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Research Article

In vitro Regeneration and ISSR-Based Genetic Fidelity Evaluation of *Stevia rebaudiana*

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ABSTRACT

Steviol glycoside, which is a natural sweetener extracted from *Stevia rebaudiana*, is globally recognized. For consumers, this compound is widely utilized by diabetic patients and demonstrates numerous therapeutic effects. However, the escalating demand for this natural sweetener and medicinal herb impacts the availability of stevia. Conventional propagation methods, such as seed and stem cutting, frequently result in low germination rates. To address these limitations, the present research explores the potential of *in vitro* clonal propagation to ensure a consistent supply of planting material. Therefore, the objective of this study was to develop an efficient protocol for tissue culture of *S. rebaudiana* accession MS007. The highest regeneration frequency (85.19% & 86.67% for shoot tips & nodes, respectively) and maximum shoot number (14.30 & 12.77 shoots/explant, respectively) were observed on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP). Half-strength MS medium supplemented with 0.5 mg/L indole-3-butyric acid (IBA) was the optimal medium for rooting, exhibiting the highest rooting percentage, root number, and root length. Subsequently, the plantlets were acclimatised in plastic cups containing peat moss, and it was observed that 86.67% of plantlets survived when transplanted to the field. The outcome of inter-simple sequence repeat (ISSR) analysis using 38 ISSR primers confirmed the genetic fidelity between the *in vitro* regenerated plants and the mother plant. This study successfully developed an *in vitro* propagation technique for stevia to produce true-to-type clonal plants. The obtained results can be used to mass-produce stevia accession MS007 to meet market demand.

Key words: Direct organogenesis, genetic fidelity, *in vitro*, ISSR markers, stevia

INTRODUCTION

People who consume sweetened beverages with high sugar content face the risk of suffering from metabolic disorders (Seo *et al*., 2019). There are varied types of sweetener compounds available in the market as alternatives to cane sugar (sucrose), such as natural and artificial sweeteners (Saraiva *et al*., 2020). According to Saad *et al*. (2014), natural sweetening compounds are safer than artificial sweeteners, as the latter can cause negative side effects or health problems, such as blurred vision, multiple sclerosis, kidney and liver effects, weight gain, depression, muscle dysfunction, skin eruptions, and headaches. Natural sweeteners from plants have benefits in many ways, yet most of them contain calories (Kim & Kinghorn, 2002). Thus, a non-caloric natural sweetener (with no negative effect on human health) is a better alternative than natural sweeteners with calories that can cause metabolic disorders.

Stevia rebaudiana, or stevia, is an herb from the Asteraceae family and is best known as sugar or sweet leaf. According to Rolnik and Olas (2021), the Asteraceae family consists of a large variety of shrubs and herbs from 1,600 genera and approximately 25,000 species, and *S. rebaudiana* is the only one of the two species that produces steviol glycosides (Madan *et al*., 2010). The leaves possess high non-caloric sweetening compounds, mainly stevioside and rebaudioside (Samsulrizal *et al*., 2019), with sweetness levels higher than sugar from sugarcane (Razali *et al*., 2020). Moreover, stevia's diterpene glycosides have no adverse effects on human health. Hence, it has been used as a replacement for sugar, especially for diabetic patients (Ajami *et al*., 2020). Besides stevioside and rebaudioside, stevia extracts are also composed of rebaudiosides C, D, F, H, I, J, K, M, N, O, T, newly discovered dulcosides C, D, F, and rebaudiosides FX1 and FX2 (Watanabe *et al*., 2023). Due to these bioactive compounds, stevia exhibits numerous therapeutic activities, such as anti-inflammatory (Myint *et al*., 2023), antiviral (Ceole *et al*., 2020), antioxidant (Papaefthimiou *et al*., 2023), antihyperglycemic (Abdel-Aal *et al*., 2021), bactericidal (Deonas *et al*., 2022), and anti-hypertensive (Saadh *et al*., 2023) effects. According to Verma and Panda (2018), stevia is widely consumed as a sweetening compound worldwide, especially in Asian and European countries. Therefore, stevia is the optimal candidate

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to fulfill the increasing global demand for high-potency sweeteners. Currently, numerous countries worldwide focused on stevia research activities and commercial propagation, which will potentially reduce the number of diabetic patients and address sugar shortage challenges.

Stevia is conventionally propagated via stem cuttings and seeds. However, the seed germination rate is poor (Simlat *et al*., 2020), while multiple factors influence stem cutting propagation (Castañeda-Saucedo *et al*., 2020). Propagation using seeds will also produce a population of seedlings with genetic variations due to their heterogeneity, which might affect the chemical composition and sweetness of stevia. Thus, cultivation of stevia via *in vitro* clonal propagation will serve as a superior technology to address the limitations of conventional propagation and meet the demand. *In vitro* techniques constitute an essential biotechnological tool used for regenerating and conserving valuable plant genetic resources. *In vitro* propagation can be defined as culturing any plant part on a nutritive medium under controlled environmental conditions. This technique offers numerous advantages compared to conventional breeding or propagation, for examples, rapid multiplication/production of plants using a small part of plant cell/tissues (Mattick, 2018), generation of disease-free plants (Tegen & Mohammed, 2016), independence from seasonal constraints (Ahmad & Anis, 2007) and conservation of plant species from potential extinction (Ferris, 2023). The steps in *in vitro* propagation consist of surface sterilisation of selected explant(s), induction of shoots, rooting, acclimatisation/hardening, and finally planting in the field (Thakur *et al*. 2024). Shoot tips (Ahmed, 2022) and nodes (Asande *et al*., 2020) are the most desirable explants for *in vitro* propagation as their usage has been shown to minimise genetic variation. To overcome the limitations of stevia conventional propagation and to mass-produce stevia to meet the demand, *in vitro* propagation is an alternative technique. The accession MS007, a local accession developed by the Malaysian Agricultural Research and Development Institute was chosen due to its superior qualities, namely a high number of leaves and larger leaf size (Othman *et al*., 2018). Hence, this study aimed to develop an efficient *in vitro* clonal propagation method for regenerating true-to-type *S. rebaudiana* accession MS007.

