agarwal *et al.*, (1989) reported the use of high level of cytokinins (BAP and Kn) for shoot proliferation in *Capsicum annuum*. Besides, enhanced number of shoots per explant has also been reported in *Tylophora indica* by using Ascorbic acid (Sharma and Chandel, 1992).

The induced multiple shoot buds elongate when continued to incubate on the same medium and resulted in elongated shoots (Figure 1b & c). Induction and development of roots at the base of the *in vitro* grown shoots is an essential step to establish tissue culture derived plantlets on the soil. For this purpose 3-4cm micro- cuttings were inoculated on ½ MS medium supplemented with 0.25-1.0µm of either IBA/IAA/NAA or without any auxin. In medium supplemented without auxin no rooting was found. While comparing three types of auxin IBA was found to be most effective than NAA and IAA. The highest degree (100%) of rooting was obtained when *in vitro* proliferated shoots were transferred on ½ MS medium supplemented with IBA (0.5µm) (Figure 1d) (Table 3). The highest number (12.3±1.3) and maximum length (7.40±0.00) of the roots was obtained on the same medium. In this experiment percentage of root induction and number of roots per-shoot were greatly influenced by the concentration and type of auxin. For rooting IBA was found to be comparatively more effective than IAA and NAA. The findings are in agreement with those observed in other plants such as *Guizotia* abyssinica (Sujatha, 1997), *Datura metel* (Muthukumar *et al*., 2000) and *Elaeocarpus robustus* (Rahman *et al*., 2001) and *Portulaca oleracea* L. (Sharma *et al.*, 2011).

Incontrast, in some members of asteraceae such as *Carlina acault* (Trajgella, 2009) and *Eclipta alba* (Baskaran and Jayabalan, 2005) auxins in combination with ½ MS inhibited rhizogenesis particularly root elongation.

The success of micropropagation is determined by the ability to transfer plantlets to potting medium and to acclimatize them successfully to free living conditions. Thus, a successful micropropagation protocol ends with the proper hardening of the *in vitro* derived plantlets. Plantlets with well developed roots were removed and transferred to pot containing Soilrite ^{TC}: garden Soil: Compost in 1:1:1 v/v/v after 3 weeks of root initiation (Figure 1 e) in a rectangular box and later to mist chamber for establishment. Overall survival rate was recorded 70±7%.The plant developed *in vitro* showed normal morphology with respect to growth habit, however the size of leaves were reduced which indicates decreased production of stevioside (medicinally important secondary metabolite) so to increase its production efforts are going on in our lab to increase the size of leaf by inducing polyploidy. In addition to the above work done we had successfully raised synthetic seeds from *in vitro* leaves.

In conclusion, the present work describes a method for successful plant regeneration and mass multiplication of *Stevia rebaudiana* from nodal segments.

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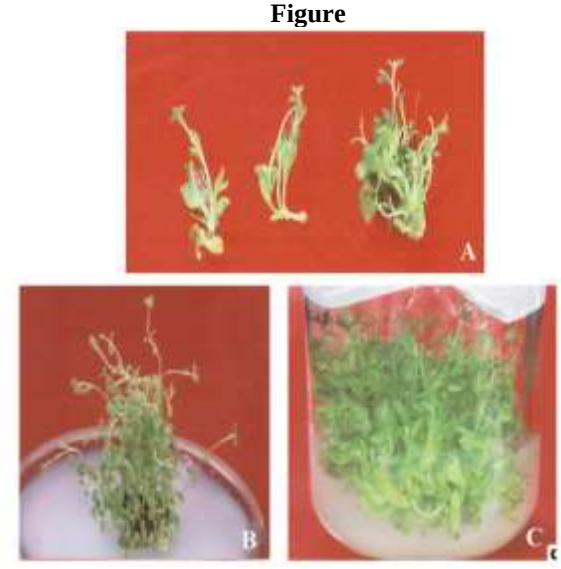
Figure



Stages in micropropagation of Stevia A: Different stages of shoot development after initiation in Stevia cultured on MS medium supplemented with BAP (5.5 µm) and ascorbic acid (50 mg/l) from nodal explant B&C: Further multiplication and elongation of shoots on same media D: Rooting of in vitro derived Stevia shoot achieved on half-strength MS medium containing IBA (0.5 µm) E: Stevia plants hardened in pot containing Soilrite TC, soil and compost.

