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Efficient regeneration of *Stevia rebaudiana* Bertoni transformants through hairy root culture technique

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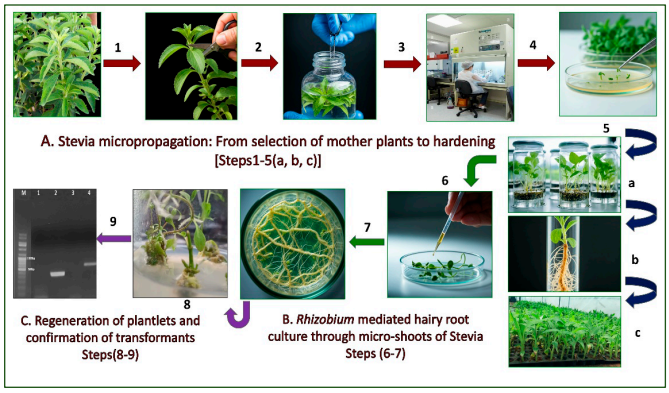
Graphical abstract



Summary

Stevia rebaudiana is a small herb from the Asteraceae family. The plant produces tetracyclic diterpenes, steviol glycosides (SGs) that makes it Stevia sweet. Stevia can act as a natural sweetener as it does not interfere with insulin metabolism in humans when consumed. Amid the prevailing paradigm of health-focused living, Stevia can be produced and used worldwide by individuals and plant breeders. Yet, it is frequently challenging to cultivate stevia conventionally due to low germinating rate and viability of its seeds. Hence, raising plants through tissue culture becomes a viable option. The present study aims to regenerate transformed Stevia lines through *Rhizobium rhizogenes* mediated hairy root culture for investigating the possibility of increasing SGs.

This study also focuses on increasing *Stevia* plant lines through an efficient and cost-effective micropropagation technique. For micropropagation, MS media supplied with 1 mg L^{-1} BAP in combination with 0.2 mg L^{-1} NAA gave the best result for shooting with nodal segments as explants. MS medium supplemented with IBA at a concentration of 1 mg L^{-1} was most effective in promoting the highest number of roots, while 2 mg L^{-1} IBA was optimal for achieving the longest root length. The highest number and frequency of hairy roots were observed with co-culture period of 48 h. Growth of transformed plantlets was observed best with 1 mg L^{-1} BAP + 0.3 mg L^{-1} NAA after 8 weeks of micro-shoots placed on solid MS media. Stevia plants raised through these biotechnological approaches will be a boon to plant breeders and researchers as these are efficient and easy methods to raise Stevia plants. The growing demand for natural sweeteners will be met at lower cost to pharmacology industries and individuals suffering from various life style diseases such as diabetes, hypertension and obesity.

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Keywords: *Stevia rebaudiana*, steviol glycosides (SGs), natural sweetener, micropropagation, Rhizobium rhizogenes, hairy roots, transformed plantlets.

Introduction

Stevia rebaudiana BERTONI, a perennial herb from the Asteraceae family indigenous to South America, is renowned for its leaves that contain steviol glycosides (SGs), a type of diterpenoid plant secondary metabolite (PSM) that is approximately 300 times sweeter than sucrose (Peteliuk et al., 2021). These non-caloric SGs have been seemingly reported to lower blood pressure and blood glucose levels, making them valuable as natural sweeteners and food additives, especially amidst the rising global incidence of diabetes and obesity (Libik-Konieczny et al., 2021). In addition to their sweetening properties, SGs might exhibit various therapeutic effects, including antibacterial, anti-inflammatory, antihypertensive, and immunomodulatory activities (Gaweł-Bęben et al., 2015; Carbonell-Capella et al., 2013).

Conventional propagation of Stevia is hindered by low seed viability and germination rates, as well as cross-pollination challenges that impede the cultivation of elite, homogenous plant populations with high glycoside concentrations (Al-Taweel et al., 2021). To meet industrial demands, tissue culture techniques such as micropropagation and *Rhizobium rhizogenes*-mediated genetic transformation have been employed to enhance SG production (Sharma et al., 2023).

Micropropagation through *in vitro* culture ensures year-long demand of plant material to meet the commercial requirement. *In vitro* cultures have significantly contributed to the synthesis of pharmaceutically and industrially relevant PSM (Teixeira Da Silva et al., 2019). *In vitro* cultures of plant tissue, cells, and organs can be used to quickly produce disease-free, homogeneous plants with high quantities of persistent PSM that are specifically targeted (Pasternak and Steinmacher, 2024). *In vitro* plant tissue culture techniques offer a practical alternative for the production of pharmaceutically important plant based PSM (Deshpande et al., 2014).

Rhizobium rhizogenes-mediated (formerly *Agrobacterium rhizogenes*) hairy root culture is an efficient method for genetic transformation and PSM production in Stevia (Michalec-Warzecha et al., 2016). Infection of explants induces hairy roots through the integration of root-inducing (Ri) T-DNA into the plant genome, leading to rapid growth and high PSM production. The root loci (*rol*) genes, particularly *rolB*, are crucial for hairy root induction and influence the phenotype of regenerated plants (Mauro and Bettini, 2021).

