

Original Research Article

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## Thidiazuron Induced Direct Shoot Organogenesis in *Stevia rebaudiana* and Assessment of Clonal Fidelity of Regenerated Plants by RAPD and ISSR

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### ABSTRACT

*Stevia rebaudiana* is a herbaceous perennial plant of Asteraceae family and an excellent substitute of sugar. Increasing demand of quality planting material has emphasized the need of rapid and mass multiplication through plant tissue culture technology. Therefore, high frequency mass multiplication of *Stevia* was established using *in vivo* and *in vitro* nodal explants. MS basal medium supplemented with 2.0 mg/l TDZ induced maximum shoot induction (68.3%) from *in vivo* nodal segments and 2.5 mg/l TDZ showed 100% shoot induction from *in vitro* nodal explants. The highest shoot multiplication (7.33 shoots/explant) was found on MS medium supplemented with 1.0 mg/l BAP+ 0.5 mg/l Kin. Root induction was performed on MS basal medium supplemented with different concentration of IAA, IBA and NAA. Quality root formation with respect to root length and root number per microshoots was observed on hormone free MS basal medium. Rooted plantlets were hardened in vermiculite and cocopeat in the ratio of 1:1 for 3 weeks and successfully acclimatized in field condition. Assessment of clonal fidelity of *in vitro* raised plantlets was performed by RAPD and ISSR molecular analysis which revealed that all the regenerated plants of *Stevia* through tissue culture were true-to-type compared to mother plants. The protocol developed in the present study could be used for production of approximately 2400 true-to-type plants in 12 week from single microshoot from 4 subcultures.

#### Keywords

Clonal fidelity, Molecular marker, *Stevia*, Tissue culture, TDZ (Thidiazuron).

#### Article Info

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### Introduction

*Stevia rebaudiana* Bertoni is a perennial herb of Astereace family which is native to the valley of Rio Monday in highlands of north-eastern Paraguay in South America. Leaves of *Stevia* produce diterpene glycosides (stevioside and rebaudiosides) which serve as high-potency sweeteners and substitute to sugar (Yadav *et al.*, 2011). The potential uses of *Stevia* which produces stevioside, a non-caloric sweetener that does not metabolize in the human body, hence control blood sugar

level (Gantait *et al.*, 2015). This is more important, especially in the context of the current social movement towards more natural foods.

In nature, seed germination in *Stevia* is poor and unsuccessful due to infertile seed and small endosperm (Shock, 1982; Carneiro *et al.*, 1997; Goettemoeller and Ching, 1999). Even some plant selections produce virtually no viable seed due to their self-

incompatibility (Yadav *et al.*, 2011). Numbers of reports are available on successful propagation of Stevia via stem cuttings. But direct planting of stem cuttings in the field has limited success due to poor rooting (Yadav *et al.*, 2011). Moreover, a huge number of stem cuttings are required for mass propagation of plants which is an obstacle as number of mother plant need to be vanishing. Hence, conventional methods are not adequate for commercial exploitation of Stevia plants and fulfill the current demand.

Micropropagation protocols of Stevia have been established using leaf (Sivaram and Mukundan, 2003; Sreedhar *et al.*, 2008; Jain *et al.*, 2009), nodal (Sung, 2006; Ahmed *et al.*, 2007; Laribi *et al.*, 2012; Modi *et al.*, 2012; Thiyagarajan and Venkatachalam, 2012; Hassanen and Khalil, 2013; Lata *et al.*, 2013; Singh and Dwivedi, 2013; Singh *et al.*, 2014) and shoot tips explants (Tamura *et al.*, 1984; Ibrahim *et al.*, 2008a; Ibrahim *et al.*, 2008b; Hassanen and Khalil, 2013), however, a major problem associated with *in vitro* culture is the possible occurrence of somaclonal variation through indirect organogenesis (Modi *et al.*, 2012; Singh *et al.*, 2014). Literatures revealed the occurrence of variability in tissue culture raised plants which did not produced true-to-type plants. Moreover, urgent need to the study about the effect of TDZ on *in vivo* and *in vitro* nodal explants of stevia for direct organogenesis and assessment of genetic fidelity of regenerated plants by molecular markers for commercial exploitation require prime consideration.

Therefore, the present investigation was emphasized on development of an efficient *in vitro* micropropagation protocol for high frequency mass multiplication of true-to-type plants and to detect the genetic uniformity of micropropagated plants of Stevia using molecular markers.

