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Rapid Clonal Propagation and Stevioside Profiles of *Stevia rebaudiana* Bertoni

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ABSTRACT

Shoot tips of *Stevia rebaudiana* Bertoni were cultured on Murashige and Skoog (MS) or B5 medium, supplemented with 6-benzyladenine (BA), indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) in different combinations. A maximum of 28 shoots per shoot tip explant were produced on B5 medium supplemented with 4.44 μM BA and 0.80 μM NAA. Significantly more shoots could be obtained from *in vitro* shoot tips of *Stevia* by repeating the cycle. Elongation of microshoots was significant on B5 medium devoid of plant growth regulators. MS medium containing 0.05 μM-0.25 μM gibberellic acid (GA_3) led to elongation of shoots up to 10-12 cm in length with 7-8 nodes each. *In vitro* leaves derived from primary shoots could regenerate 3-4 shoots on the same medium. *In vitro* rooting of microshoots (~4 cm in length) was efficient on MS basal medium with shoots 12-13 cm long having 5 nodes and 11-12 roots in one month. After hardening in micropots for one month 80% of the rooted plants survived. In another study, incorporation of 13.62 μM thidiazuron (TDZ) in modified MS medium induced 11-12 multiple shoots from shoot tip explants when inoculated in reverse polarity i.e. shoot tips in downward direction (inverted mode). Both these methods could be useful for mass multiplication of *S. rebaudiana.* The stevioside content of both *in vitro* leaves and hardened three-months-old *ex vitro* plants was analysed by HPLC. The highest stevioside content (6.72% steviolbioside and 0.11% rebaudioside-A on a dry weight basis) were found in *ex vitro* and *in vitro* leaves, respectively.

Keywords: elongation, *in vitro* rooting, micropropagation, shoot tip, steviosides
Abbreviations: BA, 6-benzyladenine; **GA_3**, gibberllic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **MES**, morpholino-ethylsulphonate; **NAA**, α-naphthalene acetic acid; **TDZ**, thidiazuron

INTRODUCTION

Stevia rebaudiana Bertoni (family Asteraceae) is a perennial shrub and for hundreds of years, indigenous people in Brazil and Paraguay have used the leaves of *Stevia* as a sweetener (Strauss 1995). The leaves of *Stevia* are known to contain diterpene glycosides, viz. stevioside and rebaudioside, which are estimated to be 100-300 times sweeter than sucrose (Ishima and Katayama 1976). From its obscure discovery in Paraguay during the last century, *Stevia* has blossomed into a major export crop for better economic prospective and is now cultivated in India, China, Japan, Taiwan, Thailand, Korea, Brazil, Malaysia (Tanaka 1982) some European countries and all over South America (Lyakhovikin *et al.* 1993). Consistently the reviews on *Stevia* have highlighted the attractiveness of steviosides as sweetener and endorsed the establishment of *Stevia* as a growing industry (Shock 1982; Midmore and Rank 2002; Meireles *et al*. 2006).

