



## Rapid regeneration and molecular assessment of genetic stability using RAPD markers in stevia

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### ABSTRACT

The present study was carried out to monitor somaclonal variations in *Stevia rebaudiana* using random amplified polymorphic DNA (RAPD) markers. Nodal and shoot tips were used as explants. Maximum shoot induction ( $98.0 \pm 2.00\%$ ) in ( $4.7 \pm 0.20$ ) days was reported in nodal explants and ( $74.0 \pm 2.45\%$ ) in ( $7.1 \pm 0.34$ ) days in shoot tip explants on MS medium supplemented with BAP 2.0 mg/l ( $EM_4$ ). The regenerated shoots were cultured on multiplication media and maximum ( $40.5 \pm 0.26$ ) shoots were observed on MS medium fortified with 0.3 mg/l BAP + 0.3 mg/l KIN + 0.1 mg/l NAA + 15 mg/l PEG on 30th day of culturing. Maximum 100% rooting was reported on 1/2 MS medium supplemented with 0.5 mg/l NAA with ( $21.2 \pm 0.3$ ) roots/shoot in  $7.4 \pm 0.26$  days. Rooted shootlets were separated individually and hardened in green house. The hardened plants of Stevia were screened for genetic stability using RAPD primers. Total twenty four RAPD primers were used which produced 82 distinct and scorable bands, with an average of 3.4 bands per primer and the amplification products range was from 100-1100 bp. The number of scorable bands for RAPD primer varied from 1 to 7. RAPD profiles from micropropagated plants were monomorphic and similar to mother plants, confirming their genetic stability. The results corroborate the fact that *in vitro* multiplication is the safest mode for production of true-to-the-type plants.

**Key words:** Genetic fidelity, *Stevia*, micropropagation, PEG, RAPD analysis.

### INTRODUCTION

*Stevia* (*Stevia rebaudiana* Bert.), belonging to the family "Asteraceae", is an important medicinal herb used as an alternative to artificially produced sugar. The leaf contains the various compounds called glycosides, viz. stevioside (9.1%), rebaudioside (3.8%), rebaudioside C (0.6%) and dulcoside (0.3%) (Bhosle, 3). Amongst these compounds, stevioside used in health concerns related to dental cares, diabetes and obesity (Das *et al.*, 4). The diterpene glycosides of stevia are being used in array of food products and beverages. Seed germination of stevia is often poor. Vegetative propagation is also too slow and having the possibilities of pathogen accumulation in the tissues (Mishra *et al.*, 12). Therefore, there is need to develop an efficient multiplication method for stevia. Tissue culture technique can be used for production of high quality planting material of stevia, however, under the influence of several factors such as the species, donor genotypes, explant type, composition of the culture medium, conditions of the physical culture and the duration between successive sub-cultures, there are chances of somaclonal variations (Larkin and Srowcroft, 9).

Since the sustainability of the regeneration system depends upon the maintenance of metabolic uniformity and genetic integrity of micropropagated

plants, therefore, it is imperative to confirm the quality of the plantlets for its commercial utility. Hence, genetic fidelity testing using preferably molecular marker techniques is essential to ensure the production and supply of true-to-the-type quality planting material. Among the markers, random amplified polymorphic (RAPD) is widely employed for the detection of genetic diversity because it has the advantage of being technically simple, quick to perform and requires only small amounts of DNA. Therefore, in the present study experiments were conducted to test the genetic fidelity of micropropagated plants using RAPD primers.

### MATERIALS AND METHODS

The experiments were conducted at the Centre for Plant Biotechnology, Hisar. Sterilized nodal and shoot tip explants were cultured on MS medium (Murashige and Skoog, 14) supplemented with different concentrations of BAP and kinetin (Table 1). Regenerated shoots were cultured on MS medium fortified with 0.3 mg/l BAP + 0.3 mg/l KIN + 0.1 mg/l NAA and different concentrations of polyethylene glycol (PEG) for *in vitro* multiplication (Table 2) and data were recorded for number of shoots/ culture. Sub-culture was carried out at 30-day intervals and were maintained on multiplication medium up to 25<sup>th</sup> sub-culture. All treatments were performed in triplicates with 10 explants in an experiment

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**Table 1.** Effect of different growth regulators (BAP and kinetin) on *in vitro* establishment of stevia (% shoot induction & average days required for shoot induction).