Table 1. Effect of different concentrations of BAP and Kn either separately or combined on MS medium on direct regeneration of multiple shoots from nodal explants of *Stevia rebaudiana* Bert. after four weeks of incubation.

Cytokines (µm)			Frequency of response (%)	Mean No. of shoots/explant	Average length of shoot(cm.)
BAP	Kn	BAP+Kn			
0.00	0.00	0.00	0.00	0.00	0.00
4.0	0.00	0.00	74	53.5±0.1	5.5±0.7
5.5	0.00	0.00	100	73.2±1.9	7.5±0.2
7.0	0.00	0.00	75	54.0±0.5	4.8±0.4
	3.0	0.00	42	48.0±0.2	3.7±0.7
	4.5	0.00	63	55.5±0.1	4.5±0.3
	5.5	0.00	100	58.2±0.5	5.7±0.1
		5.5+3.0	50	30.1±0.1	2.7±0.5
		5.5+5.0	80	25.4±0.6	3.2±0.4
		5.5+6.5	53	19.1±0.3	3.5±0.2

Results are mean ± SD of 20 replicates.

All the values are significant at the 0.01 level (Student's t-test).

Table 2. Effect of different concentrations of Ascorbic acid on *in vitro* multiple shoot induction from nodal explants of *Stevia rebaudiana* Bert after four weeks of inoculation.

Treatments	% Regeneration	Mean No. of shoots/explant	Mean shoot length (cm.)
Control	0.00	0.00	0.00
Ascorbic Acid (10.0mg)+BAP(5.5µm)	100.0	69.3±0.7	3.50±0.4
Ascorbic Acid (30.0mg)+BAP(5.5µm)	100.0	75.6±2.4	5.00±0.3
Ascorbic Acid (50.0mg)+BAP(5.5µm)	100.0	90.6±0.5	9.56±0.5
Ascorbic Acid (70.0mg)+BAP(5.5µm)	100.0	52.6±1.3	6.63±0.1
Ascorbic Acid(90.0mg) +BAP(5.5µm)	100.0	40.3±0.9	3.64±0.6
Ascorbic Acid(110.0mg) +BAP(5.5µm)	100.0	20.0±1.2	2.16±0.8

Results are mean ± SD of 20 replicates.

All the values are significant at the 0.01 level (Student's t-test).

Table 3. Effect of different auxins in half strength MS medium on root induction of *in vitro* derived nodal segments of *Stevia rebaudiana* Bert.

Auxins (µm)			Frequency of response (%)	Mean No. of roots/shoot	Mean root length(cm.)
IAA	IBA	NAA			
0.25			13.2	2.65±0.5	2.53±0.5
0.50	0.20		15.5	3.51±0.4	3.98±0.6
0.75		0.20	18.2	3.71±0.7	3.46±0.8
1.00	0.20		22.2	5.80±0.2	3.80±0.7
	0.20		71.2	9.57±0.2	4.48±0.6
	0.50		97.0	12.3±1.3	7.40±0.0
	0.75		71.5	7.52±0.1	4.20±0.7
	1.00	0.20	54.5	3.40±0.4	3.82±0.6
		0.50	38.2	6.06±0.4	3.76±0.5
		0.75	28.2	2.65±0.2	1.52±0.8
	0.2	1.00	34.4	2.16±0.3	1.68±0.3

Results are mean ± SD of 20 replicates.

All the values are significant at the 0.01 level (Student's t-test).

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