High Frequency Clonal Multiplication of *Stevia rebaudiana* Bertoni, Sweetener of the Future

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ABSTRACT

The present study describes an efficient, rapid clonal propagation protocol for a natural sweetener herb, Stevia rebaudiana. Shoot tips and nodal segments were inoculated on Murashige and Skoog's medium [MS 1962] containing different cytokinin and auxin alone or in combinations. Shoot tips were proved to be better in comparison to nodal segments having higher rate of shoot induction and multiplication. Best shoot multiplication from both the explants was obtained on MS medium supplemented with 5.0 µM 6-benzyladenine (BA) and 1.0 µM á-naphthalene acetic acid (NAA). A maximum of 19.60 shoots per shoot tip and 16.00 shoots per nodal segment with an average shoot length of 4.70 and 4.28 cm were obtained. Subculturing of the regenerating tissue on the optimized treatment showed further enhancement in shoot proliferation without any decline in multiplication rate. The regenerated microshoots showed the most efficient rooting on half strength MS medium augmented with 2.5 µM indole-3-butyric acid (IBA). Plantlets went through a hardening phase prior to ex vitro transfer and established in earthen pots containing garden soil and green manure (1:1). The established plantlets were uniform and identical to mother plant with respect to growth characteristics and vegetative morphology.

Keywords: Micropropagation, Natural Sweetener, Stevia rebaudiana, Stevioside

INTRODUCTION

Stevia rebaudiana Bertoni is a perennial herb belongs the family Asteraceae. It is a natural sweetener plant known as "Sweet Weed", "Sweet Leaf", "Sweet Herb", "Honey Leaf" etc. It is native of certain regions of South America particularly in Paraguay and Brazil.

The leaves of *Stevia* are the rich sources of diterpene glycosides, such as steviolbioside, rubsoside, rebaudioside A, B, C, D, E and F, dulcoside and stevioside *[Starratt et al., 2002]*. Among them stevioside stands first and is non-caloric, thermo-stable, intense sweetener which imparts about 300 times higher sweetness than sucrose. Now a day's use of this sweetening compound has increased dramatically due to health concerns related to sucrose usage, such as dental caries, obesity and diabetes. This sweetening compound passes through the digestive process without chemically breakdown, making safe to control sugar level *[Strauss, 1995]*. Refined extract of *Stevia* leaves are officially approved as food additives in Brazil, Korea and Japan *[Choi et al., 2002; Mizutani and Tanaka, 2002]*. Japan was the first country in Asia to market stevioside as a sweetener in food and drug industries as a substitute for synthetic sweetener, aspartame. Since then cultivation of the plant has expanded to several countries in Asia including India, China, Malaysia, Singapore, South Korea, Taiwan and Thailand. By all the means, recently *S. rebaudiana* has attracted economic and scientific interest.

Generally, propagation of *S. rebaudiana* is done by stem cutting but main problem involved in the cultivation of this species is its heterozygous and self-incompatibility natures which lead to the lack of fertilization *[Miyazaki and Wanteabe, 1974]*. The seeds of *Stevia* show very less vigor and propagation and do not allow the production of homogenous population which leads to variability in sweetening level and composition *[Felippe and Lucas*

1971; Miyagawa et al., 1986]. Poor seed germination percentage is the limiting factor to large scale cultivation of this species. Vegetative propagation is also limited by the low number of individuals obtained from single plant. Therefore, to overcome all these obstacles, micropropagation or *in vitro* culture technique can play a vital role for mass propagation and the production of genetically identical plants of *S. rebaudiana*. As far as the literature is concerned, few reports of micopropagtion of *S. rebaudiana* have been cited *[Sivaram and Mukundan 2003, Ahmed et al., 2007; Rafiq et al., 2007; Anbazhagan et al., 2010]* but none of them are proved to be satisfactory in term of industrial requirements as raw material. In this way, the present study was aimed at the establishment of an efficient protocol for high frequency *in vitro* mass propagation of *S. rebaudiana*.