Stevia is a highly self incompatible plant and plants propagated by seeds generally show a wide variation in the stevioside content (Tamura *et al.* 1984a; Nakamura and Tamura 1985). In general the percentage of seed germination is quite low in *Stevia*, and requires long time to establish seedlings (Kawatani *et al.* 1997) and also obtaining homozygous populations by this method is difficult (Felippe *et al.* 1971). Moreover, variation in stevioside content in *ex vitro*-grown and *in vitro*-cultured *Stevia* plants was reported (Rajasekaran *et al.* 2007), along with the detection of various steviosides by LCMS-ESI (Rajasekaran *et al.* 2008). Vegetative propagation of *Stevia* has its own limitations and it leads to poor levels of sweetener diterpene glycosides (~4%) in individual plants even after a selection programme of this economically important non nutritive sweetener plant (Nakamura and Tamura 1985). Micropropagation is commonly used to generate elite clonal plantlets in larger numbers in both economically important crops and ornamental plants. As micropropagation has significant advantages over traditional propagation techniques and also is the viable method of getting homogeneous population, the same can be applied to Stevia. *In vitro* propagation of *Stevia* has been reported from nodal (Tamura *et al.* 1984a; Ahmed *et al.* 2007; Ibrahim *et al.* 2008 Jena *et al.* 2009; Kalpana *et al.* 2009), leaf (Ferriera and Handro 1988; Sreedhar *et al.* 2008), suspension cultures (Ferreira and Handro 1988), shoot apex (Miyagawa *et al.* 1986; Nepovim 1998; Latha and Usha 2003; Hossain *et al.* 2008) explants and rooting of stevia cuttings *ex vitro* (Ingle and Venugopal 2009), although obtaining few shoots and the low frequency of shoot proliferation are constraints. In addition, reports on the stevioside content of the *in vitro* and *ex vitro* leaves upon field transfer of the tissue cultured plants are lacking. Apart from this, mass propagation of *S. rebaudiana* by using bioreactors had been reported (Akita *et al.* 1994) and most recently by Sreedhar *et al.* (2008), but having some limitations such as standardization of air mixing in bioreactors to get high biomass yield and lack of rooting as such in bioreactors that lead to ~50% mortality while transferring the plants to field (Sreedhar *et al.* 2008). In view of the above, there is a need to develop an efficient clonal propagation method that could be useful for commercial propagation. In the present communication we describe an efficient micropropagation method for mass multiplication of *S. rebaudiana* and stevioside profiles of tissue-cultured plants.

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Original Research Paper

MATERIALS AND METHODS

Chemicals

All the PGRs used in this study were obtained from Sigma-Aldrich, USA. Media chemicals were purchased from Himedia, Mumbai.

Plant materials and preparation of explants

Stevia rebaudiana Bertoni plants were obtained from the Department of Crop Physiology, Gandhi Krishi Vigyan Kendra, Bangalore, India. Nodal explants and shoot tips with 2 to 3 leaf primordia (length 1-1.2 cm) were collected from young growing plants maintained in the greenhouse at 40 µmol m^{-2} s^{-1} light intensity with a 16-hr photoperiod at 25°C and 70% relative humidity (RH). The explants were washed in running tap water for 20 min to remove dust and mud particles. Then they were treated with 70% ethanol (v/v) for 5 sec and washed three times with sterile distilled water (SDW). Further sterilization was carried out in the laminar airflow chamber using 0.5% bavistin (w/v) for 5 min, followed by three washes with SDW. The outer lower end of the explants were trimmed and used for the experiment by inoculating on MS medium (Murashige and Skoog 1962) or B5 medium (Gamborg *et al.* 1968), supplemented with 0.49 µM indole-3-acetic acid (IAA) and 2.22 µM of 6-benzyl adenine BA. This optimal combination of IAA and BA was resultant of initial trials of these two growth regulators at different concentrations for best response. After 8 weeks of culturing in a 16-h photoperiod with an irradiation of 40 µmol m^{-2} s^{-1} at 25 ± 2°C, the single long shoots of (~3 cm) obtained were transferred to plant growth regulator (PGR)-free MS basal medium to pre-condition them for 4 weeks for their subsequent use as explants for the mass multiplication experiment. The pH of the nutrient medium was adjusted to 5.8 and 0.75% agar (Himedia, Mumbai, India) was added prior to autoclaving. Into each 200 ml tissue culture bottle, 40 ml of media was dispensed. Five nodal or shoot tip explants were inoculated onto shoot induction medium within each bottle.

Initiation of culture

The cultures i.e. media bottles contains nodal and shoot tip explants were incubated at 24 ± 1°C with a 16-h photoperiod by using white fluorescent tubes (Philips, Mumbai, India). Various concentrations of PGRs viz. BA and α-naphthalene acetic acid (NAA) (**Table 1**) were tested in combination for multiple shoot induction. The primary shoots obtained on shoot induction medium were subcultured onto the same PGR-free medium for shoot elongation. Later shoots ~4.0 cm long were transferred to PGR-free MS basal medium or half-strength MS medium with PGRs (0.44-0.88 µM BA and 0.4-4.6 µM indole-3-butyric acid (IBA) for *in vitro* rooting.

