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# Regeneration of *Stevia rebaudiana* and Analysis of Somaclonal Variation by RAPD

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**Abstract:** The objectives of this experiment were to develop the optimal concentration of auxin and cytokinin for regeneration of *Stevia rebaudiana* and finally analysis somaclonal variation by RAPD. Various degrees of callus induced from the leaf segments cultured on MS medium supplemented with the different concentrations and combinations of NAA+BA and 2,4-D +BA. Among them, 1.5 mg $L^{-1}$ NAA with 1.0 mg $L^{-1}$ BA was the best for callus induction (91.67%) which also produced highest fresh weight (621.7 mg) and dry weight (79.00 mg) of callus. For shoot formation, calli were transferred on to MS medium supplemented with different concentrations and combinations of BA and NAA with control. The highest number of shoots (2.17) and the highest average length of the shoot (3.22 cm) per culture was observed at 1.8 mg $L^{-1}$ of BA with 0.12 mg $L^{-1}$ of NAA. The regenerated shoots were then transferred to MS liquid medium supplemented with same concentration of IBA and NAA. All the treatments produced roots and 1.50 mg $L^{-1}$ IBA produced highest percentage of root (93.33%), but 1.00 mg $L^{-1}$ NAA produced highest number (no. 7.66) of roots and highest length of roots (13.33 cm) per culture. The regenerated plantlets were successfully transferred into pots containing 75% soil and 25% sand and finally transferred into the field. Apparently somaclonal variations were examined among regenerated plants along with mother plant by RAPD. DNA samples from mother plant and 9 randomly selected regenerated plants were subjected to RAPD analysis. Bands generated through RAPD-PCR were scored according to whether they were present (1) or absent (0) to determine the extent of somaclonal variation. The estimation of genetic similarity coefficient based on RAPD band-sharing data analyzed indicated that some regenerated plants were 100% similar to the mother plants and some were 71, 57 or 14% similar may be due to variation *in vitro* condition.

**Key words:** Callus, shoot, root, regenerated plants, transplantation, somaclonal variation, RAPD

## INTRODUCTION

*Stevia rebaudiana* Bert. is a natural, non-caloric, sweet-tasting plant used around the world for its pleasant taste. The sweet herb of Paraguay, Stevia produces sweeteners in it's leaves are natural plant products (Starratt and Gijzen, 2004). Leaves of this plant produce zero-calorie ent-kaurene diterpene glycosides (stevioside and rebaudiosides), a non-nutritive, high-potency sweetener and substitute to sucrose, being 300 times sweeter than sucrose. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects. Stevia is likely to become a major source of high potency sweetener for the growing natural food market in the future (Starratt and Gijzen, 2004). Seed germination of Stevia is often poor. Therefore, there are basically two options for multiplication; tissue culture and steam cutting.

It is now evident that plant tissue culture as an essential component of Plant Biotechnology which offers novel approaches to the production, propagation, conservation and manipulation of plants (Thorpe, 1993). The success of *in vitro* culture depends mainly on the growth conditions of the source material (Caswell *et al*., 2000; Delporte *et al*., 2001), medium composition and culture conditions (Saharan *et al*., 2004) and on the genotypes of donor plants.

Plant may be regenerated directly or indirectly through callus formation. For callus induction both auxin and cytokinin are supplemented with media. Cytokinins are often used to stimulate growth and development kinetin, BAP, 2ip BA being in common use. They usually promote shoot differentiation, especially if added together with auxin. In higher concentrations (1-10 mg $L^{-1}$) they can induce adventitious shoot formation, but root formation is generally inhibited.

Shoot apex, nodal and leaf explants of *Stevia rebaudiana* Bertoni can regenerate shoots when culture on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) and indole-3-acetic acid (Sivaram and Mukundan, 2003). It was reported that the leaves of *S. rebaudiana* produced callus when

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supplemented with various concentrations and combinations of auxins (Khanam *et al*., 2005) and also nodal segments as explants were used for micropropagation of *S. rebaudiana* (Mitra and Pal, 2007).

Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in from organized growth, the genotype, growth regulators and tissue source (Karp, 1995). The main factors that influence the variation generated from tissue culture are the degree of departure.

Identification of possible somaclonal variants at nearly stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism (Welsh and McClelland, 1990; Williams *et al*., 1990) has found successful application in describing somaclonal variability in regenerated individuals of several plant species (Isabel *et al*., 1993; Munthali *et al*., 1996; Hashmi *et al*., 1997).

The main objectives of this present study were to develop the optimal concentration and combination of auxin and cytokinin for callus initiation and shoot regeneration and to develop optimal concentration of auxin for root formation from regenerated shoots then established the regenerated plants of *S. rebaudiana* and apparently analysis the somaclonal variation among regenerated plants obtained through in vitro culture of *Stevia rebaudiana* by RAPD.

## MATERIALS AND METHODS

This experiment was conducted at September 2006 to August 2007 in Plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh.

Leaves were taken from 2-4 month old plant of *Stevia rebaudiana* Bert. and cleaned thoroughly under a running tap water for 7-10 min. Then surface sterilization was done with 0.1% $HgCl_2$ for 5 min followed by washing (3-4 times) with sterile water.

