

# Micropropagation of *Stevia rebaudiana* Bertoni and assessment of genetic stability of in vitro regenerated plants using inter simple sequence repeat (ISSR) marker

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**Abstract** *Stevia rebaudiana* Bertoni has become a new sweetener crop in different regions in the world. *Stevia* produces few seeds, therefore, micropropagation is a prevalent method to obtain sufficient amount of uniform plants. In the present study, in vitro propagation of *Stevia* was attempted through multiple shoot regeneration from nodal segments cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyladenine (BA),  $\alpha$ -naphthalene acetic acid (NAA), indole acetic acids (IAA) and thidiazuron (TDZ). The maximum of number axillary shoots per explant (3.24) and highest shoot length (3.12 cm) were observed with MS medium supplemented with  $1.0 \text{ mg l}^{-1}$  BA +  $0.05 \text{ mg l}^{-1}$  NAA and  $2.0 \text{ mg l}^{-1}$  BA +  $0.05 \text{ mg l}^{-1}$  NAA, respectively. Roots were produced within two weeks and the highest percentage (98.72%) of root induction, the maximum number of roots (9.46 roots/shoot) and root length (9.87 cm) were recorded on MS medium fortified with  $0.5 \text{ mg l}^{-1}$  IAA and  $1.5 \text{ mg l}^{-1}$  IAA, respectively. The ex-vitro plantlets were successfully acclimatized with a survival rate of 70% at the hardening phase. Inter simple sequence repeat (ISSR) markers were used to analyze the genetic stability of micro-propagated and mother plants of *Stevia*. Four ISSR primers generated clear, distinct and reproducible bands. All ISSR profiles from micro-propagated plants were monomorphic and similar to mother plants, while low variation was induced in the next sub-culture. The results indicated that *Stevia* plantlets regenerated using micropropagation techniques standardized at our lab were genetically stable especially in the early sub-cultures.

**Key words:** Genetic stability, in vitro propagation, somaclonal variation, molecular marker, phylogenetic.

*Stevia rebaudiana* Bertoni belongs to Asteraceae family, it is a natural sweetener estimated to be 300–500 times sweeter than cane sugar (Anbazhagan et al. 2010). Stevioside is regenerated as a valuable natural sweetening agent due to its relatively good taste and chemical stability (Toyoda and Matsui 1997). Also, growing of *Stevia* plant requires mild temperature between 15–38°C degree and relative humidity of about 80%, and it is considered as tolerance crops to abiotic stress such as high temperature and drought. Thus, the cultivation of this plant could be an alternative for the new land reclamation (El-Zefzafi 2003; Ibrahim et al. 2008).

Propagation of *Stevia* by seeds shows a very low germination percentage, less than 10% are common (Toffler and Orio 1981). It is difficult due to self incompatibility which results in sterile seeds

(Jagatheeswari and Ranganathan 2012) and does not allow the production of homogeneous populations (Hossain et al. 2008). In the words of Sakaguchi and Kan (1982) it is 'also vegetative propagation is limited by lower number of individuals'. Hence, to overcome all these obstacles, micropropagation or in vitro culture technique will be a suitable alternative method to prepare sufficient amount of *Stevia* within a short span of time, the production of genetically identical plants, conservation, and enhancement of the natural levels of in vitro production of valuable compounds (Sung 2006).

There have been few reports of in vitro clonal propagation of *Stevia* using leaf, nodal, internodal segments and shoot tips (Akita et al. 1994; Salim et al. 2006; Uddin et al. 2006) but a considerable effort is still required to make it more practicable. However,

Abbreviations: ISSR, inter-simple sequence repeats; PCR, polymerase chain reaction; MS, Murashige and Skoog; BA, 6-benzyladenine; Kn, Kinetin; IAA, indol-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; TDZ, thidiazuron; TBE, tris-borate- edta; PGR, plant growth regulator.

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a major problem associated with in vitro culture is the possible occurrence of somaclonal variation among the sub-clones of parental lines (Larkins and Scowcroft 1981). This variation may cause through pre-existing genetic variation occurred in the explants and it is induced by the in vitro conditions (Modgil et al. 2005). In vitro cultivation in particular poses a problem in recovering true-to-type regenerates due to chromosome rearrangements, DNA methylations and gene mutation (Saker et al. 2000), thus genetic control remains a major problem for commercial micro-propagation of sugar plant such as *Stevia*. The micropropagation of plants through shoot tip or axillary bud culture allows recovery of genetically stable and true to type progeny (George and Sherrington 1984).