Although Stevia roots typically lack or have less SGs as compared to the leaves as the primary site of steviol glycoside accumulation (Peteliuk et al., 2021; Ghaheri et al., 2019), hairy roots can enhance water and nutrient absorption, potentially increasing secondary PSM production (Singh et al., 2023; Sathasivam et al., 2022). Regenerating whole plants from micro-shoots with hairy roots in-

volves culturing them on shoot induction media with appropriate plant growth regulators, resulting in transformants that retain desirable genetic modifications. This approach offers a scalable solution for commercial Stevia production and the enhancement of bioactive compounds, indirectly boosting SG synthesis in the plant thus benefiting both plant breeders and researchers.

This study aims to develop an efficient micropropagation strategy for the continuous, year-round regeneration of Stevia plants, ensuring a stable supply for large-scale production. Additionally, it seeks to enhance steviol glycosides (SGs) content by generating genetically transformed plants via hairy root cultures. These biotechnological approaches will provide a scalable platform for *Stevia* cultivation, optimizing bioactive compound profiles and addressing the growing global demand for natural sweeteners.

Materials and methods

Plant tissue culture

Two-month-old stevia plants were purchased from a nearby nursery in Pune, India and were authenticated by Botanical survey of India, Government of India, No. BSI/WRC/Iden. Cer. / 2022/1706220020583. Shoot tips and nodal segments from the stock plants were collected as explants for *in vitro* micropropagation. For the current tissue culture investigation, Murashige and Skoog's (MS) (Murashige and Skoog, 1962) medium was supplemented with 30 g L^{-1} sucrose as a carbon source and 8 g L^{-1} agars as a solidifying agent. In an aseptic setting, the explants were cultured onto growth media that contained varying doses of plant hormones. The plant growth regulators (PGRs) used in this study comprised of 6-benzyl amino purine (BAP), napthalene-1-acetic acid (NAA), indole 3-acetic acid (IAA) and indole-3-butyric acid (IBA). Inoculation for various concentrations and combinations were done in 6 replications. Incubation of the cultures was done at 25 ± 2 °C under 16/8 h (light/dark) photoperiod. The pH of the media was maintained between 5.6 and 5.8 and dispensed in 20/25 ml culture vials. Culture vials with media are sterilized by autoclaving at 15 psi and 121 °C for 20 min and allowed to solidify before inoculation. After immersing the transplants in surfactant for 10 min, they were surface sterilized by washing them under running water. Washed them with anti-fungal (cefotaxime 0.6%) and antibacterial (streptomycin 0.5%) solutions for 5 min each. Before being inoculated, the disinfected explants were rinsed with autoclaved double distilled water, dried on sterile filter paper and further sterilized for 10 min using 0.1% $HgCl_2$ (Rezaie et al., 2018) under a laminar flow cabinet. Surface sterilized plant materials were inoculated on various test media.

Micropropagation

Shoot tips and nodal segments with axillary buds, of 1 cm length were inoculated as explants in media supplied with varying amounts of plant growth regulators. Different media combinations used for the present studies include BAP, (0.0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}), as well as 1 mg L^{-1} BAP along with different concentrations of IAA (0, 0.2, 0.3, 0.4, 0.5 mg L^{-1}) and 1 mg L^{-1} BAP along with NAA at different concentrations (0, 0.2, 0.3, 0.4, 0.5 mg L^{-1}). Shoot growth was observed at time interval of 7 days up to 3 weeks. The resulting shoots were aseptically placed onto MS medium that had rooting hormones IAA and IBA at 0.5, 1.0, 1.5, 2.0 mg L^{-1} concentration each added to it. Explants without PGRs were used as control. After successful root development the plantlets were transferred carefully to a greenhouse in small poly bags with coco-peats for acclimatization.