Explants of approximately 1 $cm^2$ in length were cut and inoculated aseptically onto MS medium supplemented with different concentrations and combinations of auxin and cytokinin (NAA+BA as 0.1+1, 0.5+1, 1+1, 1.5+1, 2+1 mg $L^{-1}$ and 2, 4-D+BA as 0.1+1, 0.5+1, 1+1, 1.5+1, 2+1 mg $L^{-1}$) for callus induction.

The MS basal medium supplemented with different concentrations and combinations of growth regulator were prepared for callus induction, shoot formation and root formation. After mixing all stock solutions and growth regulators at appropriate volume, 3.5% sucrose was added. The pH of the medium was adjusted to 5.7-5.8 and the agar (0.7%) was added and dissolves by heat. The media were dispensed in the glass test tube in a volume of 14-16 mL. The media were sterilized by autoclaving at 121°C at 15 psi pressure for 20 min.

For shoot formation 19-22 days calli of approximately 0.5-0.75 $cm^2$ in size were cut and transferred onto MS medium supplemented with different concentrations and combinations of auxin and cytokinin (control and NAA+BA like 1:15 as 0.034+0.5, 0.054+0.8, 0.067+1, 0.1+1.5, 0.12+1.8, 0.134+2 mg $L^{-1}$).

Regenerated shoots of approximately 1.0-1.5 cm length were aseptically rescued from culture test tube and transferred to freshly prepared MS liquid (without agar) medium supplemented with various concentrations of IBA and NAA (1.0, 1.5 and 2.0 mg $L^{-1}$) for root formation.

All cultures were grown in an air-conditioned culture room illuminated by 40 w white florescent tubes light with intensity varied from 2000-3000 lux. The temperature of the culture room was maintained around 25°C. The photoperiod was maintained as 16 h light and 8 h darkness.

From the fourth day of inoculation, regular visual observation was done up to 25 days record the callus data, 7 days upto 36 days record the shoot and root data. Data presented in tables were analyzed using Dancans Multiple Range Test (DMRT) by MSTAT_C.

Rooted plantlets were kept in a room of normal temperature (30±2°C) at normally day lights for 7 days. Plantlets were taken out from the culture tubes and washed carefully under running tap water for complete removal of media. Then the plantlets were transplanted to small plastic pots containing 25% sand and 75% soil and sand were sterilized by heat. The pots were immediately placed to the small propagator which prevents desiccation. The plant established there and finally regenerated plants were transferred to the field.

**Analysis of somaclonal variation:** About 70 regenerated plantlets of *S. rebaudiana* were produced from one mother plant through in vitro callus culture in laboratory condition. Among the regenerated plant 9 samples along with their mother plant, were randomly selected and used as experiment material.

DNA was extracted from leaves of regenerated Stevia plants and the mother plant by modified CTAB method. Primers were used in this study A03 (5'-TGCCTCGCACCA-3') and S7 (5'-GGTGACGCAG-3'). Polymerase Chain Reaction (PCR) was carried out in presence of 6 ng of genomic DNA, PCR sumpermix 0.5 µL

(supplied by Invitrogen company), 0.5 µL (2.5 units) of taq DNA polymerase, 2.0 µL (400 picomol) of primer, 0.5 µL of $MgCl_2$ and 2.7 µL of sterile water (in a total volume of 10 µL). The sample was overlayed with one drop of mineral oil. The PCR amplification was performed in automated thermal cycler programmed for 45 cycles consists of 2.0 min denaturation at 94°C; 1 min annealing at 36°C, 2 min elongation at 72°C, followed by a final extension step at 72°C for 5 min. The PCR amplified products were subjected to electrophoresis in a 1% agarose gel at a constant voltage of 50 V. After electrophoresis, the gel was stained with ethidium bromide and photograph taken under UV transilluminator.

**Data scoring and analysis:** The RAPD bands were scored as the presence (1) or absence (0) of polymorphic DNA fragments. Genetic similarity, genetic distance were calculated according to Nei's (1972) by using POPGENE (Version 1.31) computer program.

## RESULTS AND DISCUSSION

**Callus induction:** A number of treatments with different concentration and combination of auxins (NAA, 2,4-D) and cytokinin (BA) were employed for callus induction. Data were recorded after 24 days of culture. Twelve test tubes were selected randomly from 36 test tubes of each treatment. Then calli were washed and remove the water molecule using tissue paper and fresh weight data was recorded. For dry weight, calli were wrapped with grown paper and store for dry. The dry weight was recorded after 25 days (Table 1). It showed that 1.5 mg $L^{-1}$ NAA with 1.0 mg $L^{-1}$ BA was the best for this purpose (Fig. 1a-f). This treatment produced 91.67% callus and also produced highest fresh weight (621.7 mg) and dry weight (79.00 mg) per culture. Other researchers, Uddin *et al.* (2006) found highest amount of callus in MS media with 3 mg $L^{-1}$ 2,4-D and Khanam *et al.* (2005) found best initiation of callus in MS media with 2 mg $L^{-1}$ BA in light or 2 mg $L^{-1}$ BA with 2 mg $L^{-1}$ NAA in dark. But this experiment showed best performance for callus induction by using low concentration of NAA (1.5 mg $L^{-1}$) with BA (1.0 mg $L^{-1}$).