The somaclonal variation can be detected by DNA finger printing using different types of markers like inter-simple sequence repeat (ISSR), (Xiaomel and Cuochen 2012). Compared with other molecular marker, ISSR primers are longer, so they offer advantages in the detection of somaclonal variation notably with a higher degree of sensitivity, increased stringency, higher reproducibility, high stability and dominant representation of polymorphic genetic alleles (Bornet and Branchard 2001).

Considering the increasing importance of *Stevia rebaudiana* Bertoni as alternative source to sugar production, and limited knowledge regarding to the genetic stability under in vitro culture conditions, the present work aimed to establish a complete in vitro protocol from direct shoot multiplication of *Stevia rebaudiana* Bertoni, and to assess genetic stability of in vitro raised culture by ISSR.

## Materials and methods

The experiments of the present study were conducted in the Tissue Culture and Biotechnology Laboratory, Maruot Research Station, Alexandria, Desert Research Center, Egypt with co-operation with Molecular Genetic Laboratory, Faculty of Science, North-Jeddah, King Abdulaziz University, Jeddah, Saudi Arabia during the period of June 2010 to February 2013.

### *In vitro* propagation of *Stevia rebaudiana* plant material and surface sterilization

Nodal cuttings (approximately 3 cm length) from 24 months-old plants of *Stevia rebaudiana* were collected. The nodal segments were surface sterilized by immersion for 15 min in 15% (v/v) sodium hypochlorite solution plus 2 drops/100 ml of Tween-20 and explants were washed 3 times with autoclaved sterile distilled water.

### Media and culture conditions

Murashige and Skoog (1962) (MS) basal medium (Caisson company) was gelled either with 0.8% (w/v) bacteriological agar or 0.3% (w/v) of gellan gum. Sucrose (30 g l<sup>-1</sup>) was used in all experiments as the source of carbohydrate. The pH of the medium was adjusted to 5.8 and the culture medium was autoclaved at 121°C for 20 min. For all experiments, cultures were maintained in growth room at 28±2°C, 16 h light/8 h dark photoperiod, light intensity at irradiance of 1000 lux was provided by white fluorescent tube lights. For shoot induction studies, a total of 30 nodal stem segments were inoculated for each of treatments imposed. The explants were cultured in culture tubes or jar containing MS basal media supplemented with 2.0 mg l<sup>-1</sup> BA or 2.0 mg l<sup>-1</sup> Kn or 1.0 mg l<sup>-1</sup> TDZ as a single or in combination with (0.05 or 1.0 mg l<sup>-1</sup> NAA) or (0.05 or 0.1 mg l<sup>-1</sup> IAA) (Table 2). After 5 weeks survival explants % (Total number of nodal stem segments cultured/no. of survival (healthy) explants), growth induction % (No. of nodal stem segments produced shoot induction/no. of healthy explants), Number of axillary shoots/survived explants and axillary shoot length (cm) were recorded. For in vitro shoot multiplication, the nodular stem sections were inoculated on MS medium supplemented with sucrose and different concentrations of BA or kinetin at 0.5, 1.0, 2.0, 3.0 and 4.0 mg l<sup>-1</sup> separately or in combinations for four weeks (Table 3). For rooting, the proliferated shoots (3.0 cm height) developed from nodal explants were singled out from the shoots and cultured on full and half-strength MS medium supplemented with various concentrations (0.5, 1.0, 1.5 or 2.0 mg l<sup>-1</sup>) of IAA or IBA as a single or in combination with NAA under light condition for four weeks (Table 4). A total of 15 elongated shoot being inoculated for each of treatments imposed.

### Acclimatization of rooted plantlets (hardening protocol)

The complete plantlets with well developed root system

Table 1. List of ISSR primers used in this study.

No.	Name	Sequence	Ann. Temp.	No.	Name	Sequence	Ann. Temp.
1	844A	(CT)8AC	44°C	8	HB 14	(CTC)3GC	44°C
2	HB 8	(GA)6GG	44°C	9	HB 13	(GAG)3GC	44°C
3	HB 9	(GT)6GG	44°C	10	HB 10	(GA)6CC	44°C
4	HB 11	(GT)6CC	44°C	11	17899B	(CA)6GG	44°C
5	HB 12	(CAC)3GC	44°C	12	TA-1	(AG)10C	60°C
6	844B	(CT)8GC	44°C	13	TA-2	(CT)10G	55°C
7	HB 15	(GTG)3GC	44°C	14	17898A	(CA)6AC	44°C

obtained by in vitro culture were gently washed under tap water to remove medium attached to the roots, then the plantlets were soaked in fungicide solution ( $2.0\text{ g l}^{-1}$  Benlate) for 5 min. Plantlets were transferred to plastic pots containing sterile soils consists of equal part of vermiculite, peatmoss and sand (1:1:1). The plastic pots were placed in the growth room and incubated at  $28\pm 2^\circ\text{C}$ , 70–90% relative humidity, 16h light/8h dark photoperiod and 1000lux light intensity. After two weeks the plants were transferred to green house, and to keep constant high humidity (90%) the pots were covered with dark polyethylene bags which were gradually removed within two weeks for proper hardening.