Establishing *Rhizobium rhizogenes* mediated hairy root culture

Rhizobium rhizogenes strain MTCC 532 was obtained from Microbial Type Culture Collection, Chandigarh and grown in YEP broth modified medium (10 g L^{-1} yeast extract, 10.0 g L^{-1} peptone, and 5 g L^{-1} sodium chloride in pH 7.0 ± 0.2, at 25 °C). From *R. rhizogenes* strain MTCC532, single colony was selected for inoculation into 5 mL of YEP broth in a 15 mL falcon tube and incubated overnight at 28 °C in a rotary shaker (150 rpm) to reach 0.6 optical density (Rezvankhah et al., 2022). Subsequently, the bacteria were centrifuged for 10 min at 6000 rpm and the pellet was then resuspended in 5 mL of liquid MS liquid medium at pH 5.8, devoid of sucrose and plant growth regulators. The bacterial cultures were maintained for growth at 28 °C for 2 h in a shaker incubator at 100 rpm, before inoculation. Plantlets originated from *in vitro* tissue culture were dissected, pricked with sterilized syringe needle at nodal areas and midveins of the leaves and co-cultured with the 2 h old bacterial culture with continuous agitation in dark for 5, 10, 30 and 45 min each and 1 h. Explants treated as control were kept in double distilled water for the same time. Tissue paper dried explants were transferred to agarified 1/2MS media (Yang et al., 2020) without any growth regulators and incubated for a period of 1, 2, 3, 4, 6, 24, 48 and 72 h. Antibiotic cefotaxime is used to wash the explants, to get rid of excess bacteria and dried and transferred to 1/2MS solid media comprising 250 mg L^{-1} cefotaxime, without adding any hormones. After inoculation the cultures were maintained in dark at 26 °C, throughout the experimental period. Three replications were taken for each co-cultivation duration and concentration of antibiotic was subsequently reduced.

Regeneration of plantlets.

The explants with hairy roots were aseptically transferred to MS media supplemented with 0, 0.1, 0. 2, 0.3, and 0.4 mg L^{-1} NAA plus 1 mg L^{-1} BAP. Media contained 30.0 mg L^{-1} sucrose and 8 g L^{-1} agar and cultures were maintained under 16/8h (light/ dark) photoperiod. Subculturing was often done to avoid excess rooting. Transformed plantlets regenerated completely after 60 days of inoculation of micro-shoots.

Confirmation of transformation

Specific primer pairs were employed to amplify the root loci *rolB* and *rolC* genes, as well as to control for bacterial contamination by targeting the *virD2* gene. The *rolB* gene fragment (779 bp) was amplified using the forward primer 5'GCTCTTGCAGTGCTAGATTT3' and the reverse primer 5'GAAGGTGCAAGCTACCTCTC3', while the *rolC* gene fragment (540 bp) was amplified with the forward primer 5'GAAGACGACCTGTGTTCTC3' and reverse primer 5'CGTTCAAACGTTAGCCGATT 3'. To detect the *virD2* gene fragment (630 bp), the forward primer 5´-CGGAGGCTCAGAGTGATTG-3´ and the reverse primer 5´-AGCAGCTGTGATCGATGTG-3´ were used. All primers were ordered and purchased from GK Biosciences, Pune, Maharashtra. Genomic DNA was extracted from the control non transformed roots, hairy roots and transformed plantlets of *Stevia rebaudiana* using Thermo Scientific GeneJet Plant Genomic DNA Purification Mini Kit. DNA content and purity were assessed spectrophotometrically at both wavelengths of 260 and 280 nm. PCR reaction was set up with Taq polymerase buffer comprising 1.5 mM $MgCl_2$, 200 pmol of each dNTP, 200 pmol primers, 100 ng of genomic DNA, and 2U Taq polymerase and the amplification reaction volume 25 µl with thermostat settings at (Initial denaturation, 94 °C-3 min; denaturation, 94 °C-60 s; extension, 55 °C-60 s; extension, 72 °C-60 s; final extension, 72 °C-10 min: 35 cycles). UV light was used to detect the amplified products following 1.2% of agarose gel electrophoresis that was visualized using ethidium bromide stain.

Statistical analysis

Twelve explants were chosen for each treatment, and the studies were run in six replications in plant tissue culture and three replications in

hairy root culture. The result was expressed as mean value ± standard error. SPSS (Statistical Package for Social Science; version 17) was used for statistical analysis. To assess significance of the mean values using Tukey's Honestly significant difference (HSD) test at ($p<0.05$), the one-way ANOVA was used to examine the significance of each group, and the Post Hoc test was applied to confirm the results.

Results

Micropropagation

The *in vitro* micropropagation of *Stevia rebaudiana* was conducted using nodal explants. Fig. 1 and 2 provide a clear representation of the steps involved in the micropropagation process. These figures also highlight the most effective shooting and rooting responses achieved with different combinations of plant growth regulators used in the study. Observations were systematically recorded at regular intervals throughout the experiment to monitor the growth and development of the explants.

Shoot regeneration

For Stevia *in vitro* propagation, phytohormones such as BAP in isolation and BAP in combinations with IAA and NAA were used for the shoot development using nodal segments (Fig. 1: A) and shoot tips on MS media (Tab. 1). Optimum shoot numbers observed from nodal segments as explants was 13.29 ± 0.14 by using 1 mg L^{-1} BAP. Similarly, maximum plant height (4.08 ± 0.07 cm) was also recorded with BAP in 1mg L^{-1} concentration (Fig. 1: B-D). BAP also gave best result with shoot tip explants regarding the quantity of shoots (7.13 ± 0.09) at 1.5 mg L^{-1} concentration and shoot length (3.47 ± 0.11 cm) at a concentration of 1 mg L^{-1}. A combination of BAP at 1 mg L^{-1} and 0.2 mg L^{-1} NAA displayed highest number of shoots (13.67 ± 0.17) and maximum plant height (5.00 ± 0.05 cm) in nodal segment explants. Whereas in shoot tips, a combination of BAP (1 mg L^{-1}) and NAA (0.1 mg L^{-1}) recorded the highest number of shoots (8.25 ± 0.17). Best plant height was obtained (4.47 ± 0.10 cm) when BAP (1 mg L^{-1}) was used along with NAA (0.2 mg L^{-1}). From the observation, BAP (1 mg L^{-1}) + IAA (0.5 mg L^{-1}) indicated the lowest response in various parameters studied as shown in (Tab. 1) by using both nodal and shoot tip explants. From the various parameters studied by using different plant growth regulators, Stevia nodal explants recorded better response.