In another set of experiments, shoot tip explants were inoculated in normal and inverted mode (reverse polarity) as reported earlier for *Bixa orellana* (Parimalan *et al.* 2007) onto modified MS medium (i.e. medium containing MS salts and B5 vitamins; Gamborg *et al*. 1968) containing 4.54-18.16 µM thidiazuron (TDZ). Elongation of TDZ-induced primary shoots was performed by subculturing onto half-strength MS medium containing 0.05, 0.1 and 0.25 µM of GA_3 (**Fig. 2**). Subsequently elongated shoots were rooted on half-strength MS medium.

In vitro leaves of newly formed shoots were separated and cut into explants (approx. 1 cm^2) and placed onto shoot induction medium. The microshoots of 2-3 cm long obtained after 4-5 weeks of culture were transferred to MS basal medium for elongation in the following 3 weeks. The elongated shoots rooted successfully on MS basal medium.

Transfer of *in vitro* plants to soil

After 4 weeks of culture, rooted plantlets were taken out from the tissue cultured bottles and potted in nursery pots containing a mixture of soil, peat and vermiculite (2: 1: 1 and grown in green house with 60-70% RH. They were irrigated twice a day for one month, and finally transferred to the field.

Extraction and HPLC analysis of steviosides

The extraction of steviosides from two-months-old *in vitro* leaves and tissue-cultured field-grown 3-months-old plant leaves was performed by High Performance Liquid Chromatography (HPLC) as reported earlier (Rajasekaran *et al*. 2008). In brief, *S. rebaudiana* leaves were separately dried in a hot air oven at 60°C for 2 hrs. A known weight of the dried sample was powdered and used for extraction of steviosides. These samples were defatted three times with petroleum ether (30 ml). The marc-deffated powder was boiled with SDW (2 ml) filtered and again extracted with SDW (25 ml) three times. To the pooled filtrates, potassium hydroxide (5 ml) was added and the mixture was refluxed on a steam bath for 1.5 hrs. In order to make the reaction mixture acidic (pH 5.0), glacial acetic acid was added and extracted three times with chloroform: methanol (2: 1) (6+3 ml). The combined organic extract was dried over anhydrous sodium sulphate and later the filtrates were evaporated to dryness. The residue was dissolved in methanol (10 ml) and 0.01% methanolic potassium hydroxide (pH 8.5). The reaction mixture was refluxed in a water bath at 65°C for 1 hr and evaporated to dryness to yield a residue, which was dissolved in methanol for HPLC analysis. A 90% recovery of extraction of steviosides from leaves by methanol extraction was achieved using this method.

A commercial *Stevia* powder obtained from M/S Varshapriya, Agrotech Pvt. Ltd. Bangalore, India, was used as the standard for analyzing steviolbiosides, dulcoside-A, rebaudiosides and stevioside. An aliquot of methanol extract of the experimental and standard samples was injected into a C_{18} column of 25 × 4.6 mm in size (HPLC model no: LC-10A, Shimadzu Corp., Kyoto, Japan) and run at isocratic condition using a solvent mixture of acetonitrile: water (3: 2) with a flow rate of 0.5 ml/min and λ = 258 nm. Quantitative estimation of steviosides was done based on the peak area of specific concentrations of the sample according to Rajasekaran *et al.* (2008). Stevia leaf powder that was used in this study was analyzed for the presence of different sweet glycosides viz. steviolbioside, dulcoside-A, rebaudioside-B, stevioside and rebaudioside-D.

Statistical analysis

For shoot induction, 5 explants of either nodal or shoot tip explants were used. Similarly for elongation of microshoots and also for *in vitro* rooting 5 explants were inoculated into each bottle and the experiment was repeated twice. Mean ± SE values are given in **Tables 1-4**. For analysis of steviosides 5 samples each of *in vitro* and *ex vitro* leaves were used. The data were analyzed by one way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corporation, Washington), and mean separations were performed by Duncan's Multiple Range Test at $P < 0.05$.