### ***Molecular analysis using ISSR marker genomic DNA extraction***

Total DNA was extracted, from 0.2 g of fresh young leaf tissues of seven plantlets chosen randomly for each subculture which consider as a population, by acetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990). The quality of genomic DNA was examined by agarose (1%) gel electrophoresis and quantified spectrophotometrically at A260 and A280 nm (Ying *et al.* 2011).

### ***ISSR-PCR analysis***

DNA amplification was performed in a PerkinElmer, Inc. Cetus 480 DNT Thermal Cycler (PerkinElmer, Inc. Cetus, Norwalk, Conn, USA). Out of fourteen different primers initially tested (Operon technologies INC., Alameda, California, USA), only four primers no. 2, 5, 7 and 10 produced clear PCR product were screened for ISSR analysis (Table 1). The optimized PCR for ISSR amplification reactions were performed in a total volume  $25\mu\text{l}$  contained: 10 mM Tris-HCl (pH 8.4),  $200\mu\text{M}$  of each dNTP, 1.5 mM  $\text{MgCl}_2$ ,  $50\mu\text{l}$  Primer,  $1\text{ U}\mu\text{l}^{-1}$  Taq-polymerase and 25 ng of DNA template. All the PCR components used in this study were purchased from Sigma company. Total DNA was initially denatured at  $94^\circ\text{C}$  for 2 min followed by 35 cycles of 1 min at  $94^\circ\text{C}$ , 1 min at  $57^\circ\text{C}$ , and 2 min at  $72^\circ\text{C}$ , and final incubation step for 10 min at  $72^\circ\text{C}$  to insure that the primer extension reaction proceeded to completion. All amplification products were resolved by electrophoresis on 1.5% (w/v) agarose gels on  $0.5\times\text{TBE}$  buffers (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) under a constant voltage of 80 V for 2 h, stained with  $1\mu\text{g ml}^{-1}$  ethidium bromide. 100bp DNA Ladder (Promega) was used as DNA marker and bands were visualized in a UV transilluminator at 300 nm and photographed using gel documentation equipment (Bio Rad). Only clear and reproducible band was scored as (1) for the presence and (0) for the absence of DNA bands of each mother and micropropagated plants. The rates of in vitro DNA polymorphism were calculated and given as percentage of total number of bands for the Stevia clones. Genetic similarity matrix values among mother plant and different subculture were calculated using SPSS statistical analysis program (software version 10). Using genetic similarity matrices, a dendrogram was constructed based on unweighted pair-group method with

arithmetic average (UPGMA).

### ***Statistical analysis***

The experiments were set up in completely randomized block design (CRBD), with 3 replicates for each treatment, each treatment contain 15 tubes or Jar (5 jar for each replicate), and 2 explants was cultured in Jar. Results on the percentage of survival explants, growth induction, shoots forming roots, number of shoot per explants and rooting was observed at regular intervals. Data were collected from three independent experiments and subjected to two-way ANOVA. The difference between treatments mean was separated using the least significant distance (LSD) test followed by Duncan's Multiple Range Test (Duncan 1955) at significant level of 5% was used to compare mean. All data were analyzed by Costat statistical program.

## **Results**

In the present study stem nodal were excised from healthy young shoots of mature *Stevia rebaudiana* to establish optimize protocol for their in vitro propagation.

### ***Shoot induction***

In our study, MS medium with 22 different combination of plant growth regulator (PGR) were tested. Data presented in Table 2 indicated that Stevia plants were efficiently regenerated from nodal explants and MS medium supplemented with ( $1.0\text{ mg l}^{-1}$  BA+ $0.05\text{ mg l}^{-1}$  NAA or  $1.0\text{ mg l}^{-1}$  BA+ $0.5\text{ mg l}^{-1}$  TDZ+ $0.05\text{ mg l}^{-1}$  NAA) gave the highest value of survival percentage (98%; 91%), growth induction percentage (97%; 89%) and number of axillary shoots/explant (3.42; 3.38), respectively, with no significant different between them. Also, in the present study, average axillary shoot length was ranged from 0.28 to 3.12 cm in control medium (MS medium without addition any PGR) and MS medium supplemented with  $2.0\text{ mg l}^{-1}$  BA+ $0.05\text{ mg l}^{-1}$  NAA, respectively. In the same time, the lower percentage of survival (28%) and growth induction (13%) as well as the significantly lower number of shoot induced per explants (0.21) was observed in control medium (Table 2, Figures 1 and 2).