Root regeneration

MS media was supplemented with IBA and NAA at equimolar concentrations of 0.5, 1.0, 1.5, 2.0 (mg L^{-1}) (Tab. 2). Optimum numbers of roots (9.67 ± 0.17) were formed with IBA at 1.0 mg L^{-1} whereas 2 mg L^{-1} IBA exhibited best result in terms of root length (6.28 ± 0.10 cm). NAA showed highest response at 1.0 mg L^{-1} in terms of both root numbers (7.13 ±0.18) and root length (4.15 ± 0.05 cm). NAA at 2.0 mgL^{-1} showed the lowest response. In general, IBA was

Tab. 2: Root regeneration stage of micropropagation in different combinations of plant growth regulators (PGRs) in MS medium.

Treatments	Plant growth regulators	Concentration of plant growth regulators (mg L^{-1})	Root Number	Root length (cm)	Regeneration frequency
T0	IBA	0.5	7.13±0.09^{a}	3.98±0.14^{e}	100
T1		1.0	**9.67±0.17^{b}**	5.17±0.08^{f}	100
T2		1.5	6.17±0.17^{c}	3.27±0.16^{g}	90
T3		2.0	8.08±0.08^{d}	**6.28±0.10^{h}**	75
T0	NAA	0.5	6.88±0.09^{a}	3.98±0.14^{c}	90
T1		1.0	**7.13±0.18^{a}**	**4.15±0.05^{c}**	95
T2		1.5	6.83±0.12^{a}	3.65±0.16^{c}	80
T3		2.0	4.21±0.16^{b}	3.27±0.16^{dc}	66.66

*Values are the mean of roots number and the length of the roots with different set of explants and different concentration of PGRs ± determined by treatments mean value using statistical package SPSS, with one way ANOVA that shows all data are statistically significant (<0.05). Post Hoc test of multiple comparison was done via Tukey HSD. Different letters represent statistically significant data and the same letters represent statistically non-significant data. The experiment was performed in 6 replicates.

Tab. 1: Shoot regeneration data of Stevia micropropagation, in MS media supplied with different combinations of plant growth regulators (PGRs)

Plant growth regulator	Concentration in (mg L^{-1})	Number of shoots (Nodal segment)	Plant height in cm (Nodal segment)	Number of shoots (shoot tips)	Plant height in cm (shoot tips)
BAP	0	4.33±0.21^{a}	2.32±0.16^{f}	2.25±0.17^{i}	2.31±0.11^{m}
	0.5	7.83±0.11^{b}	3.80±0.17^{g}	5.83±0.17^{j}	3.25±0.10^{n}
	1.0	**13.29±0.14^{c}**	**4.08±0.07^{g}**	6.17±0.11^{j}	**3.47±0.11^{n}**
	1.5	6.67 ± 0.17^{d}	3.15±0.17^{h}	**7.13±0.09^{k}**	2.41±0.09^{m}
	2.0	3.50±0.22^{e}	3.07±0.17^{h}	3.25±0.17^{l}	2.29±0.18^{m}
BAP (1 mg L^{-1}) + IAA	0.1	11.17±0.08^{a}	4.35±0.11^{e}	7.67±0.17^{i}	3.69±0.17^{n}
BAP (1 mg L^{-1}) + IAA	0.2	8.75±0.13^{b}	4.20±0.14^{e}	6.08±0.08^{j}	3.65±0.11^{n}
BAP (1 mg L^{-1}) + IAA	0.3	5.50±0.18^{c}	2.52±0.16^{fg}	5.17±0.10^{k}	2.08±0.04^{no}
BAP (1 mg L^{-1}) + IAA	0.4	3.08±0.08^{d}	1.80±0.09^{h}	2.17±0.10^{l}	1.70±0.10^{o}
BAP (1 mg L^{-1}) + IAA	0.5	3.33±0.17^{d}	2.07±0.08^{fh}	3.08±008^{m}	1.80±0.05^{o}
BAP (1 mg L^{-1}) + NAA	0.1	10.79±0.10^{a}	4.18±0.15^{f}	**8.25±0.17^{k}**	3.70±0.14^{p}
BAP (1 mg L^{-1}) + NAA	0.2	**13.67±0.17^{b}**	**5.00±0.05^{g}**	7.83±0.11^{l}	**4.47±0.10^{q}**
BAP (1 mg L^{-1}) + NAA	0.3	4.33±0.08^{c}	2.53±0.11^{h}	3.46±0.16^{m}	2.31±0.14^{r}
BAP (1 mg L^{-1}) + NAA	0.4	6.33±0.17^{d}	3.53±0.09^{i}	4.88±0.12^{n}	2.72±0.04^{s}
BAP (1 mg L^{-1}) + NAA	0.5	8.33±0.17^{e}	4.24±0.13^{j}	7.25±0.06^{o}	3.76±0.17^{t}

