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# A rapid and efficient protocol for *in vitro* multiplication of genetically uniform *Stevia rebaudiana* (Bertoni)

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*Stevia rebaudiana* (Bertoni), commonly called candy leaf or sweet leaf, endemic to South America, is an important medicinal plant. As a source of low calorie natural sweetener 'stevoside', it is used in obesity, diabetes, treatment of heartburn and tooth decay, and also serves as a food supplement. Large scale commercial propagation of *S. rebaudiana* demands a suitable protocol. Here, we propose an improved protocol for *in vitro* multiplication of *S. rebaudiana* from nodal explants. In this protocol, the effect of laboratory grade urea on multiple shoot induction from nodal explants was studied. The nodal explants were initially cultured on Murashige and Skoog (MS) basal media for 2 weeks which facilitated the axillary bud break. Further, culturing of these explants on MS medium fortified with 6 benzyl aminopurine (BAP) (2 mg/L) and Naphthalene acetic acid (NAA) (1 mg/L) with and without urea (5 mg/L) for a period of 40 days revealed maximum shoot production of 44.56 from a single nodal explant in media supplemented with urea as compared to 22.44 without urea. The differences in the number of shoots produced were significant and these shoots readily rooted in MS media with NAA (4 mg/L). Primary and secondary hardening was successful in these plants. There were no visible morphological abnormalities observed in the micropropagated plantlets. Genetic analysis from random samples also revealed that these plants are genetically uniform. The advantage of the present protocol is that the complete process of multiple shoot induction, rooting and hardening could be completed within a period of 6 months as compared to the existing protocols.

**Keywords**: Candy leaf, Genetic uniformity, *In vitro* regeneration, Micropropagation, RAPD, Urea

The prevalence of diabetes and obesity among people worldwide has fuelled the increased interest of researchers on alternatives to sugar. In this regard, the Candy leaf or Sweet leaf, *Stevia rebaudiana* (Bertoni) (Fam.: Asteraceae), of South American origin gained attention as source of the safe natural sweetener 'stevoside' known to be 200-300 times sweeter than sucrose[1]. The plant is used by the tribes of Paraguay and Brazil for the treatment of heartburn[2]. Its dried leaves are also mixed with beverages or bakery products instead of sugar[3]. Due to its non carbohydrate nature, it is safe for diabetes and obese people who require low calorie diet[3]. Further, stevoside ameliorates tooth decay, a common health issue associated with sugary foods[3]. Countries, such as Japan, Korea and Brazil have already approved the *Stevia* extract as food supplement[3]. In 2008, US Food and Drug Administration (FDA), has declared *Stevia* leaves as safe (GRAS, Generally regarded as safe) food additive and table top sweetener[4].

Global acceptance of stevoside as a safe sweetener has enhanced its demand and prompted its large scale cultivation. Khalil *et al*.[5] reported that *Stevia* seeds show poor germination (25%) and also loses its viability on storage. Moreover, cross pollinated nature of the plant does not favour the uniform stand of plants, and therefore, rooted cuttings are used for propagation. Propagation by cuttings showed 33% survival rate, but with the tagged seasonal effect on the regeneration of cuttings[5]. These problems can be overcome by micropropagation using tissue culture. However, in the *in vitro* culture of plant tissues, high concentrations of plant growth regulators cause somaclonal variation[6], and tools must therefore be used to test genetic changes. Although these variations are sometimes deliberately sought to obtain new varieties or desirable traits[6], genetic instability is undesirable when the aim is to multiply the genetically homogeneous selected elite plants. Genetic homogeneity of the tissue cultured plants can be ascertained using DNA markers. Several DNA markers are available and have been used in various crops and one such marker, random amplified polymorphic DNA (RAPD) offers the advantage of being simpler to use, less expensive and less time-consuming without requiring any prior sequence information. It has been successfully used to detect somaclonal variation in tissue cultured plants[7,8]. *Stevia* micro propagation has been reported by several researchers[9,10]. When we compare our results

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with those that of others we find that the process reported by us gives a high shoot multiplication rate within less time. The objective of the study is to find out the effect of urea on multiple shoot induction and genetic fidelity analysis of regenerated plants.