RESULTS

Initiation of multiple shoots

In our study, of the two media tested, B5 medium was found to be better than MS for shoot induction from shoot tip and nodal explants of *S. rebaudiana*. All the PGR combinations in B5 medium produced more shoots than with MS medium. The combination of BA (4.44 µM) and NAA (0.80 µM) on B5 medium induced more shoots i.e. 3.3 ± 0.5 and 28 ± 0.1 shoots per nodal (**Fig. 1A**) and shoot tip explant, respectively (**Fig. 1B**) than on MS medium (**Table 1**). A higher concentration of NAA (1.07 µM) in combination with BA (4.44 µM) produced only 4-5 shoots in B5 medium.

Elongation of micro-shoots and *in vitro* rooting

All the primary microshoots elongated into 7-8 cm long shoots on PGR-free B5 or MS media (**Fig. 1C**) in the following 4 weeks of culture. Alternatively, culturing the microshoots on full- or half-strength MS medium with 0.44 µM BA and 0.4 µM IBA supported not only shoot elongation but also promoted effective *in vitro* rooting (**Table 2; Fig. 1D**). However, in both the cases shoot length was 5-7

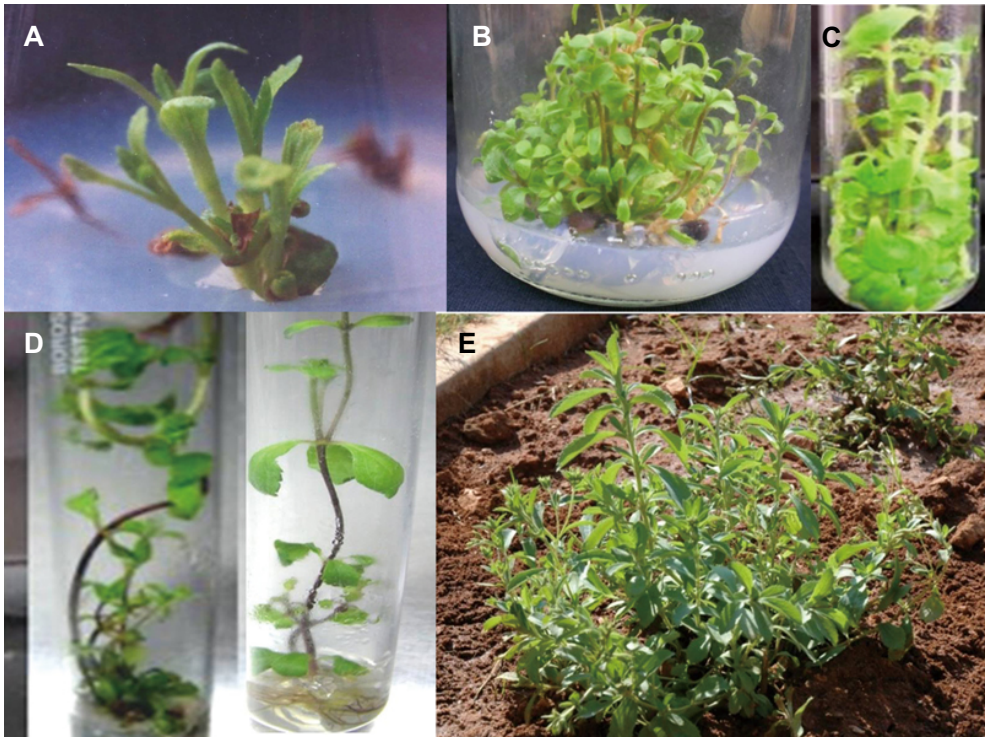

Fig. 1 ***In vitro*** **shoot multiplication of** ***S. rebaudiana*****.** (**A**) Emergence of 2-3 shoots from nodal explants in presence of BA (8.87 µM) + NAA (1.07) µM on MS medium; (**B**) Formation of up to 28 multiple shoots from shoot tip explant in presence of 1µM BA + 0.92 µM NAA B5 medium; (**C**) Elongation of microshoots on B5 medium; (**D**) Simultaneous micro-shoot elongation and *in vitro* rooting on MS basal medium with (BA 0.44 µM) + (IBA 0.4 µM); (**E**) Tissue cultured ex *vitro* grown plants.