### ***Shoot multiplication***

In vitro regenerated axillary shoot from nodal explants were cultured in order to induce multiple shoots on MS medium supplemented with different concentrations of BA or Kn either applied alone or in combination together (Table 3). Among the treatments used, BA ( $2\text{ mg l}^{-1}$ ) followed by  $2\text{ mg l}^{-1}$  BA in combination with  $5\text{ mg l}^{-1}$  Kn were the best of multiplication shoot (Figure 3), with 9.48 and 8.68 shoots/explant, respectively. While, the rate of multiplication shoots were decreased when Kn concentration was  $1.0\text{ mg l}^{-1}$ . On the other hand, the shoot length (4.58 cm) produced in MS medium

Table 2. In vitro establishment of nodal segments cultured on MS medium supplemented with 6-benzyladenine (BA), kinetin (Kn), thidiazuron (TDZ), indol-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) of *Stevia rebaudiana* Betroni.

Plant growth regulator (mg l <sup>-1</sup> )					Survival explants (%)	Growth induction (%)	Number of axillary shoots/survived explants	Axillary shoot length (cm)
BA	Kn	TDZ	IAA	NAA				
0.0	0.0	0.0	0.0	0.0	28	13	0.21±0.046 <sup>e</sup>	0.28±0.011 <sup>f</sup>
0.5	0.0	0.0	0.0	0.05	68	52	1.48±0.127 <sup>cd</sup>	2.25±0.051 <sup>bc</sup>
1.0	0.0	0.0	0.0	0.0	72	69	1.36±0.080 <sup>d</sup>	1.98±0.127 <sup>c</sup>
0.5	0.5	0.0	0.1	0.0	62	53	1.25±0.115 <sup>d</sup>	1.00±0.115 <sup>d</sup>
1.0	1.0	0.0	0.0	0.05	60	87	2.50±0.057 <sup>bc</sup>	2.48±0.167 <sup>b</sup>
1.0	0.5	0.0	0.0	0.1	68	62	2.00±0.10 <sup>c</sup>	3.05±0.144 <sup>a</sup>
0.5	1.0	0.0	0.05	0.05	58	49	1.15±0.086 <sup>d</sup>	1.65±0.202 <sup>c</sup>
1.0	0.0	0.0	0.0	0.05	98	97	3.42±0.144 <sup>a</sup>	2.98±0.069 <sup>ab</sup>
2.0	0.0	0.0	0.0	0.05	87	82	2.92±0.046 <sup>b</sup>	3.12±0.103 <sup>a</sup>
1.0	1.0	0.0	0.05	0.0	66	63	2.79±0.121 <sup>b</sup>	2.77±0.190 <sup>b</sup>
0.0	1.0	0.0	0.0	0.05	55	51	1.32±0.043 <sup>d</sup>	1.58±0.127 <sup>c</sup>
0.0	2.0	0.0	0.1	0.0	68	60	1.25±0.167 <sup>d</sup>	1.45±0.087 <sup>c</sup>
0.0	2.0	0.0	0.1	0.1	74	68	1.00±0.050 <sup>d</sup>	0.82±0.161 <sup>f</sup>
0.0	0.0	0.2	0.0	0.0	80	78	1.58±0.069 <sup>cd</sup>	0.98±0.011 <sup>e</sup>
0.0	0.0	0.5	0.0	0.0	83	80	1.65±0.028 <sup>cd</sup>	1.35±0.184 <sup>d</sup>
0.0	0.0	0.5	0.05	0.0	78	72	1.45±0.202 <sup>cd</sup>	1.38±0.127 <sup>d</sup>
0.0	0.0	0.5	0.0	0.05	84	82	1.28±0.072 <sup>d</sup>	1.30±0.173 <sup>d</sup>
1.0	0.0	0.5	0.0	0.05	91	89	3.38±0.264 <sup>a</sup>	2.92±0.040 <sup>ab</sup>
0.0	0.0	1.0	0.1	0.0	89	85	2.54±0.2.7 <sup>bc</sup>	1.88±0.069 <sup>c</sup>
0.5	0.5	0.5	0.05	0.0	77	69	2.12±0.161 <sup>c</sup>	1.56±0.023 <sup>c</sup>
0.5	0.5	0.5	0.0	0.1	79	71	2.43±0.213 <sup>b</sup>	1.48±0.184 <sup>c</sup>
1.0	1.0	0.5	0.0	0.1	76	75	2.48±0.127 <sup>b</sup>	2.85±0.086 <sup>ab</sup>

Results are mean ± SD of 3 replicates. Mean followed by the same letters are not significantly different at  $p \leq 0.05$ .