*Values are the mean of shoot growth and shoot height with two different set of explants and different concentrations of PGRs ±SE and determined by treatments mean value, with one way ANOVA that shows all data are statistically significant (<0.05). Post Hoc test of multiple comparison was done via Tukey HSD. Different letters represent statistically significant data and the same letters represent statistically non-significant data. The experiment was performed in 6 replicates.

a better PGR in terms of both root number and root length. Regeneration frequency of roots was best with IBA as compared to NAA. Fig. 1: E illustrates the maximum shoot response.

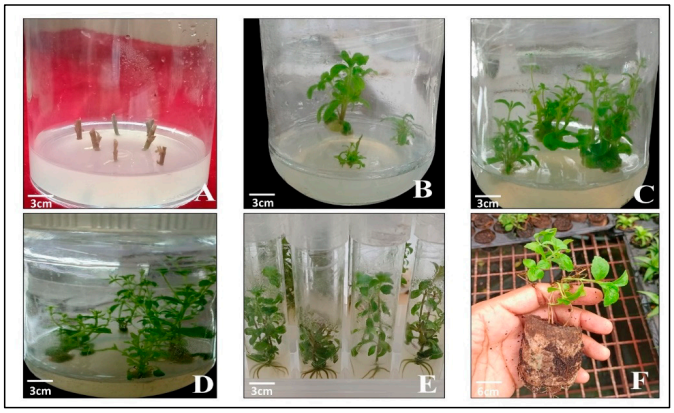

Fig. 1: *Stevia rebaudiana* Micropropagation. A: Inoculation of nodal Segment. B, C, D: Regenerated shoots from nodal segment after 2, 3 and 4 weeks of the culture. E: Rooting observed after 8 weeks. F: Hardening of the Stevia plants in coco-peat maintained in green house.

Hairy root culture establishment in Stevia

Hairy root formation depends on the amount of time the bacteria take to infect the wounded plants, that is the co-culture period. In the current study, the co-culture period recorded was 1, 2, 3, 4, 6, 24, 48 and 72 h. The formation of hairy roots in micro-shoots was checked and recorded from time to time at duration of 8, 16 and 24 days. The number of hairy roots increased in exponential manner with duration and was observed maximum after 24 days (Fig. 2). Taking co-culture period into consideration, 48 h showed the best response and on further increasing the co-culture period to 72 h, it showed a sharp decline in the number of hairy roots. The transformation frequency also followed a similar pattern. In the present studies, highest hairy root formation (11.83 ±0.17) was observed after 24 days and with 48 h co-culture period. Twelve explants were used in each replicati on and 98.61 ± 2.4% transformation frequency was obtained as illustrated in Fig. 3.

Plantlets regeneration from hairy roots

Micro-shoots with hairy roots were incised and aseptically transferred to MS media augmented with 1 mg L^{-1} BAP at concentration of in combination with NAA at different varying concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1}). MS media augmented with 1 mg L^{-1} BAP + 0.4 mg L^{-1} NAA indicated optimum plant regeneration (Tab. 3). The plantlets show ariel hairy root growth at the incised nodal areas. (Fig. 5). The number of shoots recorded was highest at 11.3 ± 0.17 and maximum height observed was 12.2 ± 0.09 cm. An increment in regeneration frequency was recorded as the NAA concentration increased up to 0.4 mg L^{-1}; highest (90%) with BAP (1 mg L^{-1}) + NAA (0.4 mg L^{-1}). On further increasing the concentration of NAA, plants regeneration rate declined.

Confirmation of transformation by rol genes

Crucial Ri plasmid genes in *R. rhizogenes, rol* genes, play a critical role in hairy root formation. Hence, *rol* genes are frequently utilized as reference genes to confirm the hairy roots. The *virD2* gene serves as a

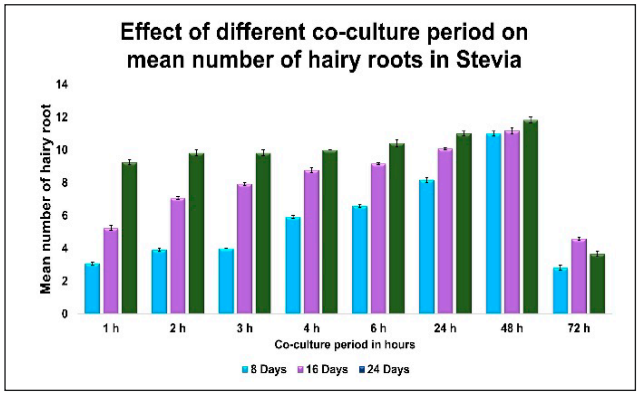

Fig. 2: Representation of effect of different co-culture periods with *R. rhizogenes* strain MTCC 532 on mean number of hairy roots induction in Stevia micro-shoots. 8,16 and 24 days represents the time interval between each observation. The highest mean number of hairy roots was recorded after 24 days with co-culture period of 48 h.