Table 1 Multiple shoot induction in *Stevia rebaudiana* on medium containing BA and NAA.

Growth regulator concentration (µM)	**Average number of shoots per explants**	
	Shoot tip explant	**Nodal explant**
MS basal medium	2.66 ± 0.57 cd	1.33 ± 0.57 cd
MS + BA (4.44) + NAA (1.07)	3.33 ± 0.57 cd	2.00 ± 0.25 c
MS +BA (8.87) + NAA (1.07)	3.00 ± 0.35 cd	2.00 ± 1.00 c
MS +BA (13.32) + NAA (1.07)	3.66 ± 0.57 cd	1.66 ± 0.57 cd
MS +BA (4.44) + NAA (0.80)	6.66 ± 0.57 b	2.33 ± 0.57 b
MS +BA (8.87) + NAA (0.80)	3.33 ± 0.57 cd	2.00 ± 0.00 c
MS +BA (13.32) + NAA (0.80)	3.66 ± 0.57 cd	3.00 ± 1.00 a
B5 basal medium	3.00 ± 0.35 cd	1.66 ± 0.57 cd
G + BA (4.44) + NAA (1.07)	4.66 ± 1.5 c	2.00 ± 0.25 c
G + BA (8.87) + NAA (1.07)	6.00 ± 1 b	2.00 ± 1.00 c
G + BA (13.32) + NAA (1.07)	5.00 ± 1 c	2.00 ± 0.25 c
G + BA (4.44) + NAA (0.80)	28.0 ± 1 a	3.33 ± 0.5 a
G + BA (8.87) + NAA (0.80)	8.0 ± 1 b	2.66 ± 0.5 b
G + BA (13.32) + NAA (0.80)	7.3 ± 1 b	2.33 ± 0.5 b

MS: Murashige and Skoog medium, G: B5 medium, Results are mean ± SD of 5 replicates
Means followed by the same letters are not significantly different at P = 0.05

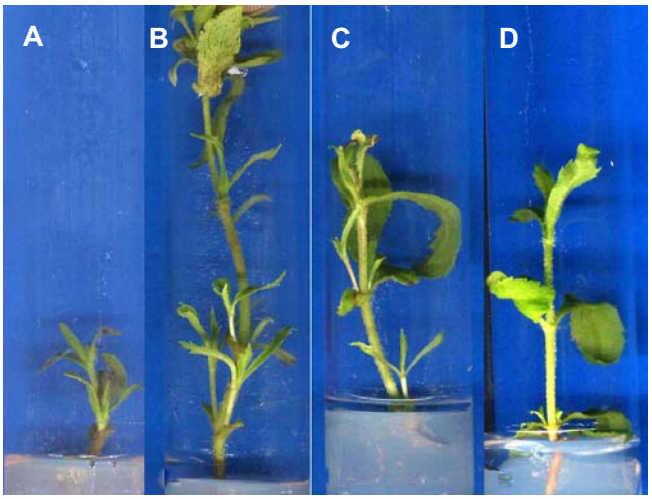

Fig. 2 Elongation of the microshoots that was primarily obtained on TDZ contained medium from hypocotyls explants inoculated in reverse polarity when on to media containing different concentrations of GA_3. (**A**) Control (without GA_3); (**B**) 0.05 µM; (**C**) 0.1 µM; (**D**) 0.25 µM.

cm. Further culturing of these microshoots on MS basal medium for one month produced shoots of 12-13 cm long, with 5 nodes and 11-12 number of roots. But half strength MS medium containing 0.44 µM BA and 0.4 µM of IBA supported shoot length of 10-11 cm with 5 nodes and 10-11 roots were noticed (**Fig. 1D**). The rooted plants were successfully hardened and the field survival was 70%. The tissue cultured plants grown *ex vitro* appeared to be normal and uniform in morphological characters (data not shown) (**Fig. 1E**).