MATERIALS AND METHODS

Source and surface sterilisation of explants

Healthy and juvenile shoot tips and nodal explants (up to 3 nodes) were harvested from 2-3 months old field-grown *S. rebaudiana* accession MS007 at the Glasshouse and Nursery Complex, Kulliyyah of Science, International Islamic University Malaysia. The explants were washed under running tap water for 30 min and pre-treated with 0.2% Carbendazim (fungicide) for 30 min.The explants were then rinsed with distilled water for 15 min, immersed for 10 min in water containing two drops of Tween 20, and washed with distilled water five times. In a laminar airflow hood, the explants were immersed in 70% ethanol for 30 s, followed by two rinses with sterile distilled water. Subsequently, the explants were treated with 5% NaOCl for 10 min and finally rinsed with sterile distilled water.

Multiple shoot bud induction

After surface sterilisation, shoot tips and nodes were inoculated in varying concentrations of cytokinins, BAP and kinetin (Kn), alone or in combination. An auxin, 1-naphthaleneacetic acid (NAA) was also employed together with BAP and Kn for shoot induction and multiplication. MS basal medium supplemented with sucrose (3%) and agar (0.65%) served as the control. The culture media pH was adjusted to 5.8. For each experimental batch, 9 shoot tips and 30 nodes were used. Each treatment was replicated thrice. Magenta vessels containing explants were incubated at a temperature of 25 ± 2°C, with a 16/8-hr day/light cycle under a light intensity of 2500 lux (white luminescent bulb).

Rooting

For root induction, healthy shoots regenerated from shoot tips and nodes were cultured in either full- or half-strength MS medium supplemented with various concentrations of indole-3-butyric acid (IBA), NAA, or indole-3-acetic acid (IAA) (0.5, 1.0, and 1.5 mg/L). The control medium comprised full- or half-strength MS medium without plant growth regulator (PGR). Each concentration of growth regulators consisted of 30 replicates, and each treatment was replicated across three batches. All cultures were incubated under similar conditions for shoot bud induction.

Acclimatization

After 1 month of rooting, the roots of plantlets were gently extracted from the media and were rinsed using running tap water for agar elimination. Then, the plantlets were transferred to polystyrene cups containing sterile peat moss. The cups were moistened with water and enclosed with clear plastic bags to maintain high humidity conditions. The cups were subsequently transferred to the incubation room to acclimatise the plantlets. After 14 days, the transparent plastic bags were completely removed, and the plants were maintained in the same environment for an additional 3 days to facilitate natural growth adaptation. The plantlets were transferred to polybags (15 cm × 24 cm) filled with approximately 7 kg of substrate. All polybags were positioned under shade, and each was irrigated daily with water.

Genetic fidelity analysis using inter-simple sequence repeats (ISSR)

Genomic DNA was extracted from young leaves of both parental and *in vitro* plants using a modified cetyl-trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987). The extracted DNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific) at A_{260}. A total of 38 ISSR primers were used for this analysis (Table 1). The 25 µL PCR mixture comprised 200 ng DNA, 12.5 µL Mytaq Red™ Master Mix (Bioline), 1 µL of 25 µM primer, and ultrapure water. PCR was performed using an Eppendorf Mastercycler following the protocols of initial denaturation for 5 min at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 36-55°C for 1 min, extension at 72°C for 1 min and final extension for 10 min at 72°C. PCR products were subjected to gel electrophoresis on 1.5% agarose using 1× TAE buffer and visualised under a UV trans-illumination system (Alpha Imager™ 2200).

Table 1. ISSR primer list with the sequence of primer and annealing temperature (Hadia *et al*. 2008 & Abdul Rashid *et al*. 2013)

Primer code	Sequence of primer (5'-3')	Annealing temperature (°C)
S15	GTG GTG GTG GC	36
S12	CAC CAC CAC GC	36
S11	GTG TGT GTG CC	36
S10	GAG AGA GAG AGA CC	36
S5	CAC ACA CAC ACA GT	36
S4	CAC ACA CAC ACA AC	36
S3	CTC TCT CTC TCT CTC TGC	36
IS 94	ATG ATG ATG ATG ATG ATG	55
IS 90	AGA GAG AGA GAG AGA GG	55
IS 85	CTC TCT CTC TCA CC	51
IS 83	AGA GAG AGA GTT G	46.9
IS 78	AGA AGA AGA AGA AGA AGA AGA	55
IS 70	GAA GAA GAA GAA GAA GAA	52.9
IS 58/2	GTG TGT GTG TGT GTG TTC	46.9
IS 58/1	GTG TGT GTG TGT GTG TCC	46.9
IS 57	GAG AGA GAG AGA GAG ACT	51
IS 56	TCT CTC TCT CTC TCT CC	51
IS 55	AGA GAG AGA GAG AGA GA	55
IS 54	AGA GAG AGA GAG AGA GC	55
IS 52/2	TCC TCC TCC TCC TCC GY	48.6
IS 52/1	TCC TCC TCC TCC TCC AY	48.6
IS 50	GAA GAA GAA GAA GAA GAA	54.2
IS 45/2	TGT GTG TGT GTG TGT GGT	46.9
IS 45/1	TGT GTG TGT GTG TGT GAT	46.9
IS 44/2	ACA CAC ACA CAC ACA CTT	52.9
IS 44/1	ACA CAC ACA CAC ACA CCT	52.9
IS 42/2	ACA CAC ACA CAC ACA CTG	48.6
IS 42/1	ACA CAC ACA CAC ACA CCG	48.6
IS 34/2	GAG AGA GAG AGA GAG ATT	45
IS 34/1	GAG AGA GAG AGA GAG ACT	45
IS 30	ACA CAC ACA CAC ACA CC	55
IS 26	GTG TGT GTG TGT GTG TGT C	48.6
IS 25	TCT CTC TCT CTC TCT CA	51
IS 23	GTG TGT GTG TGT GTG TC	55
IS 21	CAC ACA CAC ACA CAC AG	51
IS 20	CAC ACA CAC ACA CAC AA	51
1S 19	CTC TCT CTC TCT CTC TT	51
IS 12	AGA GAG AGA GAG AGA GT	51

Statistical analysis

The experimental design of this study was completely randomised. All the results obtained were analyzed using ANOVA and either Tukey HSD (homogeneity of variances for data was assumed) or Games-Howell (homogeneity of variances for data was not assumed) test at ≥95% confidence interval ($P \leq 0.05$) using PASW Statistics 18 (SPSS Inc., Chicago). Results were presented as the mean and standard error of the mean (SEM) from all three independent experiments.