## Material and Methods

Authenticated plants of *Stevia rebaudiana* were obtained from National Bureau of Plant Genetic Resources (NBPGR), New Delhi and were maintained in the green house of IARI, New Delhi. Explants were collected from 6-month old plants. The 2nd and 3rd nodal portions (from the top) of size 2-3 cm with axillary bud were taken. The explants were first washed under running tap water for half an hour followed by washing in double distilled water containing 2-3 drops of Tween 20. The explants were further sterilised with 0.1% (w/v) mercuric chloride for 5 min under the laminar flow after which they were rinsed thrice with sterile distilled water. Subsequently, explants were into pieces ranging into pieces of 0.5-1.5 cm long and inoculated on to Murashige and Skoog (MS) media[11] with agar (Himedia) 7 g/L and sucrose (Himedia) 30 g/L in wide mouthed bottles and each bottle contained 40 mL media. The cultures were maintained in this media for 2 weeks. All cultures were incubated at 25±2°C with light (2000-3000 lux) and a photoperiod of 16 h per day for two weeks. Later, the explants were sub-cultured on two different media *viz*., MS media supplemented with BAP (2 mg/L) and NAA (1 mg/L) with and without urea (5 mg/L) for multiple shoot induction and allowed to remain in the same media for a period of 40 days. There were 3 replications for each treatment with 6 explants per replication. Regular observations were made every 10 days on the number of shoots developed, and the data was subjected to standard statistical analysis using MSTAT C version 1.41 software and are expressed as mean $\pm$ standard error. The well established shoots after 50 days were sub-cultured on MS media supplemented with NAA (4 mg/L) for rooting and maintained for 30 days. Later, plants with well developed roots were transferred to bottles containing autoclaved mixture of cocopit, perlite and vermicompost (2:1:1) for primary hardening and incubated in the same culture conditions for 2 weeks. Subsequently, they were transferred to plastic pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and shifted to the green house where in a temperature of 28±2°C and relative humidity of 65-75% was maintained. The plants were irrigated with tap water twice in a week. The plantlets were acclimatized after a period of 14 days.

After a month, 10 plants were taken at random from the greenhouse and the total DNA was extracted from leaf material following the CTAB method[12]. Quality and quantity of DNA was determined by spectrophotometer (Nanodrop, Thermo). Each sample was diluted to 5 ng/µL with TE (Tris–EDTA) buffer and stored at 4°C. Initially, a gradient PCR was performed with 30 random primers. Ten random primers which were showing consistent results were selected, and randomly amplified polymorphic DNA (RAPD) PCR was performed in a volume of 25 µL containing 1 µL DNA (15 ng), 10X reaction buffer (2.5 µL), 2.5 mM $MgCl_2$, 15 ng of 10-mer deoxynucleotide primer (Sigma), 0.5 units of Taq polymerase and 10 mM of dNTP's and 16.5 µL nuclease free water. Random primers from operon series OPA, OPB, OPG, and OPE were used for indexing polymorphism in tissue culture raised clones. The list of primers and the annealing temperature used is given in Table 1. The amplification reaction consisted of an initial denaturation step at 94°C for 5 min and 35 cycles comprising of denaturation at 94°C for 1 min, annealing at 1 min (temperature according to the primer) and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR amplifications were performed in a thermocycler (Eppendorf). The amplified product was resolved on 1.2 % agarose gel using TBE buffer and stained with ethidium bromide. Gels were visualized using a gel documentation system (Alpha Imager). The size of the amplicons was estimated by comparing with 100 bp DNA ladder (Fermentas).

Table 1—Details of random primers used in this study

| Primer Name | Sequence (5' - 3') | Temp. (°C) |
|---|---|---|
| OPB-20 | GGACCCTTAC | 38.3 |
| OPG-02 | GGCACTGAGG | 38.3 |
| OPG-03 | GAGCCCTCCA | 38.3 |
| OPG-04 | AGCGTGTCTG | 38.3 |
| OPG-06 | GTGCCTAACC | 40.9 |
| OPG-12 | CAGCTCACGA | 38.3 |
| OPG-17 | ACGACCGACA | 38.3 |
| OPE-18 | GGACTGCAGA | 38.3 |
| OPG-09 | CTGACGTCAC | 40.9 |
| OPG-19 | GTCAGGGCAA | 40.9 |