Influence of TDZ on multiple shoot formation

TDZ also supported multiple shoot formation in both modes of shoot tip explants inoculation. Incorporation of 13.62 µM TDZ in modified MS medium induced 3-4 and 11-12 multiple shoots from shoot tip explants in normal mode and inverted mode (reverse polarity) of inoculation (**Table 3**), respectively. Elongation of TDZ induced primary shoots cultured on MS medium containing 0.05 µM, 0.1 µM, 0.25 µM of GA_3 leads to elongation of shoots up to 10-12 cm with 7-8 nodes (**Fig. 2**). These shoots were rooted on half strength MS medium containing BA (0.44 µM) + IBA (1.15 µM).

Steviosides profiles in *ex vitro* and *in vitro* leaves. HPLC analysis of leaf extracts revealed that, Stevia leaves contain rebaudiosides C, A, E and D but lack dulcoside-A (**Table 4, Fig. 3**). The maximum quantities of steviosides appeared in *ex vitro* leaves. It was found to be enriched by steviolbioside (6.72%) whereas, the amounts of stevioside

Table 2 Elongation and *in vitro* rooting in *Stevia rebaudiana*.

Medium + plant growth regulators (µM)	Average shoot length (cm)	Average number of nodes	Average number of roots
MS	12.5 ± 0.58 a	5.0 ± 0.5 a	11.5 ± 0.5 a
1/2MS + BA (0.44) + IBA (0.4)	11.7 ± 0.7 a	4.6 ± 0.8 a	5.40 ± 1.5 b
1/2MS + BA (0.44) + IBA (1.15)	10.8 ± 0.7 a	4.5 ± 0.5 a	5.20 ± 1.09 b
1/2MS + BA (0.44) + IBA (2.3)	11.0 ± 0.8 a	4.4 ± 0.6 a	10.6 ± 2.7 a
1/2MS + BA (0.88) + IBA (0.4)	8.50 ± 1.1 b	3.6 ± 0.9 b	3.60 ± 0.5 c
1/2MS + BA (0.88) + IBA (2.3)	7.90 ± 0.5 b	3.6 ± 0.7 a	3.80 ± 1.0 c
1/2MS + BA (0.88) + IBA (4.6)	6.40 ± 0.6 c	3.2 ± 0.8 b	0

MS: Murashige and Skoog medium, BA: 6-benzyladenine, IBA: Indole-3-butyric acid, Results are mean ± SD of 5 replicates
Means followed by the same letters are not significantly different at P = 0.05

Table 3 Effect of thidiazuron on *in vitro* shoot multiplication of *Stevia rebaudiana*.

Treatments (concentration in µM)	Shoot tip inoculated in normal polarity		Shoot tip inoculated in inverted polarity	
	Average number of shoots	Average shoot length (cm)	Average number of shoots	Average shoot length (cm)
MMS	1.0 ± 0	2.1 ± 0.2	1.0 ± 0.0	2.3 ± 0.2
MMS + Thidiazuron (4.54 µM)	3.3 ± 0.5 b	2.2 ± 0.2	5.0 ± 1.0 b	2.7 ± 0.2
MMS + Thidiazuron (9.08 µM)	3.6 ± 0.2 b	2.4 ± 0.0	5.3 ± 0.0 b	2.3 ± 0.2
MMS + Thidiazuron (13.62 µM)	4.3 ± 0.5 a	2.2 ± 0.1	11.6 ± 0.5 a	2.3 ± 0.3
MMS + Thidiazuron (18.16 µM)	3.6 ± 0.4 b	2.4 ± 0.0	4.0 ± 1.0 b	2.2 ± 0.2

n=5, MMS: Modified MS (MS salts+B5 vitamins), Results are mean ± SD of 5 replicates
Means followed by the same letters are not significantly different at P = 0.05

Table 4 HPLC profiles of the eight known sweet glycosides of *S. rebaudiana* (% of steviosides on dry wt. basis).