RESULTS AND DISCUSSION

Multiple shoot induction

In most species, shoots are being induced and multiplied when their explants are cultured on medium in the presence of PGRs. For the shoot induction experiment, shoot tips and nodes from 2-3 months old stevia were placed on MS medium augmented with two different types of cytokinins, BAP and Kn, either alone or in combination. NAA was also used with BAP and Kn for shoot induction and multiplication. MS basal medium (with zero PGR) served as the control treatment.

In general, the results demonstrated that the inoculated shoot tips and nodes exhibited differential responses to growth medium with a variety of types and concentrations of PGRs. In the control treatment (MS medium without any PGR), no shoot induction was observed for both explants (Table 2 – Table 7). Shoot buds were induced as early as 7 days after culturing on medium with PGRs. In most cases, shoot induction occurred within 15 days of culture. The first cytokinin tested for shoot induction was BAP at various concentrations (0.5 - 3.0 mg/L). All the measured parameters increased with increasing BAP concentration up to 1.0 mg/L. After testing all concentrations, 1.0 mg/L BAP was the optimal concentration for shoot bud induction, with a response percentage of 85.19% and 86.67% for shoot tips and nodes, respectively, 14.30 shoots/explant and 5.75 cm shoot length for shoot tips, and 12.77 shoots/explant and 5.53 cm for nodes (Table 2, Figure 1a-b). Shoot tips and nodes began to produce a minimum of two multiplying shoots on MS medium containing 1.0 mg/L BAP within 7 to 12 days of culture. The least

response of shoot induction was observed when the explants were cultured on MS medium + 3.0 mg/L BAP, which also yielded the minimum number of shoots per explant and the lowest mean shoot length. Callus was observed at the base of explants in all concentrations of BAP, which increased in number at 2.5 and 3 mg/L BAP.

Table 2. Shoot bud induction on different concentrations of BAP

BAP (mg/L)	Shoot tips			Nodes		
	% of shoot induction	No. of shoots per explant	Shoot length (cm)	% of shoot induction (%)	No. of shoots per explant	Shoot length (cm)
0	0.00^{a}	0.00^{m}	0.00^{x}	0.00^{a}	0.00^{m}	0.00^{x}
0.5	81.48 ± 3.70^{b}	9.23 ± 0.30^{n}	4.56 ± 0.13^{y}	78.89 ± 1.11^{b}	7.89 ± 0.19^{n}	4.44 ± 0.70^{yt}
1.0	85.19 ± 3.70^{b}	14.30 ± 0.17^{o}	5.75 ± 0.19^{zu}	86.67 ± 0.00^{a}	12.77 ± 0.18^{o}	5.53 ± 0.10^{z}
1.5	74.07 ± 3.70^{bd}	11.45 ± 0.69^{p}	5.10 ± 0.17^{yu}	85.56 ± 1.11^{a}	9.58 ± 0.24^{p}	4.72 ± 0.10^{y}
2.0	59.26 ± 3.70^{cd}	8.38 ± 0.33^{n}	4.33 ± 0.15^{y}	78.89 ± 2.22^{b}	8.13 ± 0.15^{n}	4.16 ± 0.08^{t}
2.5	55.56 ± 6.42^{cd}	5.53 ± 0.29^{q}	3.53 ± 0.17^{t}	68.89 ± 1.11^{c}	5.47 ± 0.15^{q}	3.20 ± 0.09^{u}
3.0	48.15 ± 3.70^{c}	4.85 ± 0.22^{r}	3.01 ± 0.14^{t}	64.44 ± 1.11^{c}	4.52 ± 0.82^{r}	2.66 ± 0.07^{v}

Another type of cytokinin, Kn was evaluated for shoot bud induction and multiplication at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, and 3 mg/L. It could be observed that explants responded differentially towards varying concentrations of Kn in culture media (Table 3). Shoot bud initiation from both explants occurred within 10 to 15 days of culture, notably later when compared to shoot induction by BAP. The results obtained showed that the shoot induction response increased proportionally with the increment of Kn concentrations. The highest response for all the parameters measured was obtained with 3.0 mg/L Kn.

Table 3. Shoot bud induction on different concentrations of Kn

Kn (mg/L)	Shoot tips			Nodes		
	% of shoot induction	No. of shoots per explant	Shoot length (cm)	% of shoot induction	No. of shoots per explant	Shoot length (cm)
0	0.00^{a}	0.00^{m}	0.00^{x}	0^{a}	0^{m}	0^{x}
0.5	48.15 ± 3.70^{b}	3.38 ± 0.14^{n}	5.67 ± 0.12^{y}	54.44 ± 2.22^{b}	3.27 ± 0.06^{n}	5.02 ± 0.08^{y}
1.0	51.85 ± 3.70^{b}	3.29 ± 0.13^{n}	6.33 ± 0.08^{z}	61.11 ± 1.11^{bc}	3.53 ± 0.09^{no}	5.79 ± 0.08^{z}
1.5	59.26 ± 3.70^{bc}	3.69 ± 0.48^{no}	7.19 ± 0.09^{t}	64.44 ± 1.11^{cd}	3.86 ± 0.12^{op}	6.68 ± 0.07^{x}
2.0	62.96 ± 3.70^{bc}	4.12 ± 0.70^{o}	8.14 ± 0.11^{u}	70.00 ± 1.92^{de}	3.92 ± 0.10^{oq}	7.56 ± 0.07^{t}
2.5	70.37 ± 3.70^{c}	4.21 ± 0.86^{o}	10.39 ± 0.12^{v}	75.56 ± 1.11^{ef}	4.13 ± 0.09^{pq}	9.45 ± 0.80^{u}
3.0	81.48 ± 3.70^{c}	5.36 ± 0.27^{p}	11.95 ± 0.13^{w}	78.89 ± 1.11^{f}	5.06 ± 0.10^{r}	10.88 ± 0.82^{v}

The effects of combining two cytokinins on shoot induction were also examined in this study. The first combination was 0.5 mg/L Kn tested with different concentrations of BAP. Using this combination, shoot bud initiation from both explants occurred within 10 to 15 days of culture. For shoot tips, the pattern of shoot induction response was not consistent up to 1.5 mg/L BAP. Higher concentrations than 1.5 mg/L of BAP with 0.5 mg/L Kn showed a decrease in response to all evaluated parameters (Table 4). Meanwhile, for nodal explants, combining 0.5 mg/L Kn with increasing concentrations of BAP resulted in decrement in responses. Among the combinations tested, 0.5 mg/L Kn + 0.5 mg/L BAP yielded the best response while the lowest response was observed from explants cultured in 0.5 mg/L Kn with 3.0 mg/L BAP.