Table 1: Effect of different concentration and combination of auxin (NAA and 2,4-D) and cytokinin (BA viz 1.0 mg $L^{-1}$) on callus induction

| Growth regulators | Concentration (mg $L^{-1}$) | Callus induction (%) | Weight (mg) Fresh | Weight (mg) Dry |
|---|---|---|---|---|
| NAA+BA | 0.1+1.0 | 44.44 | 255.33e** | 39.00e |
| | 0.5+1.0 | 63.89 | 348.33cd | 47.00cd |
| | 1.0+1.0 | 88.89 | 444.33b | 59.67b |
| | 1.5+1.0 | 91.67 | 621.66a | 79.00a |
| | 2.0+1.0 | 86.11 | 591.66a | 76.00a |
| 2, 4-D+BA | 0.1+1.0 | 44.44 | 193.33f | 30.33f |
| | 0.5+1.0 | 63.89 | 231.66ef | 29.00f |
| | 1.0+1.0 | 88.89 | 310.00d | 42.00de |
| | 1.5+1.0 | 91.67 | 452.00b | 58.67b |
| | 2.0+1.0 | 86.11 | 376.66c | 50.67c |

**Values within column followed by different letter(s) are significantly different by DMRT at p = 0.05

**Shoot formation:** A number of treatments of cytokinin (BA) ranging from 0.5 - 2.0 mg $L^{-1}$ (viz., 0.0, 0.5, 0.8, 1.0, 1.5, 1.8 and 2.0 mg $L^{-1}$) with auxin (NAA) ranging from 0.034- 0.134 mg $L^{-1}$ (viz., 0.00, 0.034, 0.054, 0.067, 0.100, 0.120 and 0.134 mg $L^{-1}$) were employed for shoot formation (Table 2). Mitra and Pal (2007) obtained higher proliferation of shoot by using nodal segment of *S. rebaudiana* in MS media supplemented with 1.0 mg $L^{-1}$ IAA plus 10 mg $L^{-1}$ Kinetin and 30 mg $L^{-1}$ adenine sulphate. Sivaram and Mukundan (2003) regenerated shoots from shoot, apex, nodal and leaf explants of *S. rebaudiana* in MS medium supplemented with BA (8.87 µM) and IAA (5.71 µM). From this experiment it was evident that best result obtained from 1.8 mg $L^{-1}$ of BA with 0.12 mg $L^{-1}$ of NAA which produced 62% shoots