MATERIALS AND METHODS

SOURCE OF EXPLANT AND SURFACE STERILIZATION

The twigs (about 5.0-6.0 cm) of pot grown *S. rebaudiana* plant were collected from the Department of Botany, A.M.U., Aligarh. The twigs with shoot tip and 3-4 nodes were washed in running tap water for 30 min to remove the superficial dust particles. They were then immersed in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Ltd.), a broad spectrum fungicide followed by 5% (v/v) Labolene (Qualigens, India) each for 15 min. After each step of sterilization, the explants were washed with sterilized double distilled water (DDW). Further, sterilization procedure were carried out in laminar air flow chamber by using 0.1 % (w/v) $HgCl_2$ (Qualigens) for 3 min. The explants were then rinsed five times with sterilized DDW. From these sterilized twigs, shoot tips (0.5-1.0 cm) and nodal segments (1.0 -1.5 cm) were excised aseptically and implanted vertically on regeneration media.

SHOOT REGENERATION AND MULTIPLICATION

Murashige and Skoog's medium (MS) fortified with specific concentrations of plant growth regulators (PGRs) (BA, kinetin Kn, IAA, IBA and NAA) singly or in combinations was used for shoot regeneration and multiplication with 3% (w/v) sucrose (Qualigens) and 0.8% (w/v) agar (Qualigens). All the PGRs were purchased from Duchefa, Netherland. Basal MS medium without any PGR was used as control. For further shoot proliferation optimized treatment was used. Subculturing was regularly done at four week of interval.

IN VITRO ROOTING

For *in vitro* root induction the regenerated microshoots (3.0-4.0 cm) were transferred to half-strength MS medium supplemented with NAA and IBA (1.0, 2.5 and 5.0 µM). Half strength basal MS nutrient medium devoid of auxin was treated as control.

CULTURE CONDITIONS

All the cultures were raised in 25 × 150 mm culture tubes (Borosil, India). The pH of the medium was adjusted to 5.8 with 1N NaOH and 1N HCl before autoclaving at 1.06 kg cm^2 and 121ºC for 15 min. After inoculation all the cultures were maintained at 25 ± 2ºC in 16/8 h light/dark cycle with 50 µmol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes (Philips, India, 40 W).

EX VITRO HARDENING

Plantlets with well developed shoots and roots were removed from the culture media, washed gently under running tap water and transferred to thermocol cups (expanded polystyrene) containing sterilized soilrite (75% Irish peat moss and 25% horticulture grade expanded perlite) (Keltech Energies Ltd., India) under diffused light (16/8 h photoperiod) conditions. Plantlets were covered with transparent polythene membranes to ensure high humidity and watered every third day with normal tap water. Polythene membranes were opened progressively after two weeks in order to harden plants to culture room conditions. After four weeks, these plantlets were transferred to earthenware pots (12 cm diameter) containing normal garden soil and green manure (1:1) and maintained in a greenhouse under normal day length conditions.

STATISTICAL ANALYSIS

All the experiments were repeated thrice with 20 replicates for each treatment. The data were analyzed using SPSS Version 12 (SPSS Inc., Chicago, USA) and mean were compared using Tukey's test at 5% level of significance.

RESULTS AND DISCUSSION

In this study experiments were conducted to standardize the explant source and culture media for multiple proliferation of shoot and result in mass propagation of homogenous elite plantlets of *S. rebaudiana*. Shoot tips and nodal segments from field grown plant of *S. rebaudiana* were inoculated on MS medium supplemented with

Table 1: Effect of different concentrations of growth regulators on multiple shoot formation through shoot tip and nodal segments after 4 weeks of culture