Medium	Nodal explant		Shoot tip	
	% Shoot induction*	Av. No. of days required for shoot induction*	% Shoot induction*	Av. No. of days required for shoot induction*
EM <sub>0</sub> (control)	22.0 ± 3.74 (27.6 ± 2.72)	16.5 ± 1.10	24.0 ± 2.45 (29.2 ± 1.63)	16.5 ± 1.10
EM <sub>1</sub> (MS + BAP 0.5 mg/l)	70.0 ± 3.16 (56.9 ± 2.00)	11.2 ± 0.72	52.0 ± 3.74 (46.0 ± 2.16)	12.2 ± 0.77
EM <sub>2</sub> (MS + BAP 1.0 mg/l)	72.0 ± 3.74 (58.2 ± 2.39)	8.8 ± 0.42	52.0 ± 3.74 (46.1 ± 2.16)	12.1 ± 0.31
EM <sub>3</sub> (MS + BAP 1.5 mg/l)	72.0 ± 3.74 (58.2 ± 2.39)	6.0 ± 0.28	52.0 ± 3.74 (46.1 ± 2.16)	10.5 ± 0.38
EM <sub>4</sub> (MS + BAP 2.0 mg/l)	98.0 ± 2.00 (86.3 ± 3.69)	4.7 ± 0.20	74.0 ± 2.45 (59.4 ± 1.63)	7.1 ± 0.34
EM <sub>5</sub> (MS + BAP 2.5 mg/l)	82.0 ± 2.00 (65.3 ± 1.62)	5.9 ± 0.28	62.0 ± 3.74 (52.0 ± 2.21)	9.3 ± 0.57
EM <sub>6</sub> (MS + KIN 0.50 mg/l)	84.0 ± 4.00 (66.9 ± 2.99)	6.0 ± 0.11	62.0 ± 3.74 (52.0 ± 2.21)	9.0 ± 0.20
EM <sub>7</sub> (MS + KIN 1.0 mg/l)	86.0 ± 2.45 (68.3 ± 1.99)	5.9 ± 0.52	52.0 ± 3.74 (46.1 ± 2.16)	10.5 ± 0.11
EM <sub>8</sub> (MS + KIN 1.5 mg/l)	88.0 ± 2.45 (70.43 ± 1.99)	5.5 ± 0.27	68.0 ± 2.00 (55.6 ± 1.20)	8.9 ± 0.74
EM <sub>9</sub> (MS + KIN 2.0 mg/l)	76.0 ± 2.45 (60.8 ± 1.63)	6.2 ± 0.18	52.0 ± 3.74 (46.1 ± 2.16)	10.9 ± 0.50
EM <sub>10</sub> (MS + KIN 2.5 mg/l)	74.0 ± 2.45 (59.4 ± 1.63)	6.6 ± 0.40	50.0 ± 5.48 (44.9 ± 3.20)	11.7 ± 0.28

\*Mean of three replicates, ± = SE (mean)