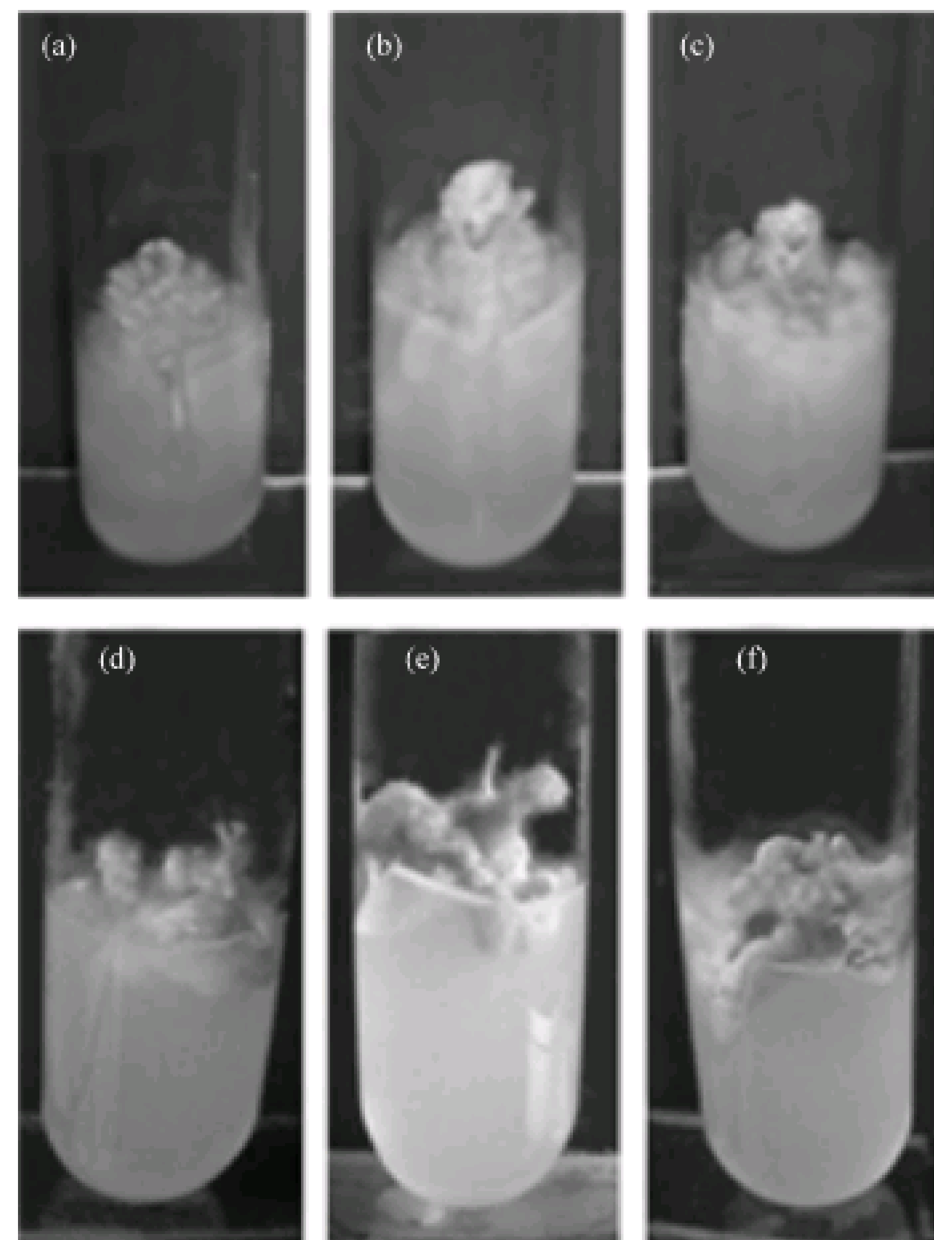


Fig. 1: Callus induction in MS medium. (a) 1.0 mg $L^{-1}$ 2,4-D and 1.0 mg $L^{-1}$ BA, (b) 1.5 mg $L^{-1}$ 2,4-D and 1.0 mg $L^{-1}$ BA, (c) 2.0 mg $L^{-1}$ 2,4-D and 1.0 mg $L^{-1}$ BA, (d) 1.0 mg $L^{-1}$ NAA and 1.0 mg $L^{-1}$ BA, (e) 1.5 mg $L^{-1}$ NAA and 1.0 mg $L^{-1}$ BA and (f) 2.0 mg $L^{-1}$ NAA and 1.0 mg $L^{-1}$ BA

Table 2: Effect of different concentration and combination of cytokinin (BA) and auxin (NAA) on shoot formation

| Growth regulators | Concentration (mg $L^{-1}$) | Shoot formation (%) | No. of total shoot/culture | Average length of shoots/culture (cm) |
|---|---|---|---|---|
| BA+NAA | Without GR | * | * | * |
| | 0.5+0.034 | * | * | * |
| | 0.8+0.054 | * | * | * |
| | 1.0+0.067 | 17 | 0.17b** | 0.28b |
| | 1.5+0.100 | 17 | 0.17b | 0.17b |
| | 1.8+0.120 | 62 | 2.17a | 3.23a |
| | 2.0+0.134 | 45 | 1.45ab | 1.34b |

*Indicates no response or result. **Values within column followed by different letter(s) are significantly different by DMRT at p = 0.05

Table 3: Effect of IBA and NAA in MS liquid medium for root formation

| Growth regulators | Concentration (mg $L^{-1}$) | Shoot formation (%) | No. of total shoot/culture | Average length of shoots/culture (cm) |
|---|---|---|---|---|
| IBA | 1.00 | 60.00 | 4.530ab** | 1.670b |
| | 1.50 | 93.33 | 7.330a | 2.200b |
| | 2.00 | 20.00 | 1.670b | 0.370c |
| NAA | 1.00 | 86.67 | 7.670a | 13.33a |
| | 1.50 | 60.00 | 10.07a | 2.030b |
| | 2.00 | 60.00 | 7.730a | 2.400ab |