## **Materials and Methods**

### **Source and preparation of explants**

Nodal explants (2.0 cm) were collected from 6 month old plants growing at Hi Tech Horticulture Farm, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and technology, Udaipur. Explants were rinsed with sterile distilled water mix with few drops of Tween 20 for 10 min and rinsed thoroughly three times with sterile distilled water. Explants were disinfected by treating with 0.4 % bavastin for 15 minutes with constant swirling at 110 rpm on shaker. The solution was drained out and explants were washed properly 3-4 times with sterile distilled water. After that, explants were disinfected with freshly prepared 0.1 % Mercuric chloride solution for 6 minutes with constant swirling at 110 rpm and then rinsed 3-4 times with sterile distilled water. Tissue culture regenerated plants were also used for obtaining *in vitro* nodal explants.

### **Nutrient medium and laboratory condition**

Nodal explants were cultured on MS basal (Murasnige and Skoog, 1962) medium fortified with different concentration of TDZ for shoot induction and BAP + Kin for shoot multiplication. Shoots were transferred on MS basal medium nourished with various concentrations of IBA, IAA and NAA for root induction. All media were supplemented with 3% (w/v) sucrose and 0.8 % (w/v) agar (Hi-media, Mumbai). The pH of MS medium was adjusted to 5.80 prior to sterilization at 121°C for 15 minutes at 15 psi. The culture was incubated at 25±1°C with 16 h light/8 h dark photoperiod under the cool white fluorescent tubes (Crompton Greaves Limited, Mumbai). Subculture was done every three weeks interval with respective media. Rooted plantlets were placed in plastic pots, containing vermiculite and cocopeat (1:1) kept in plastic box to maintain high humidity.

### **Analysis of clonal fidelity**

Total genomic DNA was extracted from fresh young leaves of randomly selected *in vitro* raised plants following the CTAB method with minor modification (Doyle and Doyle, 1987). The quality of extracted DNA after RNase treatment was evaluated on 0.8% agarose gel and finally the DNA was quantified using spectrophotometer. Each DNA sample was diluted to 50 ng  $\mu\text{l}^{-1}$  with TE (Tris-EDTA) buffer before use and stored at 4°C. Genetic fidelity was performed on *in vitro* regenerated Stevia plants by direct organogenesis. DNA samples were subjected to polymerase chain reaction (PCR) to generate fingerprinting pattern using RAPD and ISSR primers obtained from OPERON Technologies, USA. Selected 35 RAPD and twenty five ISSR primers were tested for genetic uniformity assay depending on reproducible banding pattern (Tables 1 and 2). RAPD-PCR (Modi *et al.*, 2012) and ISSR-PCR (Singh *et al.*, 2014) was performed as per standardized protocol for Stevia. PCR reactions were repeated to confirm the reproducibility of the banding pattern.

### **Statistical analysis**

For *in vitro* tissue culture experiments, statistical analysis was performed with JMP software version 9 using Tukey Kramer HSD test to determine significant differences among treatments at  $P \leq 0.05$ .

## **Results and Discussion**

### **Shoot induction from *in vivo* and *in vitro* nodal explants**

Mass multiplication protocol was established from *in vivo* nodal explants. Sterilized nodal explants (1.0-2.0 cm) were cultured on various concentrations of TDZ (0.5-3.0 mg/l). Almost at every concentration of TDZ, bud

break was observed during 3–8 days after inoculation (Fig. 1 A). After two weeks on various shoot induction medium, maximum 2-3 microshoots emerged from nodes. The highest shoot induction (68.3 %) was observed at 2.0 mg/l TDZ. At higher concentration of TDZ (2.5-3.0 mg/l), shoot induction percentage significantly decreased (Table 3).

*In vitro* nodal explants (2-3 cm) derived from *in vitro* regenerated plants were cultured on various concentrations of TDZ (0.5-3.0 mg/l). In all concentrations of TDZ, shoot induction was noted during 3–5 days after inoculation. After two weeks on various shoot induction medium, maximum 2-5 microshoots were observed from *in vitro* nodal segments (Fig. 1 B). The lowest shoot induction percentage was 61.66 % at 0.5 mg/l TDZ while 2.5 mg/l TDZ showed 100 % shoot induction. At higher concentration of TDZ (3.0 mg/l), shoot induction percentage significantly decreased (Table 4).

### **Shoot multiplication**

MS basal media were used for shoot multiplication with various concentrations of BAP (1.0-2.0 mg/l) in combination with 0.5 mg/l Kin (Fig. 1 C, D). After 3 weeks of culture on different shoot multiplication medium, maximum shoots per explants (7.3) were observed at 1.0 mg/l BAP in combination with 0.5 mg/l Kin (Table 6).