Stevioside compounds	*Ex vitro* leaves of tissue cultured 3 months old field grown plant (%)	*In vitro* leaves (%)
Steviolbioside	6.72 ± 0.5 a	0.095 ± 0.005 a
Dulcoside-A	-	0.024 ± 0.001 b
Rebaudioside-B	-	-
Stevioside	0.031 ± 0.009 c	-
Rebaudioside-C	0.024 ± 0.005 c	0.020 ± 0.003 b
Rebaudioside-A	0.040 ± 0.008 c	0.112 ± 0.014 a
Rebaudioside-E	0.094 ± 0.002 b	-
Rebaudioside-D	0.195 ± 0.045 b	-

Results are mean ± SD of 5 replicates
Means followed by the same letters are not significantly different at P = 0.05

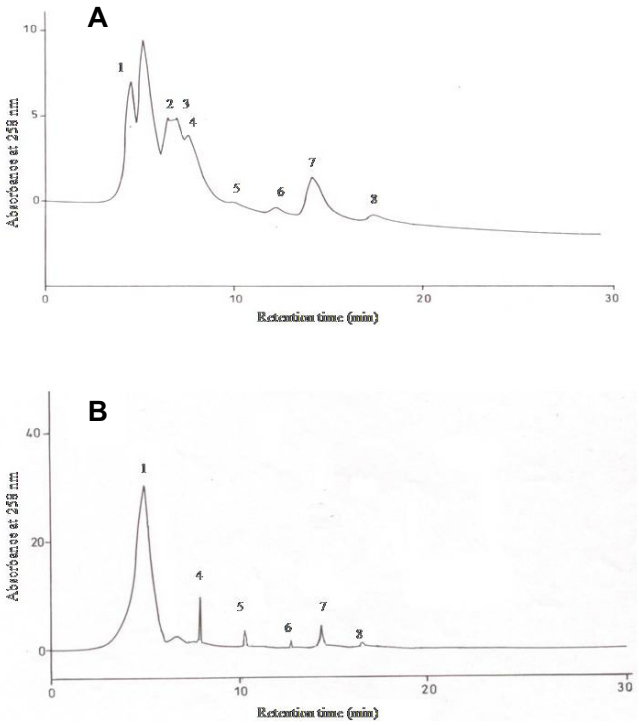

Fig. 3 Identification of major *S. rebaudiana* steviosides by HPLC. (**A**) mixture of steviosides as standard; (**B**) *ex vitro* leaves; (1) Stevioside; (2) Dulcoside A; (3) Rebaudioside B; (4) Stevioside; (5) Rebaudioside C; (6) Rebaudioside A; (7) Rebaudioside E; (8) Rebaudioside D.

and rebaudiosides C, A, E and D was quite low (**Table 4**). But stevioside content in the *in vitro* leaves was very little in contrast to *ex vitro* leaves. These observations are on par with our earlier report (Rajasekaran *et al.* 2008).

DISCUSSION

In the present study, we have shown *in vitro* shoot multiplication of *S. rebaudiana*, wherein, the experiments were very simple, testing different growth regulators, media and also explants. The results were positive wherein fully rooted plants were obtained. The significance of this study is the emergence of rapid direct organogenesis from rooted hypocotyls in presence of TDZ explants inoculated in reverse polarity. The rooted hypocotyl explants placed in an inverted polarity require MES to obtain multiplication as reported in *Capsicum annuum* (Vinod Kumar *et al.* 2005). In contrast to this, shoot bud formation from rooted hypocotyls on medium devoid of MES was reported in *Bixa orellana* (Parimalan *et al.* 2007). According Sreedhar *et al* (2008) only 4 shoots could be obtained from leaf explants of *Stevia* by using a combination of BA (8.88 µM) and kinetin (4.65 µM). In our study, up to five shoots from leaf explants obtained on medium containing cytokinin (BA) and auxin (IAA) combination. Similarly by using the shoot tip explants up to 28 shoots were obtained on B5 medium containing BA and NAA. This study confirmed that exogenous hormonal combination is specific for type of explant for appropriate response.