**Values within column followed by different letter(s) are significantly different by DMRT at p = 0.05

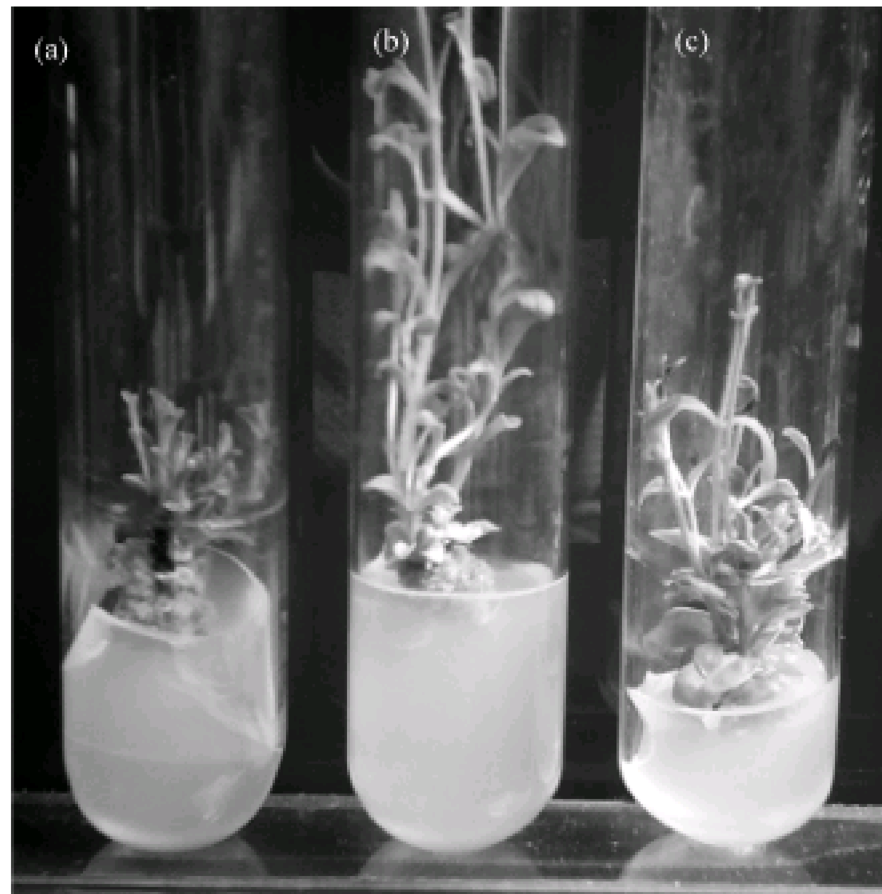


Fig. 2: Shoot regeneration on MS medium, (a) 1.8 mg $L^{-1}$ BA and 0.120 mg $L^{-1}$ NAA, (b) 2.0 mg $L^{-1}$ BA and (c) 0.134 mg $L^{-1}$ NAA

formation (Fig. 2a-c) and showed highest number of shoots (2.17) and highest average length of the shoot (3.22 cm) per culture.

**Root formation:** Shoots obtained *in vitro* have to be rooted to obtained complete plants. The regenerated shoots (1.0-1.5 cm) were transferred to MS liquid medium with various concentrations of IBA and NAA (viz., 1.0, 1.5 and 2.0 mg $L^{-1}$) for root induction. Data was recorded after 36 days of culture (Table 3). From all the treatments