Table 4. Shoot bud induction on 0.5 mg/L Kn in combination with BAP

BAP	Kn	Shoot tips			Nodes		
mg/L		% of shoot induction	No. of shoots per explant	Shoot length (cm)	% of shoot induction	No. of shoots per explant	Shoot length (cm)
0	0	0.00^{a}	0.00^{m}	0.00^{x}	0.00^{a}	0.00^{m}	0.00^{x}
0.5	0.5	81.48 ± 3.70^{b}	9.50 ± 0.36^{n}	6.20 ± 0.13^{y}	80.00 ± 0.00^{ab}	8.31 ± 0.25^{n}	5.91 ± 0.07^{y}
1.0	0.5	74.07 ± 3.70^{b}	6.95 ± 0.25^{o}	5.23 ± 0.12^{z}	75.56 ± 1.11^{b}	6.35 ± 0.22^{o}	5.13 ± 0.06^{z}
1.5	0.5	81.48 ± 3.70^{b}	5.59 ± 0.30^{p}	4.75 ± 0.14^{zt}	64.44 ± 2.22^{c}	5.38 ± 0.15^{p}	4.33 ± 0.07^{t}
2.0	0.5	70.37 ± 3.70^{b}	4.96 ± 0.28^{pq}	4.29 ± 0.15^{tu}	60.00 ± 1.92^{cd}	4.44 ± 0.14^{q}	3.41 ± 0.06^{u}
2.5	0.5	70.37 ± 3.70^{b}	4.32 ± 0.13^{q}	3.94 ± 0.10^{u}	55.56 ± 1.11^{de}	4.30 ± 0.14^{q}	3.18 ± 0.05^{u}
3.0	0.5	70.37 ± 3.70^{b}	4.05 ± 0.22^{q}	3.14 ± 0.12^{v}	51.11 ± 2.94^{e}	4.11 ± 0.10^{q}	2.58 ± 0.07^{v}

A shoot induction study was also carried out with 0.5 mg/L BAP with different concentrations of Kn. For shoot tips, both combinations of 0.5 mg/L BAP + 0.5 mg/L Kn and 0.5 mg/L BAP + 1.0 mg/L Kn yielded similar responses in shoot induction. However, the shoot induction decreased when 0.5 mg/L BAP was combined with higher concentrations of Kn. The number of shoots per explant and shoot length from shoot tips decreased with higher concentrations of Kn (Table 5). For nodal explants, all the parameters evaluated were inversely proportional to the increment of Kn concentrations. The optimum combination for shoot induction from both explants was 0.5 mg/L Kn + 0.5 mg/L BAP, which yielded the best response of shoot induction, number of shoots per explant, and shoot length.

Table 5. Shoot bud induction on 0.5 mg/L BAP in combination with Kn

Kn	BAP	Shoot tips			Nodes		
mg/L		% of shoot induction	No. of shoots per explant	Shoot length (cm)	% of shoot induction	No. of shoots per explant	Shoot length (cm)
0	0	0.00^{a}	0.00^{m}	0.00^{x}	0.00^{a}	0.00^{m}	0.00^{x}
0.5	0.5	81.48 ± 3.70^{b}	9.50 ± 0.36^{n}	6.21 ± 0.13^{y}	80.00 ± 0.00^{ab}	8.31 ± 0.25^{n}	5.91 ± 0.07^{y}
1.0	0.5	81.48 ± 3.70^{b}	6.36 ± 0.26^{o}	5.47 ± 0.12^{y}	73.33 ± 1.92^{bc}	6.35 ± 0.15^{o}	5.25 ± 0.05^{z}
1.5	0.5	70.37 ± 3.70^{bd}	6.16 ± 0.39^{op}	4.78 ± 0.13^{z}	67.78 ± 1.11^{cd}	5.45 ± 0.23^{p}	4.42 ± 0.08^{t}
2.0	0.5	51.85 ± 3.70^{c}	5.50 ± 0.40^{opq}	4.53 ± 0.11^{z}	63.33 ± 1.92^{de}	5.55 ± 0.14^{p}	4.13 ± 0.06^{u}
2.5	0.5	59.26 ± 3.70^{cd}	5.19 ± 0.19^{pq}	3.90 ± 0.10^{t}	58.89 ± 2.22^{ef}	5.02 ± 0.16^{p}	3.35 ± 0.07^{v}
3.0	0.5	48.15 ± 3.70^{c}	4.46 ± 0.33^{q}	3.43 ± 0.15^{t}	54.44 ± 2.94^{f}	5.13 ± 0.19^{p}	2.76 ± 0.08^{w}

The present study was also aimed at exhibiting the effectiveness of NAA in combination with BAP and Kn to induce shoots using shoot tips and nodes on MS medium. Combination of 0.5 mg/L NAA with either BAP or Kn (0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 mg/L) were evaluated. The obtained results demonstrated that 0.5 mg/L NAA in combination with different concentrations of BAP had significant effects on shoot induction in both shoot tips and nodes (Table 6). The optimum combination tested was 1.5 mg/L BAP + 0.5 mg/L NAA which resulted in the highest responses. The production rate started to decrease gradually with a higher concentration of BAP (beyond 1.5 mg/L). The last responses of shoot induction were observed on 3 mg/L BAP with 0.5 mg/L NAA for both explants.

Table 6. Shoot bud induction on 0.5 mg/L NAA in combination with BAP

BAP	NAA	Shoot tips			Nodes		
mg/L		% of shoot induction (%)	No. of shoots per explant	Shoot length (cm)	% of shoot induction	No. of shoots per explant	Shoot length (cm)
0	0	0.00^{a}	0.00^{m}	0.00^{x}	0.00^{a}	0.00^{m}	0.00^{x}
0.5	0.5	62.96 ± 3.70^{bd}	4.47 ± 0.26^{no}	3.13 ± 0.11^{y}	62.22 ± 2.94^{be}	3.88 ± 0.12^{nr}	2.92 ± 0.06^{y}
1.0	0.5	70.37 ± 3.70^{bc}	4.74 ± 0.21^{no}	3.47 ± 0.88^{y}	67.78 ± 1.11^{b}	4.07 ± 0.13^{np}	3.14 ± 0.04^{yt}
1.5	0.5	81.48 ± 3.70^{c}	5.23 ± 0.25^{n}	4.49 ± 0.09^{z}	72.22 ± 1.11^{b}	4.75 ± 0.19^{opq}	4.22 ± 0.05^{z}
2.0	0.5	59.26 ± 3.70^{bd}	4.50 ± 0.38^{no}	3.60 ± 0.10^{y}	65.56 ± 1.11^{be}	4.17 ± 0.14^{nqr}	3.25 ± 0.06^{t}
2.5	0.5	51.85 ± 3.70^{d}	4.14 ± 0.33^{no}	2.87 ± 0.12^{t}	55.56 ± 2.94^{ce}	3.81 ± 0.15^{or}	2.52 ± 0.08^{u}
3.0	0.5	48.15 ± 3.70^{d}	3.85 ± 0.22^{o}	2.25 ± 0.11^{u}	40.00 ± 3.33^{d}	3.61 ± 0.12^{nr}	2.11 ± 0.07^{v}