### **Rooting and acclimatization**

For root induction, shoots ( $\geq 2$  cm) were placed on MS basal medium nourished with different concentrations of IAA (0.2-1.0 mg/l), IBA (0.2-1.0 mg/l) and NAA (0.2-1.0 mg/l). First root was emerged after 8 days of culture on IAA (1.0 mg/l), NAA (1.0 mg/l) and hormone free MS basal medium. Highest rooting response (91.66 %) was noticed on

hormone free media with quality roots (Fig. 1 E), in respect to root numbers and root length (Table 5). However, certain concentration of IAA, IBA and NAA, roots emerged from callus at the base of shoots and these roots are detached from shoots during subculture. The rooted plantlets were successfully transferred in plastic pots containing cocopeat and vermiculite in the ratio 1:1. The pots were placed in a plastic box and covered with lid to ensure high humidity (Fig. 1 F). Boxes were kept in controlled condition for 3 weeks and then transferred to poly house (Fig. 1 G). All plantlets were transferred in field condition with 98.66% survival (Table 7).

### **Analysis of clonal fidelity**

RAPD and ISSR molecular markers were used to estimate the genetic stability at the DNA level of regenerated *Stevia* plants from nodal explants (Tables 1 and 2). Thirty five RAPD primers and 25 ISSR primers were screened out of which 22 primers of RAPD and 10 primers of ISSR amplified and displayed monomorphic banding pattern in all the 19 randomly selected micropropagated plants and the mother plant (Fig. 2, 3).

### **Shoot induction**

In present investigation, compared the effect of different concentration of TDZ for shoot induction from nodal explant of *Stevia*, surface sterilized nodal explants were cultured on different concentration of (TDZ 0.5-3.0 mg/l). Almost at every concentration of TDZ, bud break was noted during 3–6 days after inoculation. Highest shoot induction (68.3 %) was observed at 2.0 mg/l TDZ. It was observed that at lower and higher concentration of TDZ, shoots induction percentage was less. In the present study, no significant differences were observed at lower (1.0-1.5 mg/l) and higher (2.5-3.0 mg/l) concentration of TDZ. TDZ appears to mimic

cytokinin-like activity causing the release of lateral buds and showed better response in terms of shoot regeneration efficiency, compared to other cytokinins, similar to other findings where TDZ produced shoots comparable to or greater than that of other cytokinins (Murthy and Murch, 1998; Ramírez-Mosqueda and Iglesias-Andreu, 2016; Rout *et al.*, 2000; Singh and Dwivedi, 2013).

### **Shoot multiplication**

For shoot multiplication, induced shoots were cultured on MS basal media supplemented with different concentration of BAP (0.5-1.0 mg/l) in combination with Kin (0.5 mg/l). BAP in combination with Kin was efficient cytokinin for multiple shoot bud regeneration. Maximum shoot multiplication shoots per explants (7.3) were recorded at 1.0 mg/l BAP in combination of 0.5 mg/l Kin. Increasing the BAP concentration higher than 1.0 mg/l, plant regeneration frequency and the number of shoots per culture significantly decreased (Ibrahim *et al.*, 2008a). In previous published reports, similar trends were observed (Sivaram and Mukundan, 2003; Ahmed *et al.*, 2007; Ibrahim *et al.*, 2008a; Jain *et al.*, 2009; Atalay *et al.*, 2011; Laribi *et al.*, 2012; Modi *et al.*, 2012; Gantait *et al.*, 2015).

### **Rooting**

Shoots were placed on MS basal medium fortified with various concentrations of IAA, IBA and NAA for root induction (Rout *et al.*, 2000). After a week of culture roots emerged, beside hormone free media, shoots showed different level of callusing and false roots were noticed (Ibrahim *et al.*, 2008a; Ibrahim *et al.*, 2008b; Gantait *et al.*, 2015). Shoots having roots emerged from callus was detached during subculture. Highest rooting response was observed on hormone free media with quality roots in respect to root

numbers and root length (Table 3). Previous literature revealed the occurrence of rooting response in media supplemented with IAA, NAA and IBA (Sivaram and Mukundan, 2003; Atalay *et al.*, 2011; Modi *et al.*, 2012; Thiyagarajan and Venkatachalam, 2012; Singh and Dwivedi, 2013; Singh *et al.*, 2014). However, in present study, the quality root formation was reported on the basis of root number, root length with superior rooting response on hormone free medium.