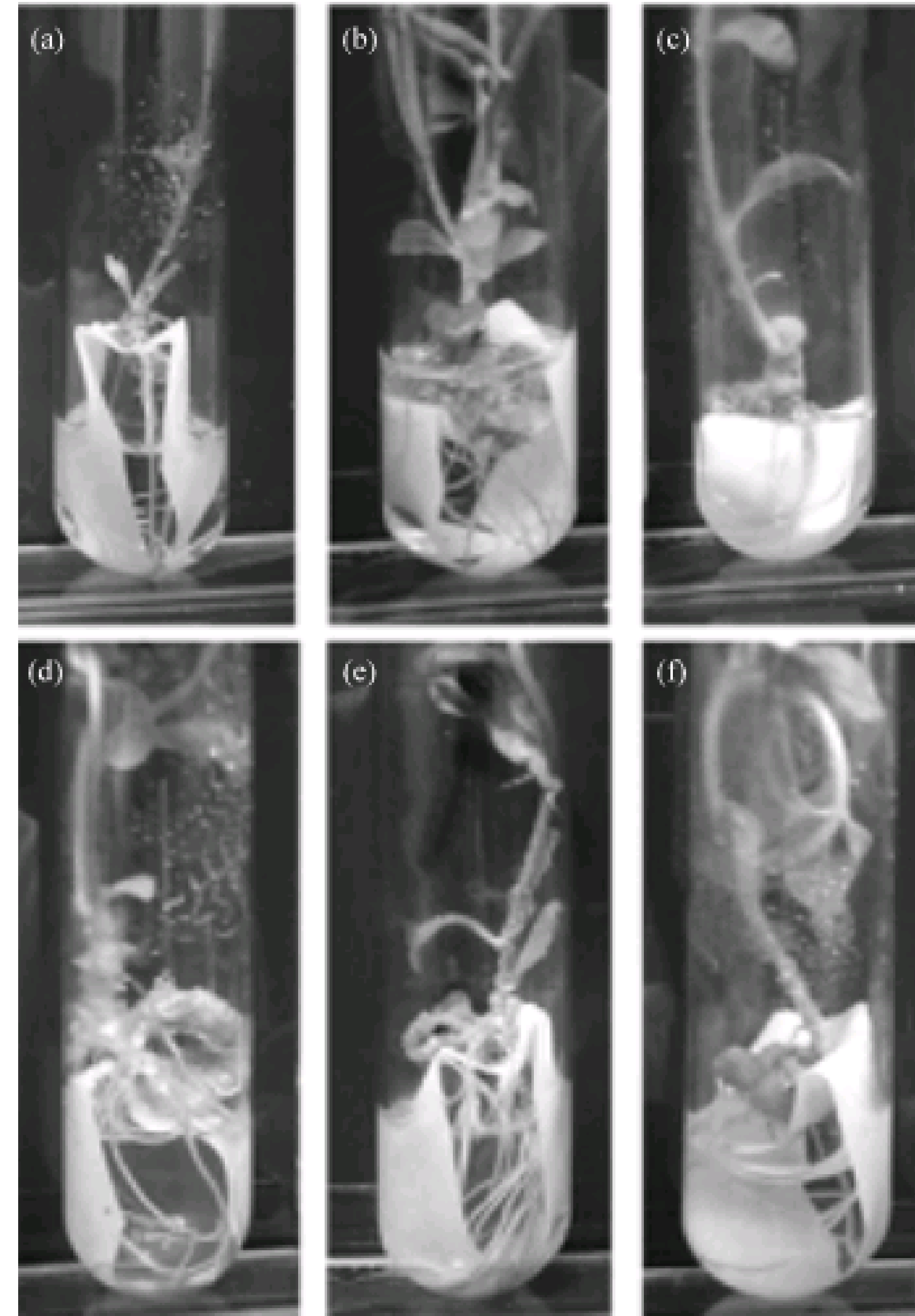


Fig. 3: Root formation on MS liquid medium supplemented with IBA, (a) 1.0 mg $L^{-1}$ IBA, (b) 1.5 mg $L^{-1}$ IBA, (c) 2.0 mg $L^{-1}$ IBA, (d ) 1.0 mg $L^{-1}$ NAA, (e) 1.5 mg $L^{-1}$ NAA and (f) 2.0 mg $L^{-1}$ NAA

1.50 mg $L^{-1}$ IBA produced highest percentage of root formation 93.33% (Fig. 3a-f), where 1.00 mg $L^{-1}$ NAA produced 86.67%. But 1.00 mg $L^{-1}$ NAA produced highest number (No. 7.66) of roots and highest length of roots (13.33 cm) per culture where 1.5 mg $L^{-1}$ IBA produced No. 7.33 and 2.20 cm, respectively (Fig. 3). Mitra and Pal (2007), obtained 90% of profuse roots from regenerated shoots within 4 weeks of inoculation on half strength of MS solid medium supplemented with 1.0 mg $L^{-1}$ IAA.

**Transplantation:** Rooted plant required transplantation, so the plantlets were transplanted to small plastic pots (Fig. 4a) containing 75% soil and 25% sand (soil and sand were sterilized by heat). The pots were immediately transferred to the small propagator (Fig. 4b) which prevents desiccation. Finally established regenerated plants were transferred to the field (Fig. 4c). About 75% plants were successfully grown.

Table 4: Genetic identity or similarity coefficient of regenerated plants and mother plants were shown based on RAPD profile

| | MP | RP 1 | RP 2 | RP 3 | RP 4 | RP 5 | RP 6 | RP 7 | RP 8 | RP 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| MP* | **** | 0.7143 | 0.4286 | 0.1429 | 1.0000 | 0.1429 | 0.5714 | 1.0000 | 0.5714 | 0.5714 |
| RP 1 | | **** | 0.4286 | 0.4286 | 0.7143 | 0.4286 | 0.2857 | 0.7143 | 0.2857 | 0.8571 |
| RP 2 | | | **** | 0.7143 | 0.4286 | 0.7143 | 0.8571 | 0.4286 | 0.8571 | 0.2857 |
| RP 3 | | | | **** | 0.1429 | 1.0000 | 0.5714 | 0.1429 | 0.5714 | 0.2857 |
| RP 4 | | | | | **** | 0.1429 | 0.5714 | 1.0000 | 0.5714 | 0.5714 |
| RP 5 | | | | | | **** | 0.5714 | 0.1429 | 0.5714 | 0.2857 |
| RP 6 | | | | | | | **** | 0.5714 | 1.0000 | 0.1429 |
| RP 7 | | | | | | | | **** | 0.5714 | 0.5714 |
| RP 8 | | | | | | | | | **** | 0.1429 |
| RP 9 | | | | | | | | | | **** |

*MP represents mother plant and RP 1 to RP 9 represents regenerated plant

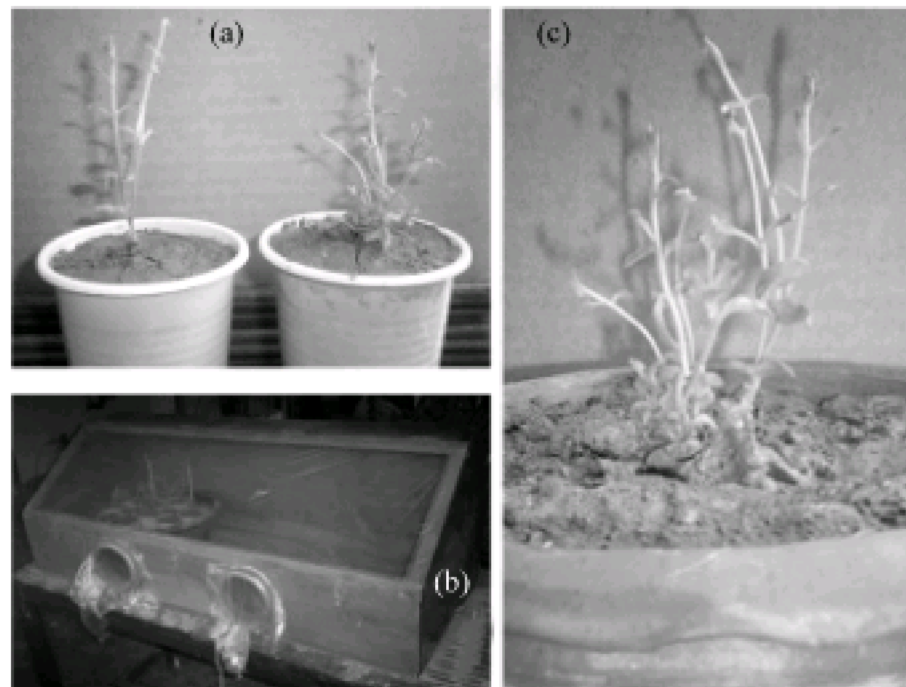


Fig. 4: (a) Transplantation of regenerated plantlets of *S. rebaudiana* Bert., (b) Transplanted plants were transferred into small propagator and (c) Established regenerated plant of *S. rebaudiana*