**Results and Discussion**

The explants after sterilisation were inoculated onto MS basal media and cultured for 2 weeks. During this period, bud break occurred in 80-90% of the explants (Fig. 1a). For testing the effect of urea on shoot multiplication, these nodal cuttings were sub-cultured on to MS media with BAP and NAA with and without urea and allowed to remain in the same media for a period of 40 days. The number of shoots that were obtained was counted regularly after a period of 10 days. The number of shoots that were formed gradually increased (Fig. 1b). After 10 days, the number of shoots that were formed in both the media i.e., with and without urea were 3.56 and 5.50 and not significantly different (Fig. 2). The significant difference in the number of shoots produced was observed after 20 days when there were 22 shoots in media without urea and 25 in media with urea. After 30 days , it was observed that the number of shoots were double in media with urea as compared to media without urea (Fig. 2). There was no increase in the number of shoots beyond 30 days in MS media with urea. After 50 days of culture, the plantlets were ready for sub-culture.

The multiple shoots were separated out and transferred to MS media with NAA (4 mg/L) for rooting. After a period of 4 weeks, the roots were developed and there was 100% rooting (Fig. 1 c-d). The rooted plantlets were taken out from the culture and washed with water carefully to remove the traces of agar and they were transplanted in glass containers (bottles of 500 ml capacity) containing autoclaved mixture of cocopit, perlite and vermicompost (2:1:1) for primary hardening and incubated in the same culture conditions. There was 100% survival of plants in this condition and the growth of the plants was also vigorous and they emerged out of the containers (Fig. 1 e-f). The well grown plants were then transferred to plastic pots containing sterile mixture of sand, soil and vermicompost (1:2:1) and transferred to the polyhouse (Fig. 1 g-h). The plantlets were irrigated with tap water twice in a week for a total period of 2 weeks to complete the acclimatization process. There was 80% survival of plants during secondary hardening.

There were no visible morphological abnormalities observed in these plants. Out of 350-400 plants that were regenerated in this experiment, 10 plants were taken at random for RAPD analysis. Out of the total 30 primers screened, 10 primers resulted in consistent

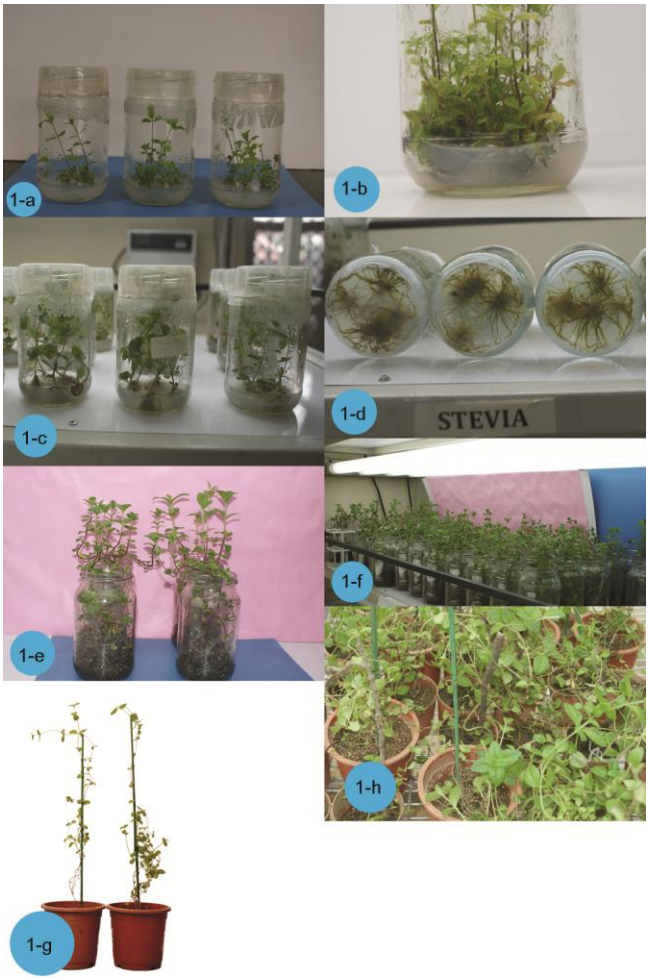


Fig. 1—Stevia tissue culture. (A) Bud break from nodal explants and multiple shoot initiation; (B) Profuse multiple shoot formation from nodal explants in MS with urea; (C and D) Rooting of *in vitro* plantlets in MS with NAA (4 mg/L); (E and F) Plantlets kept for primary hardening in jam bottles with autoclaved mixture of cocopit, perlite and vermicompost (2:1:1); and (G and H) Plantlets transferred to plastic pots and kept in poly-house for secondary hardening.