Growth regulator (µM)	Shoot tip		Nodal segment	
	No. of shoots per explant (Mean ± SE)	Shoot length (cm)(Mean ± SE)	No. of shoots per explant (Mean ± SE)	Shoot length (cm) (Mean ± SE)
control	1.00 ± .00^{g}	2.50 ± .80^{h}	1.00 ± .00^{g}	2.38 ± .05^{g}
BA (1.0)	2.40 ± .24^{fg}	2.86 ± .11^{gh}	1.80 ± .20^{fg}	2.62 ± .05^{fg}
BA (2.5)	3.60 ± .24^{ef}	3.38 ± .05^{ef}	3.00 ± .31^{f}	3.02 ± .06de
BA (5.0)	7.40 ± .40^{d}	3.92 ± .10^{bc}	5.60 ± .24^{e}	3.40 ± .04^{cd}
BA (7.5)	4.80 ± .37^{e}	3.22 ± .08^{fg}	3.20 ± .37^{f}	2.90 ± .08^{ef}
Kn (1.0)	1.80 ± .20^{fg}	3.16 ± .06^{fg}	1.40 ± .24^{fg}	2.78 ± .05^{ef}
Kn (2.5)	2.80 ± .20^{efg}	3.50 ± .03^{ef}	2.00 ± .31^{fg}	3.40 ± .07^{cd}
Kn (5.0)	4.60 ± .24^{e}	3.62 ±.05^{cde}	3.00 ± .31^{f}	3.78 ± .11^{bc}
Kn (7.5)	2.80 ± .20^{efg}	3.90 ± .04^{bc}	2.40 ± .24^{fg}	3.10 ± .04de
BA (5.0) + NAA (0.5)	13.80 ± .58^{b}	4.04 ± .05^{b}	10.60 ± .40^{c}	3.80 ± .07^{b}
BA (5.0) + NAA (1.0)	19.60 ± .50^{a}	4.70 ± .10^{a}	16.0 ± .31^{a}	4.28 ± .08^{a}
BA (5.0) + NAA (1.5)	11.60 ± .50^{c}	3.74 ± .10^{bcde}	9.2 ± .58^{cd}	4.02 ± .06^{ab}
BA (5.0) + IAA (0.5)	11.60 ± .50^{c}	3.52 ± .03^{def}	8.4 ± .50^{d}	3.78 ± .08^{bc}
BA (5.0) + IAA (1.0)	15.00 ± .31^{b}	3.88 ± .05^{bcd}	12.8 ± .73^{b}	4.08 ± .08^{ab}
BA (5.0) + IAA (1.5)	9.20 ± .86^{d}	3.24 ± .08^{f}	9.0 ± .44^{cd}	3.38 ± .10^{d}

Data pooled from 3 separate experiments each with 20 replicates. Mean values within the column with same superscript are not significantly different (P=0.05; Tukey's Test).

various cytokinins alone or in combination with auxin at different concentrations. Mean number of shoots per explant and mean shoot length varied considerably with the type of explant and treatment used (Table 1).

The proliferation efficiency of shoot tips was significantly higher than that of nodal segments when evaluated up to four weeks. Shoot tips and nodal segments remained green and elongated into single shoot on hormone free MS medium. However, supplementation of *cytokinin (BA and Kn) and auxin (NAA and IAA)* to basal MS medium induced direct shoot multiplication as a result of split in the pre existing meristem.

Shoot tips exhibited their initial response with the appearance of new leaves within 3-4 days of incubation. Apical bud started to sprout in multiple buds after one week of culture (Figure 1 A). In the present study, BA was more potent when compared with Kn for apical bud break. A linear correlation was achieved with the increase in BA/Kn concentration from 1.0 to 5.0 mM and shoot proliferation efficiency. Supplementation of 5.0 µM BA resulted in maximum proliferation as a solitary cytokinin. Shoot tips produced higher number of shoots 7.40 per explant with the mean length of 3.92 cm after four week of culture (Figure 1 B). However, nodal segments produced 5.60 shoots per explant with 3.40 cm shoot length on similar treatment. This discrepancy in result might be due to the difference in endogenous hormonal balance in different explant types. When BA concentration was increased beyond the optimum level (5.0 µM), the rate of shoot multiplication and elongation was reduced in both the explant types due to callogenesis. According , to *Preece et al., [1991]*, the formation of callus at the basal

Figure 1:

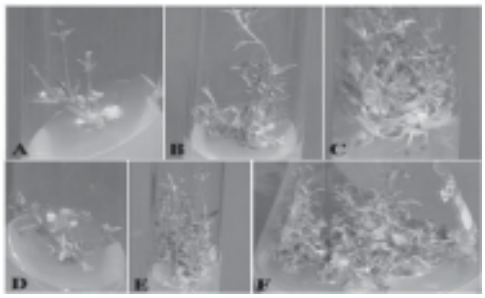

Figure 1: (A) Induction of multiple shoots on MS + BA (5.0 µM) from shoot tip after two week of incubation (B, C) High frequency of shoot multiplication on MS + BA (5.0 µM) + NAA (1.0 µM) from shoot tip after two and four week of incubation (D) Nodal segments placed on MS + BA (5.0 µM) + NAA (1.0 µM) (E) Shoot multiplication and elongation on MS + BA (5.0 µM) + NAA (1.0 µM) from nodal segment after four week of incubation (F) Culture showing shoot proliferation during subculturing

cut ends of explant on cytokinin enriched medium is frequent in species with strong apical dominance. Superiority of BA over other Kn for axillary bud promotion in *Stevia rebaudiana* has also been reported by Sivaram and *Mukundan [2003], Rafiq et al., [2007], Ahmed et al., [2007] and Anbazhagan et al., [2010]*. Our observations on the suppression of bud break at higher concentration of cytokinin was in conformity with the reports of *Ahuja et al., [1982]* in *Ocimum gratissimum* and *O. viride* and *Patnayak and Chand [1996],* in *Ocimum americanum* and *Ocimum santum*.