Shoot bud induction and multiplication efficiency were also assessed on shoot tips and nodes using 0.5 mg/L NAA with varying Kn concentrations (0.5, 1.0, 1.5, 2.0, 2.5 & 3.0 mg/L). In this study, shoot bud induction and multiplication capacity were delayed across all tested concentrations, with new shoot bud induction occurring exclusively after 12 days of culture. Results demonstrated that all responses increased progressively with Kn concentrations up to 1.0 mg/L. Increment in the concentration of Kn beyond 1.0 mg/L with 0.5 mg/L NAA adversely affected the response of shoot induction, number of shoots per explant, and shoot length in both explants used (Table 7). The maximum number of shoots per explant (5.10 & 4.58), shoot length (4.82 cm & 4.50 cm), as well as shoot response (74.07% & 65.56%), were observed in 1.0 mg/L Kn + 0.5 mg/L NAA for the shoot tips and nodes, respectively. The combination of 0.5 mg/L NAA + 3.0 mg/L Kn resulted in the least responses in shoot induction.

Table 7. Shoot bud induction on 0.5 mg/L NAA in combination with Kn

Kn	NAA	Shoot tips			Nodes		
mg/L		% of shoot induction (%)	No. of shoots per explant	Shoot length (cm)	% of shoot induction	No. of shoots per explant	Shoot length (cm)
0	0	0.00^{a}	0.00^{m}	0.00^{x}	0.00^{a}	0.00^{m}	0.00^{x}
0.5	0.5	62.96 ± 3.70^{b}	4.47 ± 0.24^{np}	3.83 ± 0.10^{yt}	58.89 ± 2.94^{bc}	3.81 ± 0.14^{n}	3.56 ± 0.07^{y}
1.0	0.5	74.07 ± 3.70^{b}	5.10 ± 0.19^{n}	4.82 ± 0.14^{z}	65.56 ± 1.11^{b}	4.58 ± 0.15^{o}	4.50 ± 0.08^{z}
1.5	0.5	59.26 ± 3.70^{bd}	4.56 ± 0.29^{np}	3.86 ± 0.12^{y}	60.00 ± 1.92^{be}	3.93 ± 0.13^{n}	3.65 ± 0.10^{z}
2.0	0.5	59.26 ± 3.70^{bd}	4.06 ± 0.23^{op}	3.45 ± 0.11^{y}	55.56 ± 1.11^{ce}	3.76 ± 0.11^{n}	3.05 ± 0.06^{t}
2.5	0.5	51.85 ± 3.70^{d}	3.79 ± 0.24^{op}	3.13 ± 0.15^{t}	51.11 ± 2.22^{ce}	3.58 ± 0.12^{np}	2.74 ± 0.04^{u}
3.0	0.5	48.14 ± 3.70^{d}	3.38 ± 0.18^{o}	2.86 ± 0.13^{t}	37.78 ± 2.94^{d}	3.33 ± 0.09^{p}	2.25 ± 0.12^{v}

From the overall results, it can be concluded that shoot tips and nodes of *S. rebaudiana* require the addition of PGRs to the MS medium to induce shoot formation since the control treatment (MS medium only) did not yield any response. Explants from other plant species, for example in *Ficus carica* (Ting *et al*., 2018), *Eurycoma longifolia* (Alttaher *et al*., 2020), and *Passiflora edulis* (Asande *et al*., 2020) also failed to induce shoots on basal media without PGRs. This result is also in agreement with other previous *in vitro* studies of stevia cultivars (Uddin *et al*., 2006; Thiyagarajan & Perumal, 2012; Ghose *et al*., 2022).

The type and concentration of PGRs were shown to affect the explants' response in shoot induction. Among the PGRs, cytokinins function to induce shoot formation, and BAP is the most widely used cytokinin in *in vitro* propagation. Among the treatments tested for shoot induction, 1.0 mg/L BAP was found to be the optimum. BAP is more effective than Kn in numerous *in vitro* clonal studies of plants including *Vanilla planifolia* (Mahardhini *et al*., 2022), *Indigofera zollingeriana* (Royani *et al*., 2021), and *Pongamia pinnata* (Sugla *et al*., 2007). Our results corroborated with other works on shoot induction of stevia, where 1 mg/L was found to be the optimal concentration (Nower, 2014; Jadid *et al*., 2024). Combinations of NAA with either BAP or Kn for

shoot induction were observed to be less responsive. This might be attributed to cell cycle arrest, which renders the explants less capable of absorbing water from the surrounding medium (van Staden *et al*., 2008). According to Qi and Zhang (2020), cell cycle arrest is affected by several factors, which include abiotic stresses (drought, temperature, salt) and biotic stress (pathogen attack).