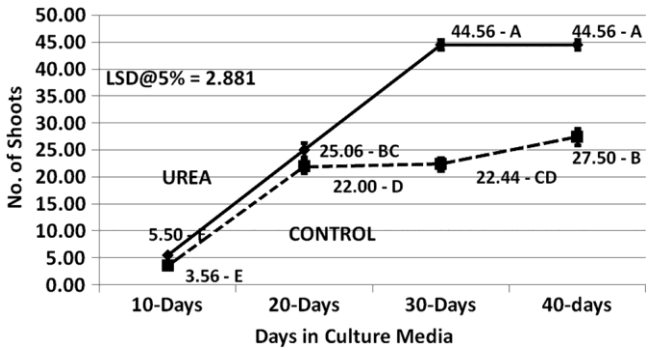


Fig. 2—Number of shoots obtained in MS with and without urea over a period of 40 days. [Data is the mean of 18 replicates per treatment. Mean followed by same letter is not significantly different]

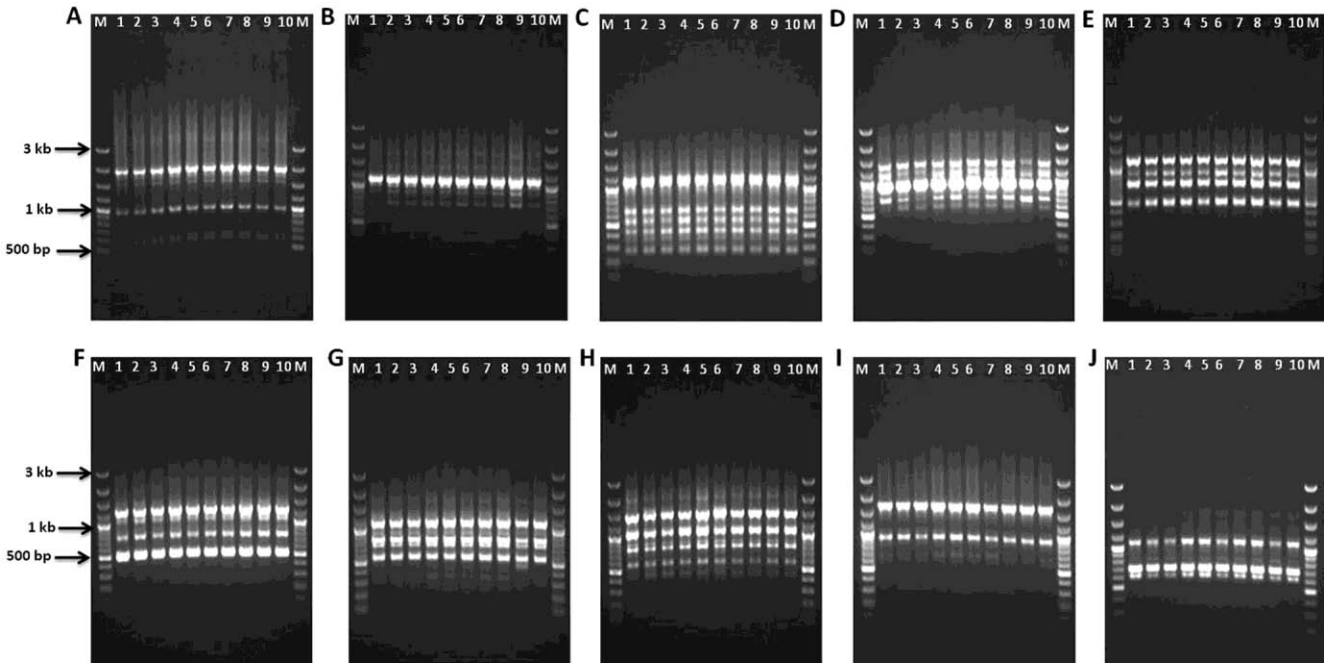


Fig. 3—RAPD for identification of somaclonal variations. [Lanes: M, 100-3000 bp DNA Ladder; 1-10, Plants regenerated from tissue culture. (A) OPG-02 (600 bp,1 kb, 2 kb); (B) OPG-03 (750 bp, 900 bp, 1.2 kb); (C) OPG-04 (250 bp, 450 bp, 550 bp, 650 bp, 1.2 kb); (D) OPG-06 (700 bp, 1 kb, 1.2 kb, 1.5 kb); (E) OPG-09 (500 bp, 700 bp, 1 kb, 1.2 kb); (F) OPG-012 (500 bp, 800 bp, 1.2 kb); (G) OPG-017 (600 bp, 800 bp, 1.2 kb); (H) OPG-019 (600 bp, 800 bp, 1 kb, 1.5 kb); (I) OPB-020 (700 bp, 900 bp, 1 kb, 1.7 kb); and (J) OPB-018 (600 bp, 700 bp, 1.1 kb)

amplification and were selected for further study. The amplification pattern obtained with all the primers is shown in Fig. 3. These 10 primers produced distinct amplification profiles and displayed monomorphic banding pattern in all the randomly selected micro propagated plants Fig. 3. A total number of 330 bands were produced in all the micropropagated plants. The number of bands per primer ranged 2-5. The band size ranged 250-1500 kb. The primer wise number of bands obtained and the size of the bands is provided in Fig. 3.

*Stevia* is a plant of commercial importance owing to its hypoglycaemic, body weight reducing and antimicrobial properties. Hence, studies on propagation of *Stevia* are being conducted and reported constantly. Recently, Khalil *et al.*[5] reported a callus mediated propagation which was successful. Here, our objective was to develop a protocol avoiding the callus phase so that the chances of genetic variability could be reduced. Most of the other studies on *Stevia* report the use of cytokinins either singly or in combination for multiplication of shoots from nodal explants. Jitender *et al.*[13] reported a maximum shoot multiplication rate of 3.42 from a single nodal explant in MS media with BAP (0.5 mg/L) and Kinetin (2.0 mg/L). Recently, Razak *et al*.