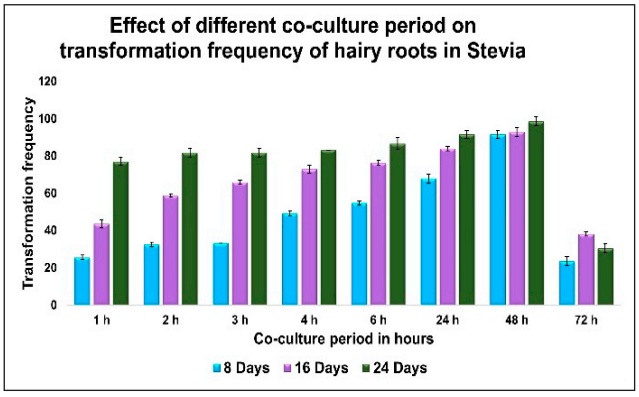

Fig. 3: Representation of effect of different co-culture periods with *R. rhizogenes* strain MTCC 532 on transformation frequency of hairy roots in Stevia micro-shoots. 8,16 and 24 days represents the time interval between each observation. The highest transformation frequency was recorded after 24 days with co-culture period of 48 h.

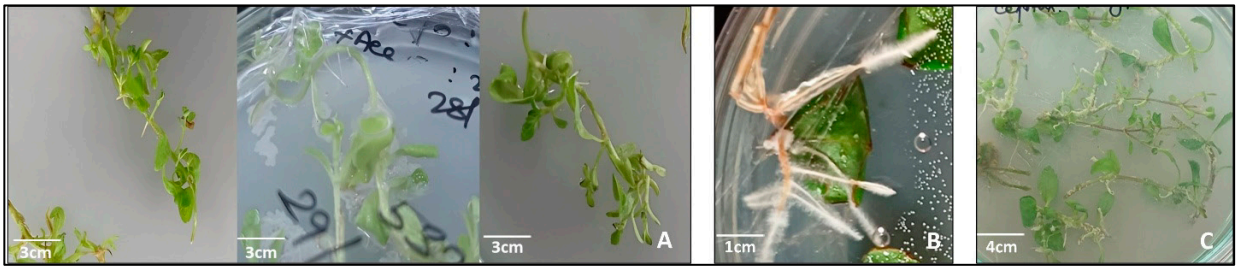

Fig. 4: Micropropagated Stevia plantlet treated with *Rhizobium rhizogenes* strain MTCC 532. A: Initiation of Hairy roots after 2 weeks of culture. B and C: Growth of hairy roots after 3 weeks.

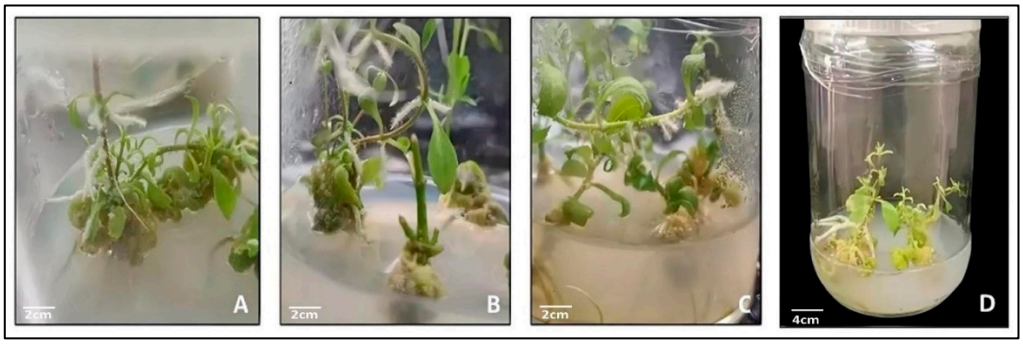

Fig. 5: Plantlets regenerate from hairy roots after 2 months of hairy root formation. Also, the aerial adventitious hairy roots form can be seen.

Tab. 3: Plantlet regeneration in plant growth regulators at varying concentrations added to MS medium.