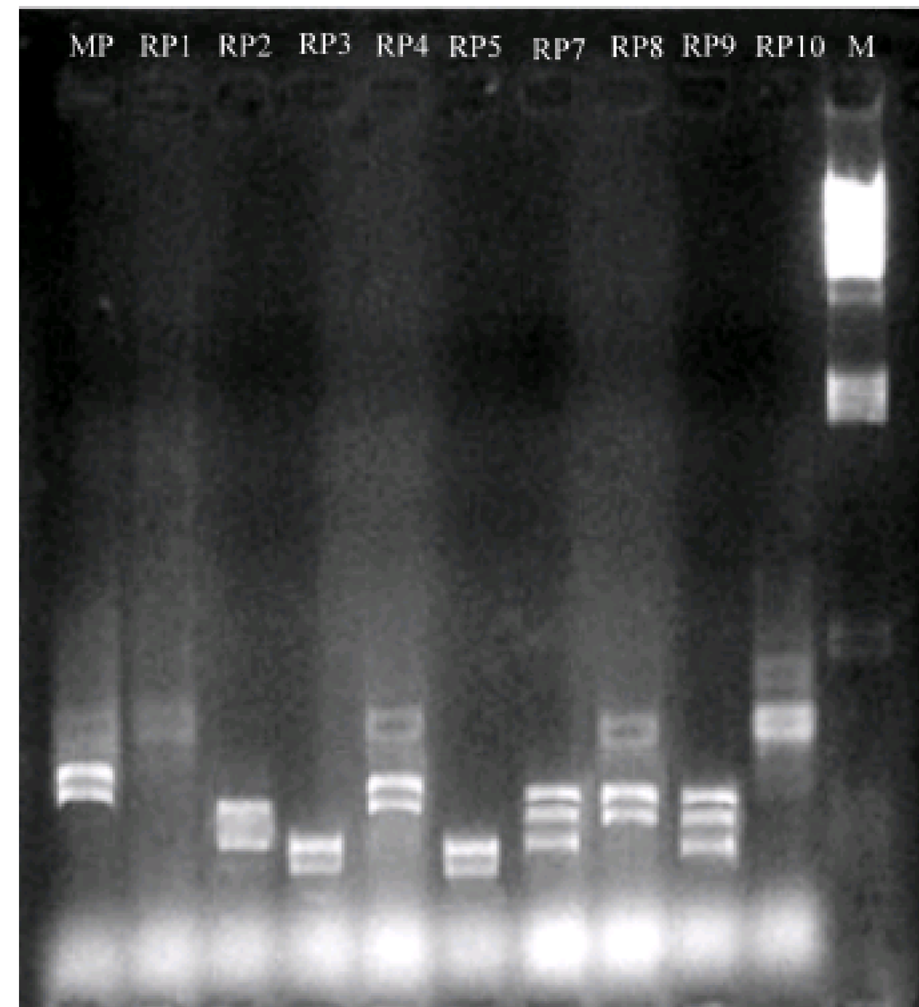


Fig. 5: Ten PCR products (9 random-selected regenerated plants and 1 mother plant) were run on the same gel. The banding patterns were produced by A03. *MP represents mother plant and RP 1 to RP 9 represents regenerated plants

**Analysis of somaclonal variation:** Detection of nucleotide sequence variability and its exploitation as a genetic marker have revolutionized many aspects of plant genetics. Several authors have applied the RAPD technique to investigate genetic variability and found it very efficient and reliable (Teixeira-Da-Silva *et al*., 2006).

We examined somaclonal variation amongst 9 random-selected regenerated plants along with mother plant by RAPD using 2 primers A03 and S7 (Supplied by Invetrogen company) Out of these primers, A03 successfully produced scoreable polymorphic RAPD bands. Ten PCR products (9 random-selected regenerated plants and 1 mother plant) were run on the same gel. The banding patterns were produced by A03. RAPD amplification is shown in Fig. 5.

Genetic similarity (Table 4) between the regenerated plants and the mother plant was scored by comparing their RAPD profile for the primer according to Nei's (1972) by using POPGENE (Version 1.31) computer program.

Analysis of the coefficient of genetic similarity among the different plants indicated that all the regenerated plants had varied degree of genetic difference from the mother plant, except for RP 4 and RP 7, which reported identical RAPD profiles. The genetic similarity between the mother and the regenerated plant was 100% for RP 4 and RP 7 and 71% for RP 1. The *in vitro* regenerated plants are maintained in Plant Biotechnology Laboratory of Khulna University, Bangladesh for further analysis.