**Table 2.** Effect of different concentration of polyethylene glycol (PEG) on *in vitro* multiplication of stevia.

Medium	Average No. of shoots/ explant		
	(7 <sup>th</sup> day)	(15 <sup>th</sup> day)	(30 <sup>th</sup> day)
SM <sub>0</sub> (control)	1.6 ± 0.07	2.5 ± 0.29	4.5 ± 0.57
SM <sub>21</sub> (MS + BAP 0.3 mg/l + KIN 0.3 mg/l + NAA 0.1 mg/l + PEG 5.0 mg/l)	5.3 ± 1.03	12.6 ± 0.54	27.8 ± 1.15
SM <sub>22</sub> (MS + BAP 0.3 mg/l + KIN 0.3 mg/l + NAA 0.1 mg/l + PEG 10.0 mg/l)	6.3 ± 1.07	17.3 ± 1.04	35.5 ± 1.69
SM <sub>23</sub> (MS + BAP 0.3 mg/l + KIN 0.3 mg/l + NAA 0.1 mg/l + PEG 15.0 mg/l)	8.5 ± 1.78	18.8 ± 0.70	40.5 ± 0.26
SM <sub>24</sub> (MS + BAP 0.3 mg/l + KIN 0.3 mg/l + NAA 0.1 mg/l + PEG 2.0 mg/l)	7.1 ± 1.43	16.7 ± 0.85	34.1 ± 1.22
SM <sub>25</sub> (MS + BAP 0.3 mg/l + KIN 0.3 mg/l + NAA 0.1 mg/l + PEG 25.0 mg/l)	5.7 ± 0.90	14.3 ± 0.52	30.3 ± 0.75

\* = Mean of five replicates, ± = Standard error (mean)

employing a completely randomized design. After 25<sup>th</sup> subculture elongated shoots were separated and cultured on 1/2 MS medium supplemented with different concentrations of IBA and NAA for rooting (Table 3). Profuse rooting was observed on many media. The plantlets were transferred in green house for hardening. The hardened plants were screened for genetic stability using 24 RAPD (Table 4) primers. Clonal fidelity of *in vitro* raised clones was tested using RAPD primers. For this purpose, 10 hardened plants were chosen randomly from the *in vitro* raised population of 522 plants and compared with the mother plant from which the explants were taken. Total genomic DNA of the mother plant and *in vitro* raised clones was extracted from young leaf tissue by using the modified cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 15). PCR amplification was carried out after optimizing

the amplification conditions, viz., concentration of template DNA, primers, MgCl<sub>2</sub>, *Taq* DNA polymerase and annealing temperature. RAPD-PCR reactions were carried out in 25 µl of reaction mix containing 1 X PCR buffer, 250 µM dNTPs mix, 0.4 µM primers, 2.5 mM MgCl<sub>2</sub>, 1.5 U *Taq* DNA polymerase and 50 ng of template DNA. PCR was initiated by a denaturation step at 94°C for 7 min. and then the reaction was subjected to 40 cycles at 94°C for 1 min., T<sub>m</sub> °C for 1 min., 72°C for 2 min., with a final elongation step of 7 min. at 72°C for RAPD primers. The amplification products were resolved by electrophoresis on 1.4% agarose gel with ethidium bromide (5 µl/100 ml). PCR amplification products were viewed under long wavelength UV light (302 nm) and photographed using Alpha Digi Doc Pro™ documentation system. RAPD amplification profiles were scored visually, based on presence or absence of bands. Clear

**Table 3.** Effect of different auxins on *in vitro* rooting of stevia (No. of days taken for root formation from regenerated shoots).