PGR (mg L^{-1})		Regeneration efficiency%	Number of shoots	Shoot height (cm)
BAP	NAA			
1	0	0	7.3 ± 0.17^{a}	5.7 ± 0.13^{e}
1	0.1	55	8.6 ± 0.08^{b}	7.3 ± 0.17^{f}
1	0.2	73	9.2 ± 0.17^{b}	8.5 ± 0.07^{g}
1	0.3	87	10.3 ± 0.25^{c}	11.2 ± 0.32^{h}
1	0.4	90	**11.3 ± 0.17^{d}**	**12.2 ± 0.09^{i}**
1	0.5	0	-	-

*Values are the regeneration frequency of plants, mean number of shoots and shoot height with different concentration of plant growth regulators (PGRs) ±SE at $p<0.05$. One way ANOVA shows all data are statistically significant. Tukey's test measures the statistical significance within each means. Different letters represent statistically significant data and the same letters represent statistically non-significant data. The experiment was performed in triplicates.

molecular marker to detect bacterial contamination in hairy root cultures, ensuring the absence of residual *Rhizobium rhizogenes*. In the present study, *rolB* and *rolC* genes from hairy roots and regenerated plantlets of Stevia were taken, Ri plasmid from the bacterium served as positive control (PC) and normal adventitious roots from germ-free *Stevia rebaudiana* were considered as negative control (NC). The PCR amplification result show *rolB* and *rolC* presence in hairy root samples as well as in hairy roots regenerated plantlets (Figure 6) which indicated transformation. However, these genes were absent in the normal roots. The *virD2* was absent in transformants.

Discussion

The present study demonstrates the efficacy of micropropagation and *Rhizobium rhizogenes* mediated hairy root culture in *Stevia rebaudiana*, highlighting the pivotal role of plant growth regulators (PGRs) in optimizing these biotechnological approaches.

Plant growth regulators become important for effective *in vitro* plant regeneration (Sehgal and Joshi, 2022). The current study demonstrated that supplementing MS media with PGRs significantly enhances shoot and root formation, emphasizing their role in regeneration. In micropropagation, 1 mg L^{-1} BAP yielded optimal shoot height and the highest shoot number from nodal segments, while 1.5 mg L^{-1} BAP produced the most shoots from shoot tip explants. Similar findings were reported by Asmuni and Hakiman (2020) for *Stevia rebaudiana*.

Our findings indicate that supplementing MS media with BAP alone or in combination with auxins such as IAA and NAA significantly enhanced shoot proliferation from nodal and shoot tip explants. Notably, a combination of 1 mg L^{-1} BAP and 0.2 mg L^{-1} NAA yielded the highest number of shoots and maximum plant height in nodal segment explants. This aligns with recent studies emphasizing the synergistic effects of cytokinin and auxins in Stevia micropropagation. For instance, Rodríguez-Páez et al. (2024) reported that optimal shoot multiplication in Stevia genotypes was achieved using BAP in combination with NAA, underscoring the importance of specific PGR concentrations tailored to genotypic responses.

The study also highlights the superior efficacy of IBA over NAA in promoting root induction. Optimal root numbers were observed with 1.0 mg L^{-1} IBA, while the longest roots were achieved with 2.0 mg L^{-1} IBA. This is consistent with findings by other researchers who have demonstrated the effectiveness of IBA in root induction protocols

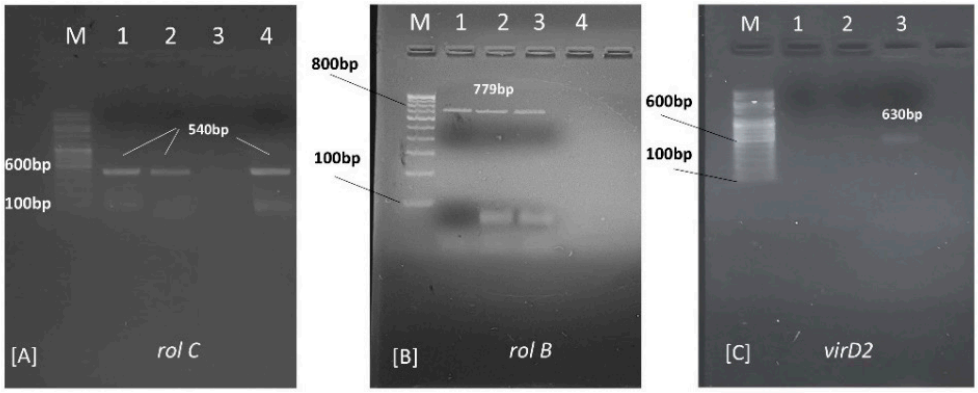

Fig. 6: PCR amplification of genes. A) lane 1 and 2: DNA from hairy roots and transformed plantlets; lane 3: DNA from non-transformed root (Negative control); lane 4: Ri plasmid from *R. rhizogenes* (Positive control). B) lane 1: DNA from non-transformed root (Negative control); lane 2-3 DNA from hairy roots and transformed plantlets; lane 4: Ri plasmid from *R. rhizogenes* (Positive control). C) lane1 and 2: DNA from hairy roots and transformed plantlets; lane 3: Ri plasmid from *R. rhizogenes*. Lane M: 100 bp DNA ladder.

for *Stevia rebaudiana*. For example, a study conducted by Bouaaza et al. (2024) on the effect of gelling agents and PGRs on *in vitro propagation* of stevia reported that IBA significantly enhanced root formation, further supporting our observations. In our study, nodal explants showed better results compared to shoot tips. The results of the present study align with previous research, highlighting the superior performance of nodal explants in regeneration. Taak et al. (2020) reported an 82.85% regeneration response in *Stevia rebaudiana* using nodal explants. Comparable outcomes were also observed in study conducted by Azad et al. (2022) in *Smilax zeylanica*, further supporting the effectiveness of nodal explants for regeneration.