### **Acclimatization**

Acclimatization of regenerated plantlets is the most important phase for a reproducible micropropagation protocol of Stevia. Hardening media is influenced by light, temperature and humidity during acclimatization (Pospisilova *et al.*, 1999; Hazarika, 2006; Vasane and Kothari, 2008; Awang *et al.*, 2009; Talukdar and Bora, 2009). Therefore, various combinations of substrates were used in hardening phase (Table 4). Highest survivals of regenerated plantlets were observed in cocopeat and vermiculite in ratio of 1:1. In this investigation, hardening procedure is well established and survival rate was found higher than in previous published reports (Sivaram and Mukundan, 2003; Sung, 2006; Jain *et al.*, 2009; Atalay *et al.*, 2011; Modi *et al.*, 2012; Thiyagarajan and Venkatachalam, 2012; Singh and Dwivedi, 2013; Gantait *et al.*, 2015; Ramírez-Mosqueda and Iglesias-Andreu, 2016).

### **Clonal fidelity**

Somaclonal variations arose due to *in vitro* stresses and are noticeable in the form of DNA methylation, chromosome rearrangements and point mutations (Lee and Phillips, 1988; Phillips *et al.*, 1994). Consequently, an assessment to ensure true-to-type propagules at an early stage of

development is considered to be very useful in micropropagation system (Mohan Jain, 2001). To assess the clonal fidelity of micropropagated plantlets several approaches were taken such as molecular approaches. Detection of genetic variation is essential for micropropagation as well as *in vitro* germplasm conservation to eradicate detrimental somaclonal variations (Kaeppler *et al.*, 2000; Mohan Jain, 2001). PCR-based techniques have been found to be greatly useful in establishing the genetic stability of cultivated as well as *in vitro* regenerated Stevia plants (Modi *et al.*, 2012; Singh *et al.*, 2014). RAPD and ISSR markers used for fidelity test and results indicated that all the tissue cultured derived plants are true-to-type and there are no somaclonal variations among those plants (Modi *et al.*, 2012; Singh *et al.*, 2014). The current report of Singh *et al.*, (2014) showed that genetic fidelity of micropropagated stevia plantlets was assessed by ISSR analysis confirming that *in vitro* plants obtained from callus showed genetic variation during the period of culturing, whereas those from nodal segments did not. In this study, we observed that RAPD and ISSR markers ensure the genetic stability without any callusing phase.

Earlier, it was reported that Stevia plants micropropagated from callus culture, showed high level of genetic variation (Singh *et al.*, 2014). Besides this, stevia crop is cross pollinated and high level of heterogeneity exists in the germplasm. Therefore, it is imperative that micropropagated Stevia plant must be screened for genetic variation along with their mother plant. In our study, the mother plants were selected on the basis of morphology and stevioside content (data not shown). Hence, micropropagated Stevia plants maintain similar characteristics with genetic uniformity.

**Table.1** RAPD primers used in genetic fidelity analysis of tissue culture raised plants of *S. rebaudiana*

| S. No. | Name of Primer | Primer sequence/<br>nucleotide sequence (5'-<br>3') |
|--------|----------------|---|
| 1      | OPM-01         | GTTGGTGGCT  |
| 2      | OPM-02*        | ACAACGCCTC  |
| 3      | OPM-03         | GGGGGATGAG  |
| 4      | OPM-04         | GGCGGTTGTC  |
| 5      | OPM-05         | GGGAACGTGT  |
| 6      | OPM-06         | CTGGGCAACT  |
| 7      | OPM-07         | CCGTGACTCA  |
| 8      | OPM-08         | TCTGTTCCCC  |
| 9      | OPM-09         | GTCTTGCGGA  |
| 10     | OPM-10         | TCTGGCGCAC  |
| 11     | OPM-12         | GGGACGTTGG  |
| 12     | OPZ-01         | TCTGTGCCAC  |
| 13     | OPZ-02*        | CCTACGGGGA  |
| 14     | OPZ-03         | CAGCACCGCA  |
| 15     | OPZ-04         | AGGCTGTGCT  |
| 16     | OPZ-05         | TCCCATGCTG  |
| 17     | OPZ-06         | GTGCCGTTCA  |
| 18     | OPZ-07         | CCAGGAGGAC  |
| 19     | OPZ-08         | GGGTGGGTAA  |
| 20     | OPZ-09         | CACCCCAGTC  |
| 21     | OPZ-10         | CCGACAAACC  |
| 22     | OPE-4          | GTGACATGCC  |
| 23     | OPH-3          | AGTCGTCCCC  |
| 24     | OPH-13         | GACGCCACAC  |
| 25     | OPG-07*        | GAACCTGCGG  |
| 26     | OPP-01*        | GTAGCACTCC  |
| 27     | OPP-02*        | TCGGCACGCA  |
| 28     | OPP-04*        | GTGTCTCAGG  |
| 29     | OPP-05*        | CCCCGGTAAC  |
| 30     | OPP-06*        | GTGGGCTGAC  |
| 31     | OPP-07*        | GTCCATGCCA  |
| 32     | OPP-08*        | ACATCGCCCA  |
| 33     | OPP-09*        | GTGGTCCGCA  |
| 34     | OPP-03*        | CTGATACGCC  |
| 35     | OPP-10*        | TCCCGCCTAC  |