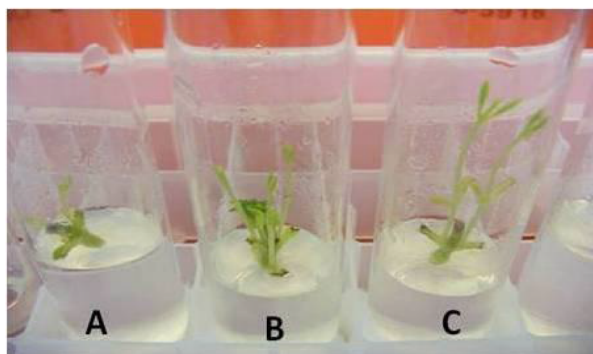


Figure 1. Shoot growing from nodal segment explants of *Stevia* on MS medium supplemented with (A) 0.5 BA mg l<sup>-1</sup> and 0.05 mg l<sup>-1</sup> NAA; (B) 1.0 mg l<sup>-1</sup> BA+0.5 mg l<sup>-1</sup> TDZ+0.05 mg l<sup>-1</sup> NAA and (C) 1.0 mg l<sup>-1</sup> BA+0.05 mg l<sup>-1</sup> NAA.

supplemented with 0.5 mg l<sup>-1</sup> BA was significantly higher than MS medium containing 4 mg l<sup>-1</sup> Kn (2.45 cm) (Table 2). Data also reflected that the shoot multiplication rate of *Stevia* increased with increase in BA up to 2 mg l<sup>-1</sup>, either applied alone or in combination with Kn, but when the BA concentration was increased up to 2 mg l<sup>-1</sup> the rate of shoot multiplication began to decline.

#### In vitro rooting of multiple shoot

The regenerated shoots, reached a height of 3–5 cm, were carefully excised and transferred to MS medium supplemented with various concentration of IBA or IAA or IBA in combination with NAA (Table 4). The first root



Figure 2. Explant proliferation and regeneration of *Stevia* on MS medium with different hormonal concentrations: (A) MS containing 2.0 mg l<sup>-1</sup> Kn and 0.1 mg l<sup>-1</sup> IAA, (B) MS containing 1.0 mg l<sup>-1</sup> BA, 0.5 mg l<sup>-1</sup> TDZ and 0.05 mg l<sup>-1</sup> NAA, (C) MS containing 1.0 mg l<sup>-1</sup> BA, 1.0 mg l<sup>-1</sup> Kn and 0.05 mg l<sup>-1</sup> NAA, (D) MS containing 1.0 mg l<sup>-1</sup> BA, 1.0 mg l<sup>-1</sup> Kn and 0.1 mg l<sup>-1</sup> NAA, (E) MS containing 1.0 mg l<sup>-1</sup> BA and 0.5 mg l<sup>-1</sup> Kn and 0.1 mg l<sup>-1</sup> NAA, (F) MS containing 1.0 mg l<sup>-1</sup> BA and 0.05 mg l<sup>-1</sup> NAA, and (G) Shoot proliferation on MS containing 2.0 mg l<sup>-1</sup> BA and 0.05 mg l<sup>-1</sup> NAA.

emerged directly from the basal part of shoot after 10 days and the data presented in Table 4 indicated that MS medium supplemented with IAA at 0.5, 1.0 and 1.5 mg l<sup>-1</sup> gave the highest percentage of root formed per explant (98.72, 92.36 and 87.85%); the best mean number of root per explant (9.46, 7.58 and 8.38) and the maximum root length (8.33, 7.42 and 9.87 cm), respectively. While the lowest rooting percentage, the minimum number of root per explant and lower root length were recorded



on the MS medium supplemented with IBA at different concentration (Figure 4). Overall, these results showed that among three Auxins tested, IAA was found to be superior for rooting in comparison to IBA alone or in combination to NAA.

### Hardening of plantlets

After following the acclimatization procedure, as mention previously in the materials and methods, Plants were irrigated with tap water every 4 days and successfully acclimatized in the green house with 70% survival rate (Figure 4).

### ISSR-PCR analysis

In this study, the genetic stability of regenerated plants derived from eight subculture was screened by ISSR markers that could be detected DNA sequence modification at the primer annealing site of genome.

Table 3. Effect of plant growth regulators on the multiplication of *Stevia rebaudiana* Betroni shoots after four weeks.