[14] reported that micropropagation of *Stevia* in MS media with a combination of BAP and Kinetin has resulted in 8 shoots per explant. Singh *et al.*[15] reported that in B5 media a maximum of 4.25 shoots could be obtained from nodal explants using cytokinins like BAP (0.5 mg/L) and Kinetin (0.5 mg/L). Modi *et al.*[10] reported a protocol with a multiplication rate of 9.56 when explants were grown in a special container. Das *et al*.[16] reported a shoot proliferation protocol and 11 multiple shoots were obtained from a single shoot tip explants. Javed *et al*.[17]also reported BAP 1 mg/L gave a maximum of 15 shoots when shoot tips were used as explants. Sivaraman & Mukundan[18] have also reported regeneration of maximum 10 shoots from nodal explants in MS media with BAP and IAA.

In our study, we observed that a maximum of 44 shoots could be produced from a single nodal explant. This may due to the subculture after the bud break into media with urea. The availability of more nitrogenous sources in the media also might be the reason for such a significant multiple shoot production. It has been reported that for shoot

production, mostly adeninic cytokinins are used. However, urea derivatives such as thidiazuron, N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU), N-phenyl-N'-benzothiazol-6-ylurea (PBU), etc.[19-21] have been reported to induce shoot multiplication in many plant species. However, these compounds are expensive as compared to the lab grade urea which we have used in our study. The multiplication rate was 44 shoots per single nodal explants, and it indicates possibility of obtaining a total of 200-300 plantlets from a single plant. Moreover, the complete process of multiple shoot induction, rooting and hardening is completed within a period of 6 months. In this protocol, the hormones were minimal and there were no frequent subcultures. Hence, the chances of somaclonal variation is also reduced which has been proved by the genetic uniformity of the regenerated plantlets. Since *Stevia* is a plant of commercial importance any protocol which shows better multiplication rate will be acceptable.

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