\* Did not amplify

**Table.2** ISSR primers used in genetic fidelity analysis of tissue culture raised plants of *S. rebaudiana*

| S. No | Name of Primer          | Primer sequence/ nucleotide sequence (5'-3') |
|-------|-------------------------|--|
| 1     | UBC-808                 | AGAGAGAGAGAGAGAGC                            |
| 2     | UBC-809                 | AGAGAGAGAGAGAGAGG                            |
| 3     | UBC-810*                | GAGAGAGAGAGAGAGAT                            |
| 4     | UBC-811                 | GAGAGAGAGAGAGAGAC                            |
| 5     | UBC 812*                | GAGAGAGAGAGAGAGAA                            |
| 6     | UBC-813*                | CTCTCTCTCTCTCTT                              |
| 7     | UBC-814*                | CTCTCTCTCTCTCTA                              |
| 8     | UBC-815*                | CTCTCTCTCTCTCTG                              |
| 9     | UBC-817*                | CACACACACACACAA                              |
| 10    | UBC-818*                | CACACACACACACAG                              |
| 11    | UBC-823                 | TCTCTCTCTCTCTCC                              |
| 12    | UBC-835                 | AGAGAGAGAGAGAGAGYC                           |
| 13    | UBC-836                 | AGAGAGAGAGAGAGACYA                           |
| 14    | UBC-891                 | HVHTGTGTGTGTGTGTG                            |
| 15    | UBC-895                 | AGAGTTGGTAGCTCTTGATC                         |
| 16    | HB08                    | GAGAGAGAGAGAGG                               |
| 17    | HB12*                   | CACCACCACGC                                  |
| 18    | HB15*                   | GTGGTGGTGGC                                  |
| 19    | (AAG) <sub>6</sub> Y    | AAGAAGAAGAAGAAGY                             |
| 20    | (GGAT) <sub>4</sub> H*  | GGATGGATGGATGGATH                            |
| 21    | ISSR 844B*              | CTCTCTCTCTCTCTGC                             |
| 22    | ISSR 17898A*            | CACACACACACAAC                               |
| 23    | ISSR 17898B*            | CACACACACACAGT                               |
| 24    | ISSR HB-11*             | GTGTGTGTGTGTCC                               |
| 25    | (GGGGT) <sub>3</sub> M* | GGGGTGGGGTGGGGTM                             |

Y=C/T, R=A/G

\* Did not amplify

**Table.3** Effect of different concentration of TDZ supplemented in MS basal media on shoot induction of *Stevia rebaudiana* data was observed after 2nd week

| TDZ (mg/l) | % Shoot induction       |
|------------|-------------------------|
| 0.5        | 20.00±2.88 <sup>c</sup> |
| 1.0        | 25.00±2.88 <sup>c</sup> |
| 1.5        | 38.33±1.63 <sup>b</sup> |
| 2.0        | 68.33±1.66 <sup>a</sup> |
| 2.5        | 38.33±1.61 <sup>b</sup> |
| 3.0        | 23.33±1.69 <sup>c</sup> |

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at p < 0.05 by Tukey- Kramer HSD by JMP @ 9.0.0 software

**Table.4** Effect of different concentration of TDZ supplemented MS basal media on shoot induction from in vitro nodal explants of *Stevia rebaudiana*. Data was observed after 2-week

| TDZ (mg/l) | % Shoot induction      |
|------------|------------------------|
| 0.5        | 61.6±1.66 <sup>c</sup> |
| 1.0        | 71.6±1.66 <sup>d</sup> |
| 1.5        | 73.3±1.66 <sup>d</sup> |
| 2.0        | 90.3±0.66 <sup>d</sup> |
| 2.5        | 100±0.0 <sup>a</sup>   |
| 3.0        | 81.6±1.66 <sup>c</sup> |

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at p < 0.05 by Tukey- Kramer HSD by JMP @ 9.0.0 software