Plant growth regulator (mg l <sup>-1</sup> )		Number of axillary shoots/ survived explants	Axillary shoot length (cm)
BA	Kn		
0.5	0.0	5.65±0.256 <sup>c</sup>	4.58±0.242 <sup>a</sup>
1.0	0.0	6.52±0.317 <sup>b</sup>	4.23±0.152 <sup>ab</sup>
2.0	0.0	9.48±0.397 <sup>a</sup>	3.87±0.075 <sup>b</sup>
3.0	0.0	5.43±0.399 <sup>c</sup>	4.3±0.092 <sup>ab</sup>
4.0	0.0	4.46±0.340 <sup>d</sup>	3.36±0.254 <sup>bc</sup>
0.0	1.0	3.42±0.270 <sup>e</sup>	3.08±0.080 <sup>c</sup>
0.0	2.0	3.94±0.119 <sup>e</sup>	3.58±0.242 <sup>bc</sup>
0.0	3.0	4.53±0.265 <sup>d</sup>	3.28±0.092 <sup>c</sup>
0.0	4.0	4.82±0.363 <sup>cd</sup>	2.45±0.317 <sup>d</sup>
1.0	0.5	6.75±0.280 <sup>b</sup>	4.48±0.300 <sup>a</sup>
2.0	0.5	8.68±0.110 <sup>ab</sup>	4.02±0.219 <sup>b</sup>
3.0	0.5	5.25±0.250 <sup>c</sup>	3.88±0.069 <sup>b</sup>
4.0	0.5	3.94±0.119 <sup>e</sup>	2.56±0.254 <sup>d</sup>
2.0	1.0	4.58±0.352 <sup>d</sup>	3.15±0.086 <sup>c</sup>
3.0	1.0	3.72±0.250 <sup>e</sup>	2.56±0.023 <sup>d</sup>

Results are mean±SD of 3 replicates. Mean followed by the same letters are not significantly different at  $p \leq 0.05$ .

Among fifteen primers tested (HB1-15 series), four primers (Table 5) responded for genomic DNA amplification and produced thirty bands ranging from 114–1451 bp. ISSR amplification pattern of mother plants and eight sub-culture of *Stevia rebaudiana* were shown by representative gel profiles of primers Hb08, Hb10, Hb12 and Hb15 (Figure 5). Of the 30 fragments scored from these primers, 24 were monomorphic and 7 were polymorphic. The highest number (3) of polymorphic bands was obtained by primer Hb12 and lowest (0) by primer Hb10. Most of the primers showed identical DNA profiles as compared with mother plant. Using the Primer Hb12 polymorphic bands with a molecular mass 660, 270 and 220 bp were present only in subculture 8 (Figure 5), while with primer Hb15 one polymorphic band at 426 bp was absent only in subculture 6, another one band at 1456 bp was absent in both of subculture 7 and 8 using primer Hb08. On the other hand, no polymorphism was detected with Hb10 primer.

The occurrence of specific bands in the *Stevia*

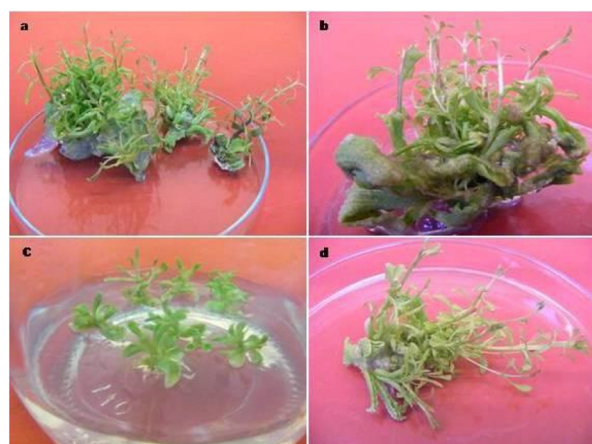


Figure 3. Show starting axillary shoots from nodal stem segments and shoot multiplication of *Stevia rebaudiana* on MS medium supplemented with; (a) 2 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> Kinetin; (b) 2.0 mg l<sup>-1</sup> BA; (c) 1.0 mg l<sup>-1</sup> kinetin and (d) 1.0 mg l<sup>-1</sup> BA and 0.5 mg l<sup>-1</sup> kinetin.

Table 4. Effects of different auxins; indol butyric acid (IBA), indol-3-acetic acid (IAA) or naphthalene acetic acid (NAA) on adventitious root formation from shoots in *Stevia rebaudiana* Betroni after four weeks.