Medium	Rooting (%)	No. of roots	Av. No. of days to rooting	Quality of roots (Visual index)
RM <sub>0</sub> (control)	12 ± 3.74 (18.0 ± 4.85)	3.0 ± 0.09	15.7 ± 1.45	+
RM <sub>1</sub> (1/2 MS)	32 ± 3.74 (34.28 ± 2.35)	5.9 ± 0.78	10.3 ± 0.23	++
RM <sub>3</sub> (1/2 MS + NAA 0.25 mg/l)	58 ± 9.69 (49.5 ± 5.86)	6.5 ± 0.30	11.2 ± 0.20	++
RM <sub>4</sub> (1/2 MS + NAA 0.5 mg/l)	90 ± 7.75 (78.4 ± 7.80)	21.2 ± 0.37	7.4 ± 0.26	++++
RM <sub>5</sub> (1/2 MS + NAA 1.0 mg/l)	72 ± 5.83 (58.4 ± 3.59)	6.4 ± 0.28	10.3 ± 0.29	++
RM <sub>6</sub> (1/2 MS + NAA 1.5 mg/l)	58 ± 9.69 (49.5 ± 5.86)	5.3 ± 0.29	11.3 ± 0.38	++
RM <sub>7</sub> (1/2 MS + NAA 2.0 mg/l)	54 ± 8.71 (47.1 ± 5.27)	5.6 ± 0.30	11.2 ± 0.28	++
RM <sub>8</sub> (1/2 MS + IBA 0.25 mg/l)	56 ± 9.27 (48.3 ± 5.60)	6.7 ± 0.20	11.6 ± 0.31	++
RM <sub>9</sub> (1/2 MS + IBA 0.5 mg/l)	86 ± 9.27 (73.6 ± 8.26)	16.6 ± 0.63	9.1 ± 0.22	+++
RM <sub>10</sub> (1/2 MS + IBA 1.0 mg/l)	58 ± 7.35 (49.6 ± 4.33)	11.3 ± 0.52	12.0 ± 0.37	++
RM <sub>11</sub> (1/2 MS + IBA 1.5 mg/l)	58 ± 7.35 (49.6 ± 4.33)	9.5 ± 0.25	11.7 ± 0.31	++
RM <sub>12</sub> (1/2 MS + IBA 2.0 mg/l)	40 ± 5.48 (39.0 ± 3.37)	8.3 ± 0.47	12.4 ± 0.38	++

\* = Mean of five replicates, ± = SE (mean); Figures in parentheses are transformed values

+ = poor, ++ = good, +++ = very good, ++++ = excellent

**Table 4.** DNA amplification profile generated for clonal fidelity testing of *in vitro* propagated plants of *S. rebaudiana* using RAPD primers.

Primer	Sequence (5'-3')	Mol. wt. (bp)	No. of scorable band(s) per primer
OPE-2	GGTGCGGGAA	100-650	2
OPE-4	GTGACATGCC	200-1030	5
OPF-14	GGCTGCAGAA	400-960	4
OPH-19	CTGACCAGCC	520-1040	3
OPD-11	AGCGCCATTG	500-1010	5
OPA-02	TGCCGAGCTG	400-970	6
OPA-03	AGTCAGCCAC	480-1020	7
OPA-05	AGGGGTCTTG	320-600	5
OPA-07	GAAACGGGTG	510-1040	3
OPA-09	GGGTAACGCC	560-900	4
OPA-11	CAATCGCCGT	700-900	3
OPA-15	TTCCGAACCC	260-1100	3
OPA-20	GTTGCGATCC	520	1
OPB-15	GGAGGGTGTT	410-900	4
OPD-03	GTCGCCGTCA	500-990	4
OPG-03	GAGCCCTCCA	490-980	4
OPG-05	CTGAGACGGA	900-1000	2
OPG-09	TCACGTCCAC	500-1860	3

*Table 4 Contd...*

Primer	Sequence (5'-3')	Mol. wt. (bp)	No. of scorable band(s) per primer
AY1	CGGTGGCGAA	400-1020	3
AY2	AGCGGCTAGG	570-1080	2
AY3	ACATCCTGCG	570-900	4
AY4	ACGGCAGTGG	790-830	2
AY5	GGTCTCCTAG	700	1
AY6	GCGAACCTCG	800-1000	2
Total			82
Mean			3.4

and unambiguous bands were scored. The size (in nucleotide base pairs) of amplified bands was determined. It was based on its migration relative to molecular size marker, *i.e.* 100 bp ladder. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel. For RAPD profiles, the well resolved and consistently reproducible fragments of 100 bp were scored as present or absent. For detecting any genetic change, all the RAPD results were compared with each other.