Addition of auxin to the optimized concentration of BA (5.0 µM) showed stimulatory effect in the enhancement of multiple shoot induction. Among different cytokinin-auxin combinations attempted, enhanced proliferation has been achieved in BA (5.0 µM) + NAA (1.0 µM) for both the explant types, thus exhibiting similar type of hormonal requirement for morphogenesis. On this treatment a maximum number of 19.60 and 16.00 shoots per explant with an average shoot length of 4.70 and 4.28 cm have been obtained through shoot tip and nodal segments respectively (Figure 1 C, D, E). Thus, incorporation of NAA to BA supplemented medium exerted rapid enhancement of shoot length with higher number of nodes per shoot which make them sturdy as compared to shoots induced on BA alone. On further increasing NAA concentration to (1.5 µM) morphogenic potential of shoot tips and nodal segments was reduced. The results substantiate with the earlier findings of several workers where the addition of low level of NAA with cytokinins promoted shoot proliferation as reported in *Ceropegia bulbosa* [Britto et al., 2003], *Salvia nemorosa [Ska³a and Wysokiñska,2004]* and *Spilanthes mauritiana* [Sharma et al., 2009]. However, some studies advocated the use of BA and IAA for satisfactory axillary bud multiplication in *Stevia rebaudiana [Sivaram and Mukundan 2003, Anbazhagan, 2010], Ocimum gratissimum [Gopi et al., 2006]* and *Scoparia dulcis [Karthikeyan et al., 2009]*.

Table 2: Effect of different concentrations of auxins supplemented to half strength MS nutrient medium on *in vitro* root induction after 4 weeks of culture

Auxin (µM)	% of microshoots rooted	No. of roots per shoots (Mean ± SE)	Root length (cm) (Mean ± SE)
Control	-	$0.00 \pm .00^{e}$	$0.00 \pm .00^{f}$
IBA (1.0)	75	$4.80 \pm .37^{bc}$	$5.24 \pm .08^{cd}$
IBA (2.5)	84	$8.00 \pm .31^{a}$	$6.36 \pm .06^{a}$
IBA (5.0)	73	$5.60 \pm .24^{b}$	$5.68 \pm .05^{b}$
NAA (1.0)	68	$3.00 \pm .31^{d}$	$4.50 \pm .08^{e}$
NAA (2.5)	75	$6.00 \pm .31^{b}$	$5.46 \pm .07^{bc}$
NAA (5.0)	70	$3.60 \pm .24^{cd}$	$5.00 \pm .06^{d}$

EFFECT OF SUBCULTURE PASSAGE ON SHOOT PROLIFERATION

As it has been observed in the present study, MS medium supplemented with BA (5.0 mM) and NAA (1.0 mM) was found to be most effective for axillary bud multiplication and proliferation from shoot tips and nodal segments, therefore this treatment has been selected as optimal and used for further shoot proliferation. By repeated subculturing of regenerating tissue, a prolific shoot culture was established (Figure 1F). The number of shoots per explant increased significantly after every subculture passage as clear from Figure 2 A. Besides, subculturing has a significant promotive effect on shoot length also Figure 2 B. Enhanced shoot multiplication in subsequent subculture was in accordance with earlier published reports on *Eclipta alba* by *Borthakur et al., [2000] and Husain and Anis [2006]*. Subculturing had a more significance effect on shoot proliferation from shoot tips as compared to nodal segments/tissue.