Auxin concentration (mg l <sup>-1</sup> )			% of shoot forming roots	Number of roots/explant	Root length (cm)
IBA	IAA	NAA			
0.5	0.0	0.0	62.35	3.45±0.144 <sup>e</sup>	5.36±0.207 <sup>d</sup>
1.0	0.0	0.0	53.24	3.13±0.213 <sup>e</sup>	5.48±0.167 <sup>d</sup>
1.5	0.0	0.0	51.25	2.85±0.086 <sup>f</sup>	5.88±0.219 <sup>d</sup>
2.0	0.0	0.0	47.38	2.78±0.127 <sup>f</sup>	5.65±0.086 <sup>d</sup>
0.0	0.5	0.0	98.72	9.46±0.311 <sup>a</sup>	8.33±0.271 <sup>b</sup>
0.0	1.0	0.0	92.36	7.58±0.109 <sup>c</sup>	7.42±0.138 <sup>c</sup>
0.0	1.5	0.0	87.85	8.38±0.069 <sup>b</sup>	9.87±0.069 <sup>a</sup>
0.5	0.0	0.5	71.39	5.32±0.103 <sup>d</sup>	6.65±0.196 <sup>cd</sup>
1.0	0.0	0.5	73.28	6.31±0.109 <sup>cd</sup>	7.45±0.184 <sup>c</sup>
1.5	0.0	0.5	70.24	5.98±0.750 <sup>cd</sup>	6.42±0.265 <sup>cd</sup>

Results are mean±SD of 3 replicates. Mean followed by the same letters are not significantly different at  $p \leq 0.05$ .

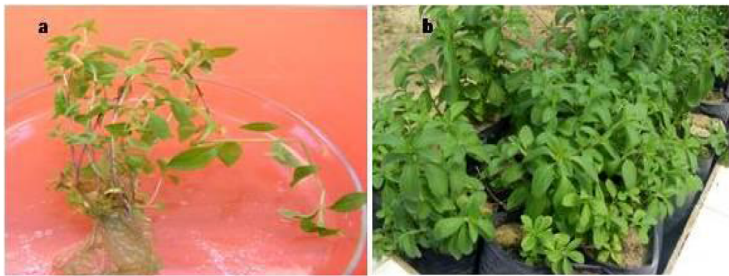


Figure 4. Root formation on MS medium supplemented with (a) 0.5 mg l<sup>-1</sup> IAA, (b) Plantlet established in pot containing vermiculite, sand and peatmoss (1:1:1).

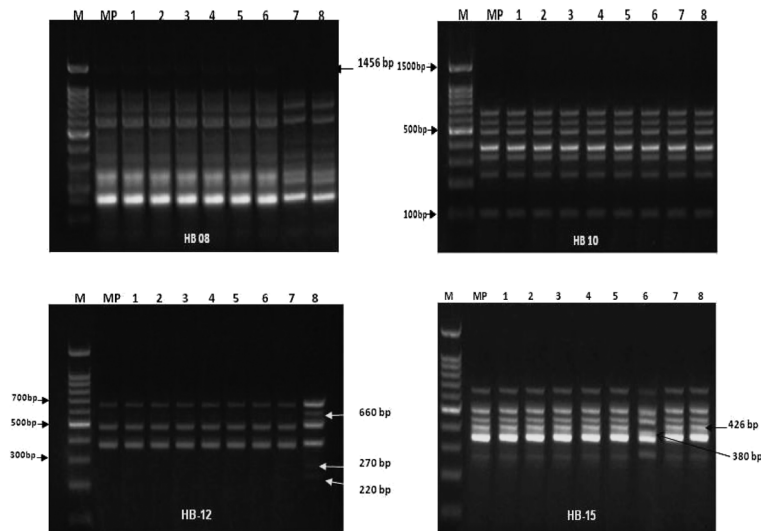


Figure 5. ISSR-PCR profiles generated by primers HB08, HB10, HB12 and HB15. Lane M, 100 bp DNA Ladder (Promega); Lane MP, the field grown mother plant; Lanes 1 to 8, different subcultures of micropropagated *Stevia rebaudiana* plants corresponding to the first subculture up to eight subculture. Arrow indicates the size of the fragments (bp).

Table 5. Details of ISSR analysis of in vivo (mother plant) and in vitro (regenerated plantlets) using four primers responded for amplification.

Serial No.	Primer code	Total amplified bands	No. of polymorphic bands	% of polymorphic bands	Size range (bp)
1	HB08	9	2	22.2	170–1451
2	HB10	7	0	0	114–697
3	HB12	6	3	50	220–674
4	HB15	8	2	25	251–651
Total		30	7	24.3	114–1451

*rebaudiana* tissue of different subcultures showed high similarity especially until subculture 5, but low genetic variations induced among in vitro plants and mother plants by increase the time period of the subculture (Figure 6).