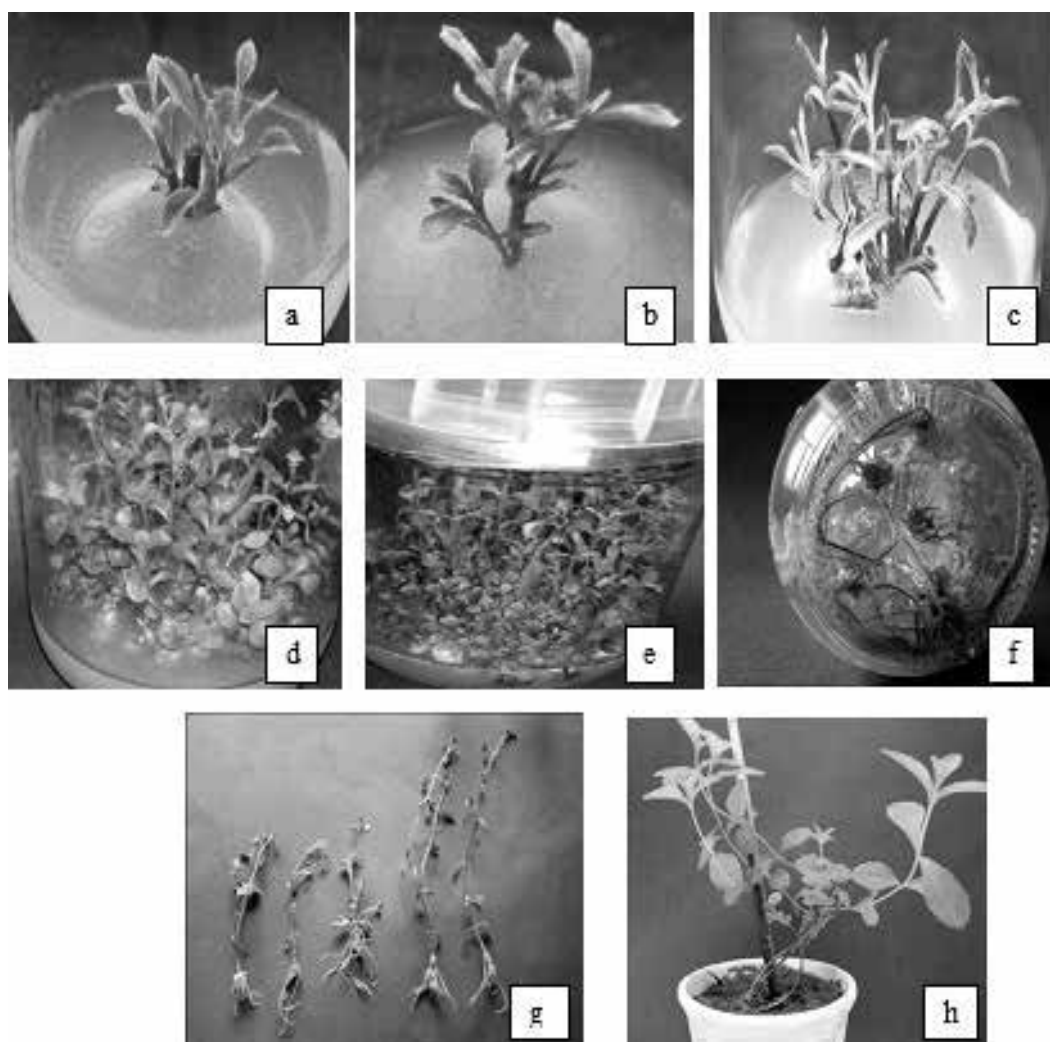
## RESULTS AND DISCUSSION

Stevia varied in shoot induction response to different media used and best response was observed

*Contd...*

on medium EM<sub>4</sub> (MS + BAP 2.0 mg/l) with maximum shoot induction ( $98.0 \pm 2.00\%$ ) in ( $4.7 \pm 0.20$ ) days using nodal explants and ( $74.0 \pm 2.45\%$ ) shoot induction in ( $7.1 \pm 0.34$ ) days using shoot tip explant (Table 1, Fig. 1a & 1b). Anbazhagan *et al.* (1) also observed the similar results on MS medium supplemented 2.0 mg/l BAP with the maximum shoot induction using nodal explant and found BAP more effective than KIN. Hassanen and Khalil (5) also reported maximum shoot induction (91.3%) on MS medium supplemented with BAP, while Laribi *et al.* (8) reported maximum shoot induction (92%) on MS medium supplemented with 1.0 mg/l BAP along with 0.5 mg/l IBA. The regenerated shootlets were sub-cultured on MS medium fortified with 0.3 mg/l