In our study, higher concentrations of BA resulted in less number of multiple shoot formation in both nodal explants and shoot tips of *Stevia*, but at lower levels BA induced multiple shoots, which was supported by earlier reports wherein, the incorporation of BA was found to be effective than kinetin (Tamura *et al.* 1984b; Benne and Davies 1986; Rogers *et al.* 1999). We found that, the combination of BA and NAA was better than BA and IBA (**Tables 1, 2**). According to Tamura *et al.* (1984b) very high concentration of kinetin (10 mg/l) was required to get 50-100 multiple shoots from shoot tip explant in 10-11 weeks culture initially and the same number of shoots upon subsequent culture on same medium in next 4-5 weeks. Similarly kinetin was used alone or in combination with BA by Ahmed *et al.* (2007) and Hossain *et al.* (2008). Latha and Usha (2003) have reported that combination 8.87 µM BA with 5.71 µM IAA induced ~11 and 10 multiple shoots from shoot apex and nodal explants, respectively. According to a recent report Jena *et al.* (2009) could obtain up to 7 shoots per nodal

explant of *S. rebaudiana* under the influence of high concentrations of BA (13.32 µM). In our study, the *in vitro* leaf explants and nodal explants of regenerated plants were able to produce 15-17 and 3-4 shoots respectively upon culturing on B5 medium containing 4.44 µM BA and 0.80 µM NAA which is better than the earlier report in this regard (Tamura *et al.* 1984b) wherein, only 3-5 multiple shoots were obtained in presence of 1-2 mg/l BA on MS medium from leaf explants. The multiplication factor reported so far for shoot formation from shoot tip explants was ten (Latha and Usha 2003). But in the present study, the maximum number of shoots (28 ± 1) were observed from shoot tip explants in modified MS medium containing 4.44 µM BA and 0.80 µM NAA. A single shoot apex regenerate into ~28 shoots, and nodal explants produced 3-4 shoots after 40 days of culture on the same medium respectively (**Table 1**). *In vitro* derived leaf explants from multiple shoots produced 15-17 shoots per explant after 4-5weeks when cultured on same medium. Shoot tip explants from these *in vitro* multiple shoots, or the nodal explants and also excised leaf explants, when transferred to a fresh medium able to produce multiple shoots in next 4-5 weeks. Since this asexual cycle of shoot multiplication can be repeated every 4-5 weeks, with a multiplication factor of 28 for shoot tip explants followed by their nodal and leaf explants as said above in a span of 7-8 months that includes one month *in vitro* rooting, it could be possible to get approximately 100,000 plants. Around 70,000 to 75,000 hardened *Steiva* plants can be obtained with a survival rate of 70-75% in the green house conditions. Hence, these results are highly useful for mass multiplication of *Stevia* for commercial propagation.

Our results in this study indicated that the contents of the steviosides in field grown plant leaves of *Stevia* appeared to be several folds more than that of *in vitro* leaves. The main interest in this study was to find different steviosides in leaves. It is observed that relatively the absence of synthesis of rebaudioside E and D along with stevioside in *in vitro* leaves. Regulation concerning the synthesis of wider spectrum of secondary metabolism compounds was established earlier for other plant cultures (Rolph and Coad 1991; Ban-thorpe and White 1995). Similarly the accumulation of the steviosides in *Stevia* cell cultures was known (Nabeta *et al.* 1976; Swanson *et al*. 1992) the variations in levels may be simply explained by the unstable level of these compounds during both prolonged plant maintenance. Steviosides proportion in *ex vitro* leaves might be greatly changed depending on the plant age and phase of plant development. The highest amount of the steviosides in leaves suggest that namely leaves serve as a main organ for both synthesis and primary accumulation of steviosides compounds. In our study we report on the comparative studies on steviosides production in both *ex vitro* and *in vitro* leaf. The main intention for attempting to grow *Stevia* leaves *in vitro* conditions is to study the variation in total stevioside content because the same will vary in different agroclimatic conditions like locations, latitude, altitude, topography and rain fall compare to the field grown plants (Chalapathi 1997). In conclusion, the clonal propagation method that we have developed in this study by using *in vitro* shoot tip explants is highly reproducible to get healthy *Stevia* plants that further substantiated by their significant steviosides composition.

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