IN VITRO ROOTING

Microshoots excised from *in vitro* proliferated cultures were implanted to half strength MS medium with or without different concentrations of auxin, NAA and IBA (1.0, 2.5 and 5.0 mM) for *in vitro* rooting. Microshoots showed similar responses regarding *in vitro* rooting irrespective of explants source. Hormone free nutrient medium failed to induce *in vitro* rooting. However, auxin supplemented media induced rooting within one week of culture Table 2. Among the treatments tried, 2.5 mM IBA was found to be the best which resulted in the induction of an average of 8.00 roots per shoot with an average root length of 6.36 cm in 84% of culture within four week of incubation (Figure 3 A). Similar hormonal requirement for *in vitro* rooting has also been reported by *Sivaram and Mukundan [2003],* in same plant species. However, they reported a slightly higher number of roots but the roots formed in the present study were good enough for their successful establishment in soil. On the other hand *Ahmed et al., [2007]* and *Anbazhagan et al. [2010],* suggested the requirement of IAA for best rooting in *S. rebaudiana* while *Rafiq et al. [2007],* found optimum rooting in NAA supplemented nutrient medium.

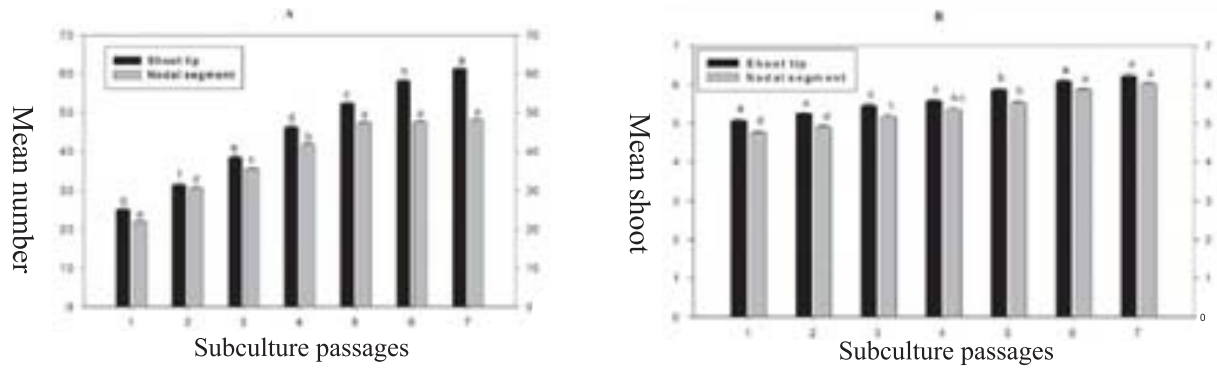

Figure 2: (A, B) Effect of subculture passages on shoot proliferation and elongation efficiency through shoot tip and nodal segments

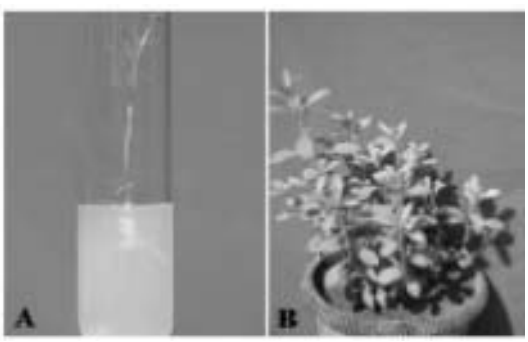

Figure 3: (A) *In vitro* rooting in half strength MS + IBA (2.5 µM) (B) An acclimatized plant of *S. rebaudiana* under field condition

EX VITRO HARDENING

Plantlets with well developed roots were acclimatized and hardened off in solirite condition as described in materials and method. The use of sufficiently porous substratum that allows adequate drainage and aeration has been recommended for fast acclimatization of *in vitro* regenerated plants *[Dunstan and Turner 1984]*. After four *weeks* under greenhouse, the potted plants were transferred to natural field conditions for better establishment and at the end of this study, around 96% of plants were thrived well in natural field environment (Figure 3 B).

ACKNOWLEDGMENTS

Anwar Shahzad gratefully acknowledges the financial support provided by the Department of Science and Technology (DST), Government of India, New Delhi in the form of SERC Fast Track Scheme Vide no. SR/FT/L-23/2006 and DST-FIST Programme 2005 (Project No. SR/FST/LSI-085/2005). Shiwali Sharma is also thankful to UGC, for the award of UGC-BSR Fellowship in Science (1st April 2010) for providing research assistance.

REFERENCES

Ahmed MB, Salahin M, Karim R, Razvy MA, Hannan MM, Sultana R, Hossain M, Islam R [2007]. An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh, Amer-Eur J Sci Res 2:121-125.