Discussion

In the present study, we have shown the successful induction of shoots from *S. rebaudiana* through excessive in vitro shoot proliferation and transfer of rooted

plant to soil. Different responses of shoot regeneration were recorded from nodal explants on different growth regulators (Tables 2, 3 and Figures 1, 2). The combination of 1.0 mg l<sup>-1</sup> BA and 0.05 mg l<sup>-1</sup> NAA was found to be effective to produce more multiple shoots than other treatments. By culturing the nodal explant on 12 different combination of PGR, up to three shoots were obtained on medium containing BA and NAA during shoot induction stage (Paravatam et al. 2010). Our results were in agreement with those reported by Bochra et al. (2012) who found that shoot bud initiation of *Stevia*

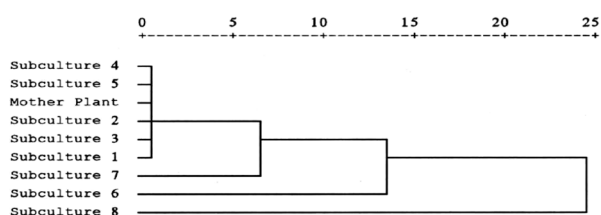


Figure 6. Phylogenetic tree for mother plant and regenerated plants of different subculture of *Stevia* based on the basis of their banding patterns with ISSR primers. Value of the x axis mean the degree of difference between different subculture.

*rebaudiana* did not respond well when the nodal explants were cultured on growth regulators free MS medium. To the contrary, Negar et al. (2012) observed that the combination of BA and IAA was more suitable for *Stevia* propagation than other used combination of PGR. While, Sabah and Rasha (2013) indicated that  $2.00 \text{ mg l}^{-1}$  BA recorded maximum number of shoots (43.9) compared with Kinetin. The presence of cytokinin in the medium was essential to induce bud break, shoot proliferation and shoot length from *Stevia rebaudiana* nodal explants (Thiyagarajan and Venkatachalam 2012).

In the same trend, the results in Table 3 and Figure 3, are in contrast with previous papers which reported that the maximum shoot multiplication from nodular stem section of *Stevia rebaudiana* was obtained by supplementing only  $2.0 \text{ mg l}^{-1}$  BA in the MS medium (Rafiq et al. 2007). Also, Shatnawi et al. (2011) reported that BA had better effect than Kinetin for increasing the number of shoot and length of *Stevia*.

Efficient methods for development roots in vitro are equally important for better *Stevia* formation. Auxins have several promotive functions in tissue culture and they have the ability to promote root initiation at early stages. The few reports on in vitro propagation of *Stevia* indicated that lower concentrations of auxin have been used to promote rooting and IAA has superiority for rooting in comparison to IBA and NAA (Anbazhagan et al. 2010).

Despite the many advantages of micropropagation through tissue culture, the presence of somaclonal variation amongst sub-clones of one parental line is a major problem encountered with the in vitro culture. Factors that account for the occurrence of somaclonal variation in vitro include chromosomal rearrangements, single gene mutations, pre-existing DNA variability, sub and supra-optimal levels of plant growth regulators, length of culture, period and method of regeneration (Martins et al. 2004). A better analysis to evaluate genetic stability of plantlets of *Stevia rebaudiana* can be made using ISSR markers (Lata et al. 2012). In this study, we succeeded to extract DNA and subjected it to ISSR analysis as a tool in survey the *Stevia rebaudiana* genome for mother plants and different subculture, the data obtained that, 78.8% of bands profiles generated using

ISSR from micropropagated plants were monomorphic and similar to those of mother plant, until subculture 5, which reflect that the maintenance of allele composition during successive cycles of vegetative propagation (Table 5 and Figure 5). Thiyagarajan and Venkatachalam (2012) reported that no genetic variability was detected among the tissue culture-raised plantlets; hence, nodal explants can be successfully employed for the commercial multiplication of *Stevia* without much risk of genetic instability. Also, absence of genetic variation using ISSR has been reported in several cases such as micropropagated plants of *Cannabis sativa* (Lata et al. 2010), *Musa spp.* (Ying et al. 2011) and *Gentiana straminea* (Tao et al. 2011).

Low genetic variations were observed among the in vitro propagated sativa plants as recorded after subculture 5 (Figure 6). This result was in agreement with those reported by Hemaïd et al. (2012), who indicated that genetic variation induced in the in vitro plants of *Moricandia nitens* increased with the time period of the subculture. This low genetic variation was reported in the micropropagated plants of *Anoectochilus formosanus* (Zhang et al. 2010) and *Hydrangea macrophylla* (Liu et al. 2011).

## Conclusion

In the present study, stable regeneration of normal shoots was successfully achieved from nodal segments of *Stevia rebaudiana*. As most of ISSR-based bands were highly monomorphic. This indicated that the clones were genetically highly stable. In agreement with this, low variation was detected in the micropropagated plants compared to the mother plants. It is concluded that our micropropagation protocol can be useful in crop improvement programs for monitoring the stability of in vitro propagated germplasm and cryo-preserved materials. Future work will be considered to assess the uniformity among the in vitro propagated plantlets, the hardened ex vitro plants and the field-grown plants.

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