BAP + 0.3 mg/l KIN + 0.1 mg/l NAA and different concentrations Poly ethylene glycol (PEG) (Table 2). The maximum ( $40.5 \pm 0.26$ ) shoots/explant were reported on medium SM<sub>23</sub> fortified with MS medium 0.3 mg/l BAP + 0.3 mg/l KIN + 0.1 mg/l NAA + 15 mg/l PEG (Fig. 1c - e) on 30<sup>th</sup> day of subculture. The media supplemented with combination of BAP, KIN, NAA along with PEG were more effective for *in vitro* multiplication of stevia amongst all media tested. The effect of PEG on production of strong and thick *in vitro* shoots was also observed with more survival rate during acclimatization as compared to other media on which shoots showed the symptoms of vitrification. This is the first report on effect of PEG on *in vitro* multiplication and inhibition of vitrification

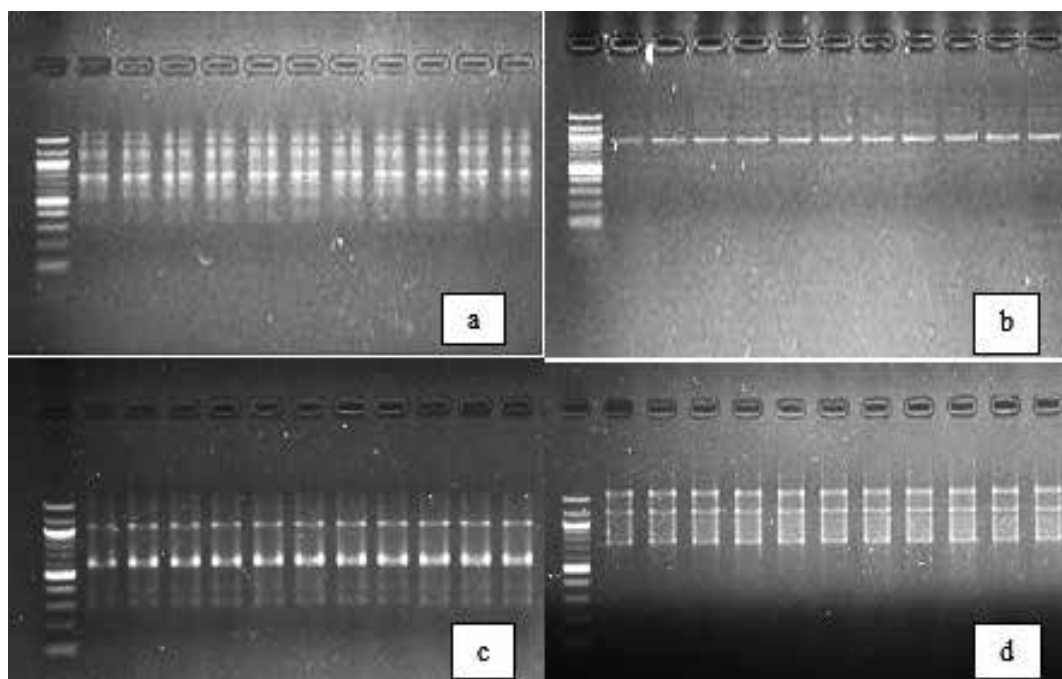


**Fig. 1.** *In vitro* propagation of stevia (a) Shoot induction from nodal explant on EM<sub>4</sub> medium, (b) Shoot induction from shoot tip explant on EM<sub>4</sub> medium, (c) *In vitro* shoot multiplication on SM<sub>23</sub> medium after 7<sup>th</sup> day of culturing, (d) Shoot multiplication on SM<sub>23</sub> medium after 15<sup>th</sup> day of day, (e) Shoot multiplication on SM<sub>23</sub> medium after 30<sup>th</sup> day, (f and g) *In vitro* rooting from regenerated shoots on RM<sub>4</sub> medium, (h) Hardened plant of stevia in pot