## CONCLUSION

A number of treatments of auxin (NAA and 2,4-D) ranging from 0.1-2.0 mg $L^{-1}$ (viz., 0.1, 0.5, 1.0, 1.5, 2.0 mg $L^{-1}$) with cytokinin (BA 1.0 mg $L^{-1}$) were employed for callus induction. Among different treatments, 1.5 mg $L^{-1}$ NAA with 1.0 mg $L^{-1}$ BA was the best for

callus induction (91.67%) which also produced highest fresh weight (621.7 mg) and dry weight (79.00 mg) of callus. The calli were transferred in to different concentrations and combinations of cytokinin (BA) ranging from 0.0-2.0 mg $L^{-1}$ (viz. 0.0, 0.5, 0.8, 1.0, 1.5, 1.8 and 2.0 mg $L^{-1}$) with auxin (NAA) ranging from 0.034- 0.134 mg $L^{-1}$ (viz., 0.00, 0.034, 0.054, 0.067, 0.100, 0.120 and 0.134 mg $L^{-1}$) were employed for shoot formation (BA : NAA = 15 : 1). Among these, 1.8 mg $L^{-1}$ BA with 0.12 mg $L^{-1}$ NAA showed best result for shoot formation which produced highest number of shoots (2.17) and highest average length of the shoot (3.22 cm) per culture. For rooting the micro-cutting shoots were transferred to MS liquid medium supplemented with IBA and NAA both at the concentration of 1.0, 1.5, 2.0 mg $L^{-1}$. Data were recorded after 36 days of culture. From all the treatments 1.50 mg $L^{-1}$ IBA produced highest percentage of root formation (93.33%), but 1.00 mg $L^{-1}$ NAA produced highest number (no. 7.66) of roots and highest length of roots (13.33 cm) per culture. The regenerated plantlets were successfully transferred into pots containing 75% soil and 25% sand and established by using a propagator and finally transferred into the field ground.

Somaclonal variations were examined among regenerated plants along with mother plant by RAPD using A03 primer. The estimation of genetic similarity coefficient and genetic distance along with dendrogram (Without root and with root) based on RAPD band-sharing data indicated that some regenerated plants were 100% similar to the mother plants and some were 71, 57 or 14% similar may be due to variation in *in vitro* condition.

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## REFERENCES

Caswell, K.L., N. Leung and R.N. Chibbar, 2000. An efficient method for *in vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. Plant Cell Tissue Organ Cult., 60: 69-73.

Delporte, F., O. Mostade and J.M. Jacquemin, 2001. Plant regeneration through callus initiation from thin mature embryo fragments of wheat. Plant Cell Tissue Organ Cult., 67: 73-80.

Hashmi, G., R. Huettel, R. Meyer, L. Krusberg and F. Hammerschlag, 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Rep., 16: 624-627.

Isabel, N., L. Tremblay, M. Michaud, F.M. Tremblay and J. Bousquet, 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.) B.S.P. Theor. Applied Genet., 86: 81-87.

Karp, A., 1995. Somaclonal variation as a tool for crop improvement. Euphytica, 85: 295-302.

Khanam, S., P.E. Rajasekharan, K. Das, D. Raman and B.G. Shivananda, 2005. *In vitro* methods for production of stevioside from Stevia rebaudiana. Indian J. Nat. Prod., 21: 14-15.

Mitra, A. and A. Pal, 2007. *In vitro* regeneration of Stevia rebaudiana (Bert) from the nodal explant. J. Plant Biochem. Biotechnol., 16: 59-62.

Munthali, T., H.J. Newbury and B.V. Ford-Lloyd, 1996. The detection of somaclonal variants of beet using RAPD. Plant Cell Rep., 15: 474-478.

Nei, M., 1972. Nei's Original measures of genetic identity and genetic distance. Am. Nat., 106: 283-292.

Saharan, V., R.C. Yadav, N.R. Yadav and B.P. Chapagain, 2004. High frequency plant regeneration from desiccated calli of *indica* rice (*Oryza sativa* L.). Afr. J. Biotechnol., 3: 256-259.

Sivaram, L. and U. Mukundan, 2003. *In vitro* culture studies on *Stevia rebaudiana*. *In vitro* Cell. Dev. Bio. Plant, 39: 520-523.

Starratt, A.N. and M. Gijzen, 2004. Stevia Rebaudiana-Its Biological, Chemical and Agricultural Properties. Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre, Sandford St., London, Ontario.

Teixeira-Da-Silva, J.A., N. Singh and M. Tanaka, 2006. *Priming biotic* factors for optimal protocorm-like body and callus induction in hybrid *Cymbidium* (*Orchidaceae*) and assessment of cytogenetic stability in regenerated plantlets. Plant Cell Tissue Organ Cult., 84: 135-144.

Thorpe, T.A., 1993. Physiology and Biochemistry of Shoot Bud Formation *in vitro*. In: Advances in Developmental Biology and Biotechnology of Higher Plants. Soh, W.Y., J.R. Liu and A. Komamine (Eds.). The Korean Society of Plant Tissue Culture, Korea, pp: 210-224.

Uddin, S.M., S.H.M. Chowdhury, M.M.H.M. Khan, B.M. Uddin, R. Ahmed and A.M. Baten, 2006. *In viro* propagation of Stevia Rebaudiana Bert in Bangladesh Afr. J. Bioechnol., 5: 1238-1240.

Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.