**Table.5** Effect of different concentration of IAA, IBA and NAA supplemented in MS basal media on root induction of *S. rebaudiana* data was observed after 2 week

| Auxins (mg/l) |     |     | No of average root/shoot | Mean root length        | Rooting response (%)    | Callusing |
|---------------|-----|-----|--------------------------|-------------------------|-------------------------|-----------|
| IBA           | IAA | NAA |                          |                         |                         |           |
| Control       |     |     | 6.32±0.15 <sup>b</sup>   | 3.72±0.20 <sup>a</sup>  | 91.66±1.66 <sup>a</sup> | -         |
| 0.2           |     |     | 3.92±0.70 <sup>bc</sup>  | 0.96±0.03 <sup>b</sup>  | 20.00±2.88 <sup>d</sup> | -         |
| 0.4           |     |     | 4.33±0.19 <sup>b</sup>   | 0.88±0.05 <sup>b</sup>  | 58.33±1.66 <sup>b</sup> | ++        |
| 0.6           |     |     | 6.19±0.25 <sup>a</sup>   | 0.32±0.01 <sup>c</sup>  | 58.33±4.40 <sup>b</sup> | +++       |
| 0.8           |     |     | 2.22±0.40 <sup>c</sup>   | 0.32±0.01 <sup>c</sup>  | 13.33±1.66 <sup>d</sup> | +++       |
| 1.0           |     |     | 2.72±0.14 <sup>bc</sup>  | 0.34±0.00 <sup>c</sup>  | 10.00±2.88 <sup>d</sup> | +++       |
|               | 0.2 |     | 5.78±0.04 <sup>bc</sup>  | 3.73±0.08 <sup>a</sup>  | 70.00±5.77 <sup>b</sup> | -         |
|               | 0.4 |     | 5.10±0.19 <sup>c</sup>   | 2.25±0.02 <sup>b</sup>  | 78.33±1.66 <sup>b</sup> | +         |
|               | 0.6 |     | 6.02±0.16 <sup>b</sup>   | 2.17±0.01 <sup>bc</sup> | 78.33±1.66 <sup>b</sup> | +         |
|               | 0.8 |     | 13.98±0.16 <sup>a</sup>  | 1.73±0.06 <sup>c</sup>  | 98.33±1.66 <sup>a</sup> | +         |
|               | 1.0 |     | 3.64±0.05 <sup>d</sup>   | 1.85±0.03 <sup>bc</sup> | 98.33±1.66 <sup>a</sup> | +         |
|               |     | 0.2 | 7.90±0.21 <sup>a</sup>   | 1.84±0.01 <sup>b</sup>  | 93.33±3.33 <sup>a</sup> | +         |
|               |     | 0.4 | 5.57±0.35 <sup>b</sup>   | 0.71±0.02 <sup>c</sup>  | 76.66±1.66 <sup>b</sup> | -         |
|               |     | 0.6 | 8.93±0.44 <sup>a</sup>   | 0.53±0.00 <sup>c</sup>  | 56.66±3.33 <sup>c</sup> | ++        |
|               |     | 0.8 | 3.41±0.12 <sup>c</sup>   | 0.53±0.00 <sup>c</sup>  | 51.66±4.40 <sup>c</sup> | +++       |
|               |     | 1.0 | 2.38±0.20 <sup>c</sup>   | 0.32±0.01 <sup>c</sup>  | 18.33±1.66 <sup>d</sup> | ++++      |

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at p < 0.05 by Tukey- Kramer HSD by JMP @ 9.0.0 software



**Table.6** Effect of different concentration of BAP and Kin supplemented in MS basal media on shoot multiplication of *Stevia rebaudiana* data was observed after 3 week

| Cytokinin (mg/l) | III Week               |                        |
|------------------|------------------------|------------------------|
|                  | No of shoots/explant   | Mean shoot Length      |
| 1.0BAP+0.5Kin    | 7.33±0.14 <sup>a</sup> | 1.63±0.08 <sup>a</sup> |
| 1.5BAP+0.5Kin    | 5.56±0.12 <sup>b</sup> | 0.56±0.05 <sup>b</sup> |
| 2.0BAP+0.5Kin    | 4.51±0.13 <sup>c</sup> | 0.57±0.06 <sup>b</sup> |