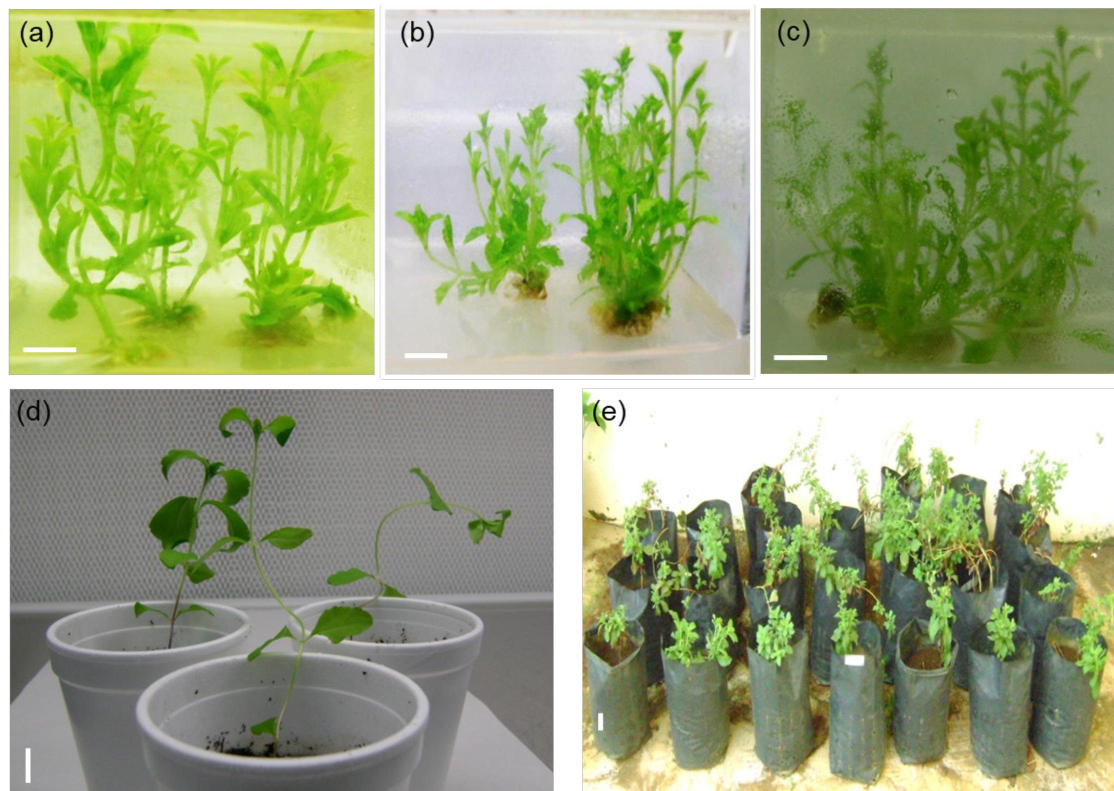

Fig. 1. Direct organogenesis of stevia from shoot tip and nodal explants: (a) Shoot multiplication from shoot tip on MS + 1.0 mg/L BAP, (b) Shoot multiplication from node on MS + 1.0 mg/L BAP, (c) Subculture of shoot tip for multiplication, (d) Acclimatisation of plantlets under *in vitro* conditions and ready to be transferred to the field conditions and (e) Mature plants after acclimatization of *in vitro* regenerated plantlets in the field. Bar = 1 cm.

Rooting

In this study, *in vitro* elongated shoots from explants were transferred to either half-strength or full-strength MS medium supplemented with IBA, NAA, and IAA for rooting. For the control, full- and half-strength basal MS media without the addition of any PGRs were used. In general, for all treatments, shoots produced roots within two weeks of culture in both full-strength and half-strength MS media, but a delay in root induction was observed at high auxin concentrations due to callus formation at the base of shoots. For the control treatment, the half-strength MS medium was more efficient in inducing roots directly from the basal part of shoots compared to the full-strength MS medium (Table 8 & Table 9).

The first auxin tested for root induction was IBA with concentrations of 0.5, 1.0, and 1.5 mg/L in half- or full-strength MS media. The earliest root induction occurred when shoots were placed on half-strength MS medium with 0.5 mg/L IBA, and the first roots appeared from the lower part of the shoots after 7 days of culture. In all cases, IBA in half-strength MS media performed better compared to IBA in full-strength MS medium (Table 8 & Table 9). The general pattern of responses obtained showed that all responses decreased with increasing concentration of IBA. The highest percentage of root induction was observed on half-strength MS medium fortified with 0.5 mg/L of IBA, with 92.22% from shoot tips and 91.11% for nodes (Table 9). The lowest response of root induction was obtained when shoot tips and nodes were cultured on full-strength MS medium with 1.5 mg/L IBA.

The use of NAA at different concentrations was also evaluated for its effectiveness in inducing root formation. The first root appeared within 10-12 days in root initiation media, which was later compared to IBA. It can be observed that the percentage of root induction, number of roots per explant, and root length increased proportionally with increments in NAA concentration up to 1.0 mg/L, either on full-strength or half-strength MS medium for both explants (Table 8 & Table 9). The highest number of roots/explant, 9.73 (shoot tips) and 9.53 (derived from nodes) were observed in half-strength MS medium supplemented with 1 mg/L NAA, and with root lengths of 3.06 cm and 2.92 cm for shoot tips and nodes, respectively. This medium also demonstrated the highest response in root induction, with 85.56% root regeneration in shoot tips and 83.33% in nodes.

The present study also investigated different concentrations of IAA, 0.5, 1.0, and 1.5 mg/L in both half- and full-strength MS media. When IAA was incorporated into both strengths of MS media, it was observed that the response for all measured parameters for both explants increased with the incremental rise in IAA concentration up to 1.0 mg/L (Table 8 & Table 9). A concentration of 1 mg/L IAA added to half- and full-strength MS media produced the highest response of root induction from the nodes, the highest number of roots per explant, and the longest root induced from both explants.

Table 8. Effect of full-strength MS with different concentrations of IBA, NAA, and IAA on rooting