Anbazhagan M, Kalpana M, Rajendran R, Natarajan V, Dhanavel D [2010]. *In vitro* production of *Stevia rebaudiana*, Emir J Food Agric 22:216-222.

Borthakur M, Dutta K, Nath SC, Singh RS [2000]. Micropropagation of *Eclipta alba* and *Eupatorium adenophorum* using a single-step nodal cutting technique, Plant Cell Tiss Org Cult 62:239-242.

Britto SJ, Natarajan E, Arockiasamy DI [2003]. *In vitro* flowering and shoot multiplication from nodal explants of *Ceropegia bulbosa* Roxb. var. *bulbosa*., Taiwan 48:106-111.

Choi YH, Kim I, Yoon KD, Lee SJ [2002]. Supercritical fluid extraction and liquid chromatographic-electrospray mass spectrometric analysis of stevioside from *Stevia rebaudiana* leaves, Chromatograph 55:617-620.

Dunstan DI, Turner KE [1984]. The Acclimatization of micropropagated plants, in: Cell Culture and Somatic Cell Genetics of Plants, Laboratory procedures and their applications, Vasil IK (ed.), vol. 1, Academic Press, Orlando, pp 123-129.

Felippe GM, Lucas NMC [1971]. Estudo da viabilidade dos fructose de *Stevia rebaudiana* Bert., Hoehnea 1:95-105.

Gopi C, Natarajasekhar Y, Ponmurugan P [2006]. *In vitro* multiplication of *Ocimum gratissimum* L. through direction regeneration, Afric J Biotech 5:723-726.

Husain KH, Anis M [2006]. Rapid *in vitro* propagation of *Eclipta alba* (L.) Hassk. through high frequency axillary shoot proliferation, Acta Physiol Plant 28:325-330.

Karthikeyan S, Prasad R, Mahendran TS, Rajagopal K, Ravendran V [2009]. Direct regeneration and *in vitro* flowering of *Scoparia dulcis* L., Ind J Sci Technol 2:55-57.

Miyagawa H, Fujioka N, Kohda H, Yamasaki K, Taniguchi K, Tanaka R [1986]. Studies on tissue culture of *Stevia rebaudiana* and its components. II. Induction of shoot primordia, Planta Med 52:321-323.

Miyazaki Y, Wantenabe H [1974]. Studies on the cultivation of *Stevia*; on the propagation of plant, Jap J Trop Agric 17:154-157.

Mizutani K, Tanaka O [2002]. Use of *Stevia rebaudiana* sweeteners in Japan, in: *Stevia*, the genus *Stevia*, Medicinal and Aromatic Plants Industrial Profile London, Kinghorn AD (ed.), vol. 19, Taylor and Francis, pp 178-195.

Murashige T, Skoog F [1962]. A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiol Plant 15:473-497.

Patnaik SK, Chand PK [1996]. *In vitro* propagation of the medicinal herbs *Ocimum americanum* Syn. Ocimum Sims Hoary basil and *Ocimum sanctum* (Holly Basil), Plant Cell Rep 15:846-850.

Preece JE, Sutter EG [1991]. Acclimatization of micropropagated plants to the greenhouse and field, in: Micropropagation: Technology and Application, Debergh PC, Zimmerman RM (eds.). Kluwer Academic Publisher, Dordrecht, pp 71-93.

Rafiq M, Dahot MU, Mangrio SH, Naqvi HA, Qarshi IA [2007]. *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni, Pak J Bot 39:2467-2474.

Sharma S, Shahzad A, Jan N, Sahai A [2009]. *In vitro* studies on shoot regeneration through various explants and alginate – encapsulated nodal segments of *Spilanthes mauritiana* DC, an endangered medicinal herb, Int J Plant Dev Biol 3:56-61.

Sivaram L, Mukundan U [2003]. *In vitro* culture studies on *Stevia rebaudiana*. *In Vitro* Cell Dev Biol-Plant 39:520-523.

Skala E, Wysokiñska H [2004]. *In vitro* regeneration of *Salvia nemorosa* L. from shoot tips and leaf explants, *In Vitro* Cell Dev Biol-Plant 40:596-602.

Starratt AN, Kirbyb CW, Pocsa R, Brindle JE [2002]. Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*, Phytochem 59:367.

Strauss S [1995]. The perfect sweetener?, Technol Rev 98:18-20.