on micropropagation of stevia. Mirniam *et al.* (11) reported the maximum shoot proliferation on the MS medium supplemented with 1.5 mg/l BAP along with 0.1 mg/l NAA. On the contrary, Hassanen and Khalil (5) reported that MS medium supplemented with 2.0 mg/l BAP recorded the maximum number of shoots of 43.9 shoots/ explant but these shoots were very thin containing many lateral shoots and low survival rate during acclimatization. The elongated shoots were transferred to 1/2 MS liquid medium fortified with different concentrations of NAA and IBA (Table 3). Profuse rooting was observed after three weeks and maximum  $90\% \pm 7.75$  rooting along with  $21.2 \pm 0.37$  number of roots and  $7.4 \pm 0.26$  av. number of days to rooting was observed on 1/2 MS liquid medium supplemented with 0.5 mg/l NAA (Fig. 1f & g). Similarly, Kittisak and Dheeranupattana (7) also reported that root numbers varied with different concentrations of IBA, IAA and NAA supporting the results. On the contrary, Jena *et al.* (6) reported maximum rooting on half-MS + 0.5 mg/l IAA. *In vitro* rooted plantlets were successfully transferred in greenhouse with 100% survival (Fig. 1h).

Clonal fidelity of *in vitro* raised clones was tested using RAPD markers. For this purpose, 10 hardened plants were chosen randomly from the *in vitro* raised population of 522 plants and compared with the mother plant from which the explants were taken. Out of which 24 primers showed amplification with the

DNA of mother plant and were used to study genetic fidelity. The number of scorable bands for each RAPD primer varied from 1 (OPA-20, AY5) to 7 (OPA-03) (Table 4). The 24 RAPD primers produced 82 distinct and scorable bands, with an av. of 3.4 bands per primer. The RAPD amplicon ranging in size from 100 (OPE-2) to 1100 bp (OPA-15). No polymorphism was detected during the RAPD analysis of *in vitro* raised plants (Fig. 2). All *in vitro* raised plants were found genetically stable and similar to mother plants in monitoring variability in the DNA sequences of the plants (Bhattacharya *et al.*, 2). No polymorphism was detected during the RAPD analysis of *in vitro* raised clones. Lata *et al.* (10) reported absence of genetic variation in stevia and found that the profiles of micropropagated plants were monomorphic and comparable to mother plants, confirming the genetic stability among micropropagated plants and mother plant. In contrast, somaclonal variations were reported in micropropagated plants of stevia by Moktaduzzaman and Rahman (13), and Hassanen and Khalil (5) using RAPD primers. Genetic fidelity testing of stevia using RAPD primers revealed that all the plants raised through *in vitro* propagation were true-to-the type and the protocol standardized for *in vitro* propagation is better than earlier findings by Hassanen and Khalil (5) as in the present study micropropagated shoots were more in number, thick, green in colour and healthy. Genetic fidelity testing



**Fig. 2.** RAPD profile of mother plant (lane-2) and ten *in vitro* raised plants (lane 3-12) of *stevia rebaudiana* Bertonii generated for Genetic Fidelity testing using primer OPA03 (a), AY5 (b), CPE-4 (c), and CPE-4 (d)

of stevia using RAPD primers revealed that all the plants raised through *in vitro* propagation were true-to-the-type and the protocol standardized for *in vitro* propagation was better than earlier findings by Hassanen and Khalil (5) as in the present study micropropagated shoots were more in number, thick, green in colour and healthy. No polymorphism was detected during the RAPD analysis of *in vitro* raised clones. Therefore, it has been concluded that this is first report that has studied the effect of PEG on prevention of vitrification in stevia and produced genetically stable plants.

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