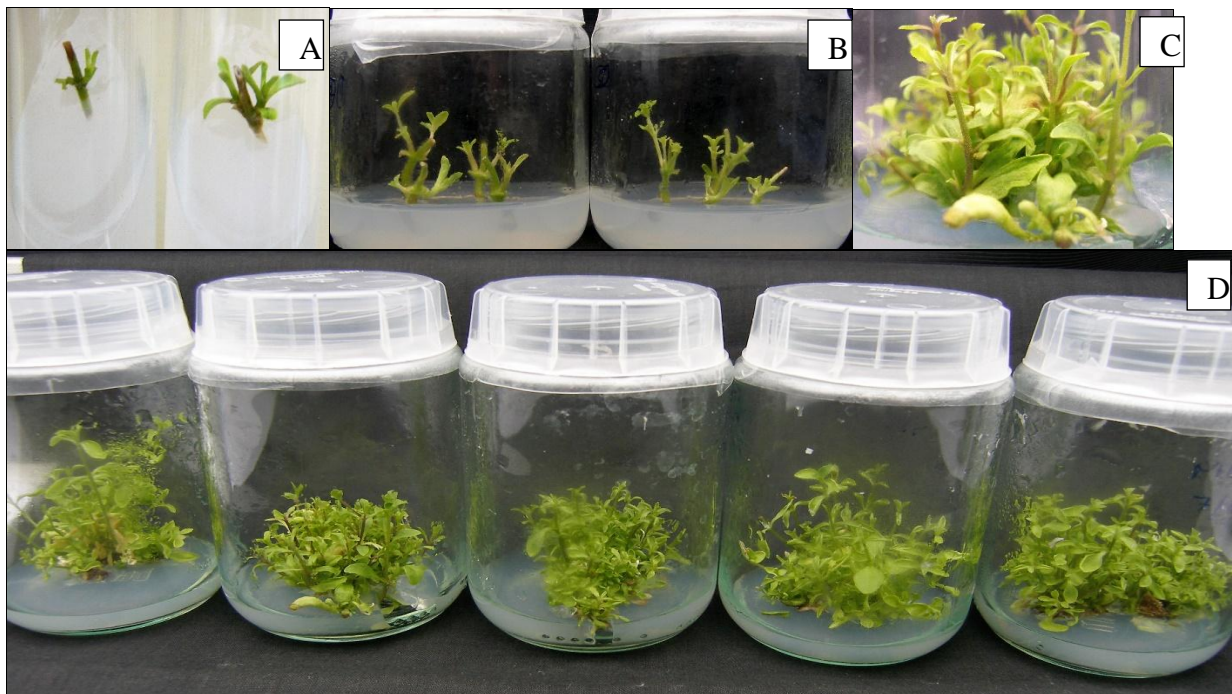
Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at p < 0.05 by Tukey- Kramer HSD by JMP @ 9.0.0 software

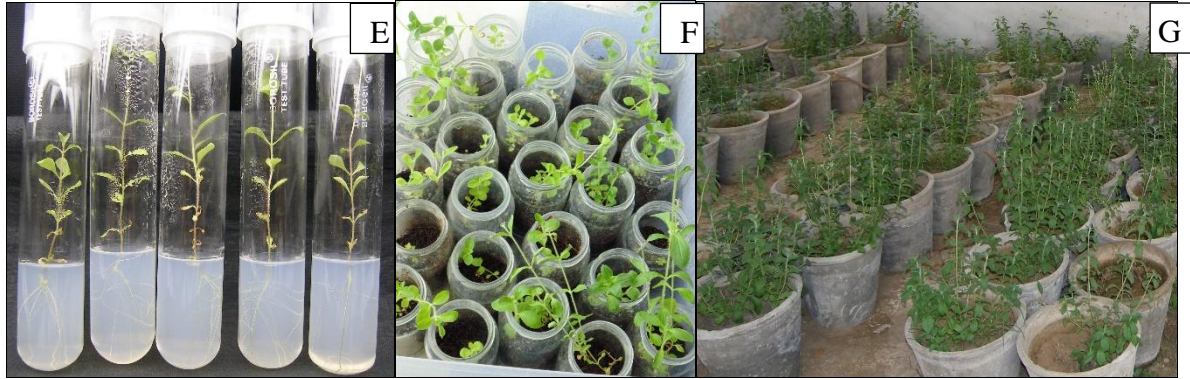
**Table.7** Effect of different type of hardening material used for survival of regenerated plantlets of *S. rebaudiana*. Data was observed after 3-weeks

| Hardening material (ratio 1:1) | % survival plants       |
|--------------------------------|-------------------------|
| Cocopeat+vermiculite           | 98.66±1.33 <sup>a</sup> |
| Cocopeat+vermicompost          | 77.33±3.52 <sup>b</sup> |
| Cocopeat+sand                  | 28.00±2.30 <sup>d</sup> |
| Cocopeat+perlite               | 52.00±4.00 <sup>c</sup> |
| Vermiculite+vermicompost       | 44.00±4.61 <sup>c</sup> |