IBA (mg/L)	NAA (mg/L)	IAA (mg/L)	Full-strength MS Shoot tips			Full-strength MS Nodes		
			% of root induction	No. of roots per explant	Root length (cm)	% of root induction	No. of roots per explant	Root length (cm)
0	0	0	68.89 ± 1.11^{a}	2.15 ± 0.08^{m}	0.87 ± 0.04^{x}	61.11 ± 1.11^{ab}	2.02 ± 0.10^{m}	0.91 ± 0.03^{x}
0.5	-	-	81.11 ± 1.11^{b}	9.23 ± 0.27^{n}	3.03 ± 0.05^{y}	80.00 ± 1.92^{b}	8.99 ± 0.23^{n}	2.96 ± 0.07^{y}
1.0	-	-	68.88 ± 1.11^{a}	7.27 ± 0.36^{o}	2.63 ± 0.07^{z}	65.56 ± 1.11^{a}	7.15 ± 0.28^{o}	2.48 ± 0.05^{z}
1.5	-	-	60 ± 1.92^{a}	5.28 ± 0.26^{p}	1.61 ± 0.08^{w}	57.78 ± 1.11^{c}	5.09 ± 0.13^{p}	1.77 ± 0.10^{w}
	0.5	-	68.89 ± 2.22^{a}	4.26 ± 0.11^{n}	1.42 ± 0.06^{y}	70.00 ± 1.92^{ab}	4.38 ± 0.11^{n}	1.36 ± 0.05^{y}
	1	-	74.44 ± 1.11^{a}	7.30 ± 0.30^{o}	2.33 ± 0.07^{z}	72.22 ± 1.11^{a}	7.28 ± 0.31^{o}	2.16 ± 0.08^{z}
	1.5	-	66.67 ± 1.92^{a}	4.60 ± 0.16^{p}	1.26 ± 0.05^{w}	65.56 ± 1.11^{b}	5.05 ± 0.17^{n}	1.15 ± 0.04^{y}
		0.5	53.33 ± 1.92^{b}	5.17 ± 0.18^{n}	1.39 ± 0.12^{y}	52.22 ± 1.11^{b}	4.80 ± 0.16^{no}	1.29 ± 0.06^{y}
		1	64.44 ± 1.11^{a}	5.41 ± 0.19^{n}	2.10 ± 0.16^{z}	62.22 ± 1.11^{c}	5.36 ± 0.18^{n}	1.88 ± 0.09^{z}
		1.5	48.87 ± 1.11^{b}	4.35 ± 0.20^{o}	1.14 ± 0.10^{w}	46.67 ± 1.92^{b}	4.29 ± 0.21^{o}	1.28 ± 0.04^{y}

Table 9. Effect of half-strength MS with different concentrations of IBA, NAA, and IAA on rooting

IBA (mg/L)	NAA (mg/L)	IAA (mg/L)	Half-strength MS Shoot tips			Half-strength MS Nodes		
			% of root induction	No. of roots per explant	Root length (cm)	%of root induction	No. of roots per explant	Root length (cm)
0	-	-	75.56 ± 1.11^{a}	2.65 ± 0.09^{m}	1.45 ± 0.06^{x}	70.00 ± 1.92^{a}	2.59 ± 0.10^{m}	1.53 ± 0.05^{x}
0.5	-	-	92.22 ± 1.11^{b}	14.92 ± 0.24^{n}	4.29 ± 0.06^{y}	91.11 ± 1.11^{b}	13.82 ± 0.19^{n}	4.16 ± 0.05^{y}
1.0	-	-	84.44 ± 1.11^{c}	11.08 ± 0.25^{o}	3.17 ± 0.05^{z}	83.33 ± 1.92^{c}	10.98 ± 0.20^{o}	2.94 ± 0.10^{z}
1.5	-	-	74.44 ± 1.11^{a}	7.75 ± 0.21^{p}	2.61 ± 0.07^{w}	72.22 ± 1.11^{a}	7.54 ± 0.22^{p}	2.41 ± 0.07^{w}
	0.5	-	76.67 ± 1.92^{ab}	6.77 ± 0.22^{n}	1.92 ± 0.05^{y}	75.56 ± 1.11^{a}	6.51 ± 0.18^{n}	1.81 ± 0.09^{y}
	1	-	85.56 ± 1.11^{b}	9.73 ± 0.32^{o}	3.06 ± 0.07^{z}	83.33 ± 1.92^{b}	9.53 ± 0.27^{o}	2.92 ± 0.06^{z}
	1.5	-	72.22 ± 1.11^{a}	6.45 ± 0.20^{p}	1.45 ± 0.06^{x}	68.89 ± 1.11^{c}	6.11 ± 0.16^{n}	1.37 ± 0.04^{y}
	-	0.5	57.78 ± 1.11^{b}	6.46 ± 0.34^{n}	1.71 ± 0.08^{y}	56.67 ± 1.92^{b}	6.12 ± 0.29^{n}	1.56 ± 0.08^{x}
	-	1	74.44 ± 1.11^{a}	8.72 ± 0.29^{o}	2.31 ± 0.07^{z}	73.33 ± 1.92^{a}	8.46 ± 0.28^{o}	2.24 ± 0.05^{y}
	-	1.5	55.56 ± 1.11^{b}	6.0 ± 0.23^{p}	1.32 ± 0.05^{w}	54.44 ± 1.11^{b}	5.31 ± 0.18^{n}	1.62 ± 0.10^{w}

The overall results revealed that the half-strength MS medium was more effective in inducing adventitious rooting in stevia compared to the the full-strength MS medium. Half-strength MS medium is more effective as relatively low salt concentrations in the growth medium were able to enhance adventitious rooting efficiency in shoots (Dönmez *et al*., 2022). The addition of auxins (IBA, NAA & IAA) produced profuse rooting. Compared to IAA and NAA, IBA is preferred for rooting because IAA is sensitive to light, while NAA can induce callus at the base of explants (Bhojwani & Dantu, 2013). Previous studies have also reported that IBA was the most effective auxin for rooting in stevia, producing the maximum number of roots (Verma *et al*., 2012; Majumder & Rahman, 2016). According to Ludwig-Muller (2000), the acceleratory effects of IBA on root development might be due to several factors, such as preferential uptake, transport, and stability over other auxins, leading to gene activation.

Acclimatization

After 30 days of rooting, the rooted plantlets were grown in polystyrene cups filled with pre-wetted sterilized peat moss and covered with transparent polybags to maintain high humidity. The plantlets were kept in a controlled environment for hardening for 2 weeks, and the polybags were gradually removed to acclimatize the plantlets under controlled conditions (Figure 1d). After two weeks, the hardened plants were transferred to large polybags filled with soil, and the plants were maintained under field growth conditions for further acclimatization (Figure 1e). The acclimatization success rate was 86.67% and successfully acclimatised plants exhibited robust growth in the field. *In vitro* regenerated plants were phenotypically consistent with the mother plant, and after one and a half months, flowering was observed in most of the plants.

Acclimatization or hardening is a crucial step in ensuring the success of plant *in vitro* culture. The survival percentage of *in vitro* regenerated plants in this study is comparable with other stevia tissue culture studies. About 63% survival was reported by Patel and Shah (2009), while the highest survival rate (95%) was reported by Majumder and Rahman (2016). Different compositions of soil influenced the survival of plantlets. For instance, 82% survival was obtained using a mixture of 1 sand:1 soil: 1 vermiculite (Anbazhagan *et al*., 2010) while lower survival (65.8%) was obtained using 1 sand:1 soil (Thiyagarajan and Perumal, 2012).