To induce hairy roots, micro-shoots of tissue-cultured *Stevia* plants were infected with *Rhizobium rhizogenes* strain MTCC 532 and cultured on half-strength MS basal media. Hairy root formation began after 8, 16, and 24 days, with the highest number and transformation frequency observed at 48 h of co-culture. Similar findings were reported by Brijwal and Tamta (2015) in *Berberis aristate* and by Phuong et al. (2023) in *Allium sativum*, where a 48h co-culture period achieved maximum transformation (93%). Prolonging co-culture to 72 h drastically reduced root formation and transformation frequency, consistent with observations in *Stevia rebaudiana* by Singh et al., (2017) and in *Duboisia leichhardtii* by Singh et al. (2018). The drastic reduction of hairy roots after 72 h could be due to excessive bacterial growth, considering its 48 h incubation period. However, Amini et al. (2024) found the highest transformation efficiency (68.18%) at 72 h in *Verbascum erianthum* and *Verbascum stachydiforme*.

Micro-shoots with hairy roots when transferred to MS media supplemented with BAP and NAA, regenerated transformed plantlets after 8 weeks of hairy root formation, suggesting the importance of plant growth regulators in shoot induction. Jedličková et al. (2024) and He-Ping et al. (2001) reported the same in *Brassicaceae* and *Pogostemon cablin* respectively. In both the studies callus stage was observed before shooting. Whereas in the present study, transformed plantlets exhibited direct shooting without callus formation. This might be due to the usage of micro-shoots with hairy roots as explants not objectively hairy roots. Similar to the current findings, Jedličková et al. (2024) reported spontaneous aerial adventitious hairy roots in transformants of some Brassicaceae plants. This methodology can also be applied for the regeneration of other plant species. If no or little regeneration occurs in other species or cultivars, the protocol would need some modification.

Rhizobium rhizogenes induced hairy root transformation is variable because of random integration of Ri T-DNA into the plant genome that can affect gene expression, secondary metabolite synthesis and root morphology (Gutierrez-Valdes et al., 2020). Variability in T-DNA integration sites and copy number can cause variation among transformed lines, that is why internal replicates are needed to verify data reliability and repeatability. This study addressed such variability by optimizing co-culture conditions to achieve high transformation efficiency and stable hairy root induction. By using mean number of hairy roots per explants to analyse data and generating multiple hairy root lines, the effect of transformation-related variability was minimized. Molecular validation through *rolB* and *rolC* gene detection confirmed transformation.

Conclusion

This study highlights the potential of combining micropropagation and *Rhizobium rhizogenes*-mediated hairy root culture for efficient regeneration of *Stevia rebaudiana* Bertoni. The findings demonstrate that optimized concentrations of plant growth regulators, such as 1 mg L^{-1} BAP combined with 0.2 mg L^{-1} NAA, significantly enhance shoot regeneration, while 1 mg L^{-1} IBA proves highly effective for rooting. The induced hairy root cultures and subsequent regeneration of transformed plantlets offer a valuable platform for improving steviol glycoside production. This method not only facilitates large-scale propagation of disease-free and genetically identical Stevia plants but also provides a foundation for enhancing secondary metabolite accumulation through genetic transformation which can pave the path for stable integration of transgenes for future use.

Given the increasing global demand for natural sweeteners, these approaches provide a scalable, cost-effective solution for industrial production. Moreover, the transformed Stevia lines with higher steviol glycoside content can serve as an alternative to conventional sugar, aiding in the management of lifestyle diseases such as diabetes, obesity, and hypertension. Future studies could explore the scalability of these methods and the feasibility of large-scale production of steviol glycosides for pharmaceutical and nutraceutical industries.

Author contributions

P.S: Conceptualization, Methodology, Formal analysis, Investigation, Writing—Original Draft. D.L: Methodology, Formal analysis, Investigation. M.C: Methodology, Formal analysis, Investigation. P.D: Methodology, Formal analysis, Investigation. B.P: Methodology, Formal analysis J.D: Review and Editing A.P: Review and Editing. H.T: Review and Editing. M.W: Validation, Resources, Writing—Review and Editing, Visualization, Supervision. All the authors carefully checked and approved the final manuscript.

Conflict of interest

No potential conflict of interest was reported by the authors.

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