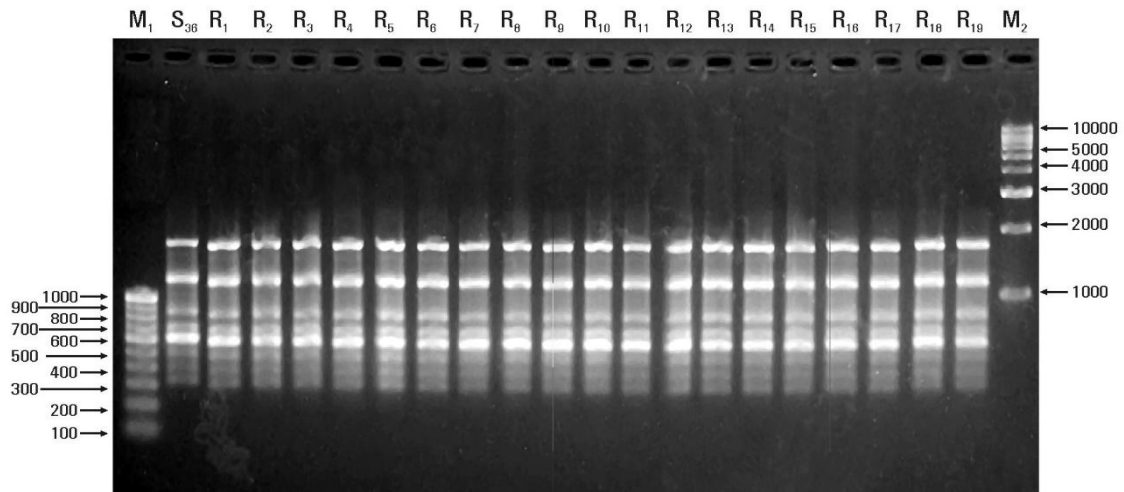
Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at p < 0.05 by Tukey- Kramer HSD by JMP @ 9.0.0 software

**Fig.1** (A) Shoot induction from in vivo nodal explants, (B) Shoot induction from in vitro nodal explants, (C, D) Shoot multiplication, (E) Rooting, (F, G) Hardening of regenerated plantlets

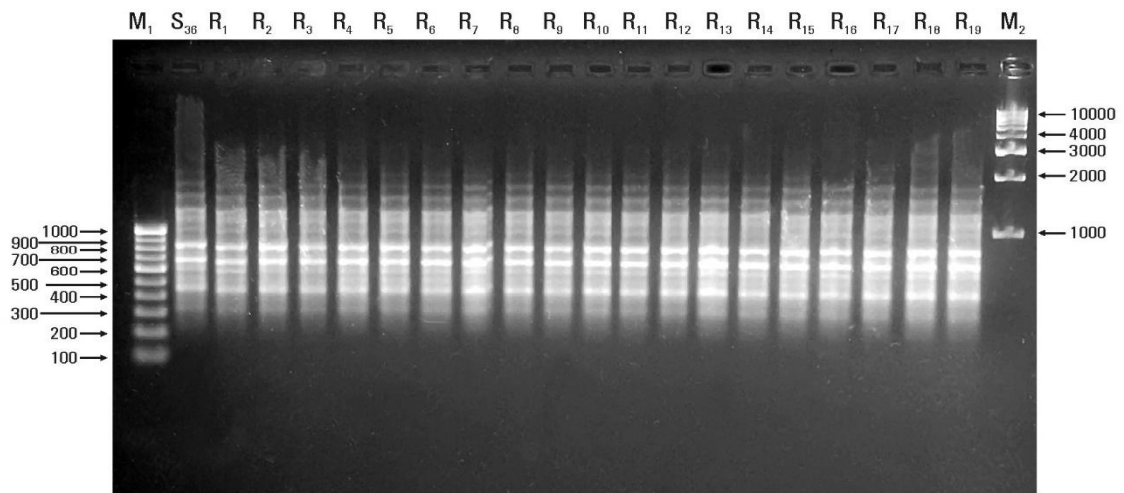




**Fig.2** DNA fingerprint pattern generated with primers OPM-06. M1 and M2-DNA marker, lane S36 - DNA from mother plant, lanes R1 to R19 - DNA from tissue culture raised plants



**Fig.3** DNA fingerprint pattern generated with primers UBC-811. M1 and M2-DNA marker, lane S36 - DNA from mother plant, lanes R1 to R19 - DNA from tissue culture raised plants



In conclusion, in this study, an efficient protocol for high frequency mass multiplication from nodal explants of *S. rebaudiana* through repeated subculture was reported. By using this protocol, minimum 2400 plants could be produced from a single microshoots within 12 weeks with 4 subculture. Therefore, multiplication of planting materials can be increased by many folds using more number of microshoots and producing true-to-type quality planting material. This protocol produce high amount of quality planting material within 12 weeks with higher survival rate. Hence, the protocol can be considered for high frequency mass multiplication of quality planting materials of stevia with high survival rate.

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