ISSR – PCR analysis

A total of 38 ISSR primers were screened on the extracted genomic DNA of mother plants and *in vitro* plants obtained from shoot and nodal explants after confirming the presence of DNA and verifying the concentration and purity. Following the PCR protocol, the PCR products were subjected to gel electrophoresis on 1.5% agarose. The result of gel electrophoresis using different ISSR primers was intended to demonstrate the capability of ISSR primers selected to assess the genetic fidelity

between mother plants and *in vitro* plants.

Results showed that all ISSR primers produced reproducible, clear, and scorable amplification products, with 35 out of 38 ISSR markers exhibiting monomorphic banding patterns, with band sizes ranging from 250 bp to 6000 bp (Figure 2). Concurrently, 3 primers, IS 42/2, IS 52/1, and IS 54 revealed polymorphic and unambiguously scorable banding patterns (Figure 2c). These 3 primers generated 7 to 12 polymorphic bands with a size range of 250 bp to 3000 bp. Based on the results obtained where 35 out of 38 ISSR markers gave monomorphic banding patterns, it can be concluded that the genetic fidelity of *in vitro* stevia plants is being maintained and that the ISSR marker is an effective marker for assessing genetic fidelity between mother plants and *in vitro* plants.

Some plants produced through *in vitro* propagation exhibit unpredictable variation, termed somaclonal variation, which can be influenced by both genetic and epigenetic mechanisms (Wijerathna-Yapa *et al*., 2022). Currently, numerous powerful and sophisticated molecular marker techniques have been developed and employed to assess the genetic fidelity and stability of *in vitro* propagated plants. The commonly used markers include inter-simple sequence repeats (ISSR) and randomly amplified polymorphic DNA (RAPD) markers because they are sensitive, straightforward, and cost-effective. Both of these markers have been successfully applied to detect genetic similarities or differences in tissue-cultured materials (Yuan *et al*., 2009). The genetic fidelity of *in vitro* regenerated plants of *Orthosiphon stamineus* (Ali *et al*., 2019), *Dendrobium transparens* (Joshi *et al*., 2023), *Solanum khasianum* (Chirumamilla *et al*., 2021) and *Rubus fruticosus* (Borsai *et al*., 2020) were confirmed using ISSR marker system. Hassanen and Khalil (2013) stated that ISSR markers yielded the highest percentage of monomorphic bands compared to RAPD and AFLP markers. The reliability of ISSR markers in assessing genetic fidelity was also confirmed by Pradhan *et al*. (2023), where more reproducible and scorable bands were obtained using ISSR compared to RAPD markers in tissue culture of orchid. This makes ISSR an ideal genetic marker for various studies, especially genetic diversity, DNA fingerprinting, and phylogenetics, through which the ISSR marker was able to determine the true-to-type nature of *in vitro* regenerated plants of *S. rebaudiana* accession MS007.

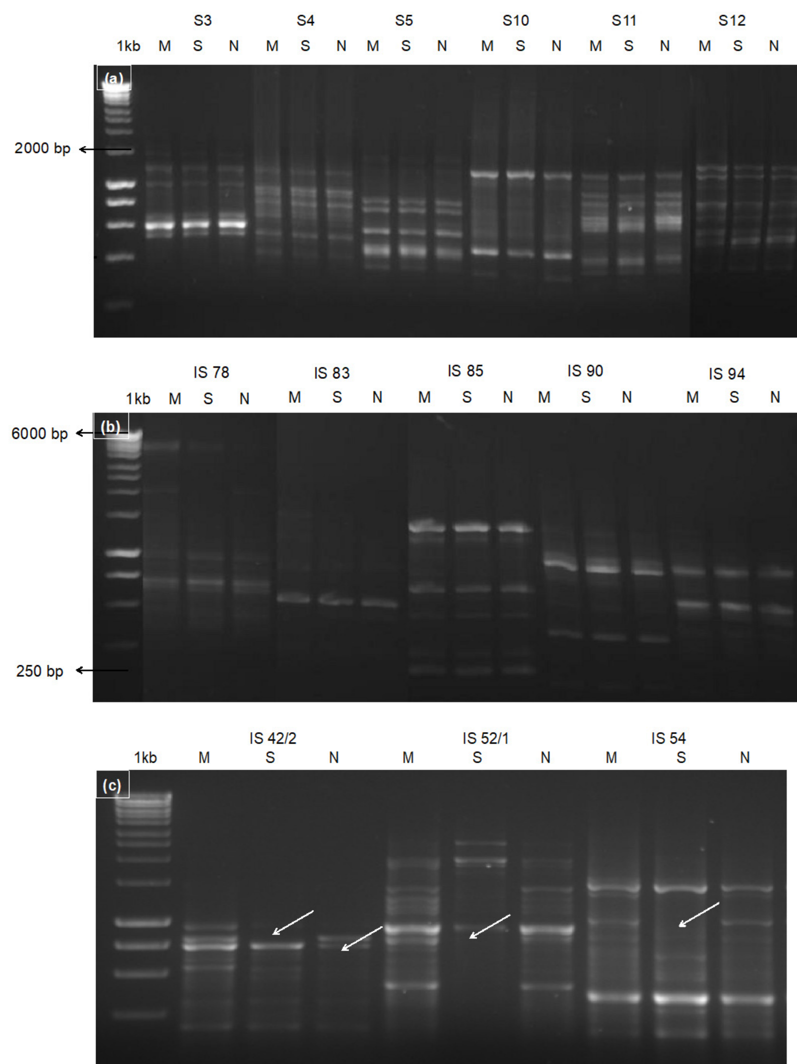

Fig. 2. Banding patterns of ISSR fragments of *Stevia rebaudiana* by using primers (a) S3, S4, S5, S10, S11 and S12, (b) IS78, IS83. IS85, IS90 and IS94 (c) IS 42/2, IS 52/1 and IS54. M = mother plant, S = sample from *in vitro* plant obtained from shoot explant, and N = sample from *in vitro* plant obtained from nodal explant. Banding patterns were sized using Vivantis 1 kb DNA ladder.

CONCLUSION

This study successfully demonstrates *in vitro* clonal propagation of *S. rebaudiana* accession MS 007 using shoot tip and nodal explants, producing the optimal responses in shoot and root induction, as well as survival rates. The *in vitro* regenerated plantlets were phenotypically identical to the mother plant where the homogeneous banding patterns confirmed that genetic fidelity was maintained during *in vitro* culture of the stevia plant. This plant regeneration system is reliable for large-scale propagation in a relatively short time and may be used in future breeding and conservation programs for large-scale stevia production.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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