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Influence of Plant Growth Regulators on Glandular Trichome Density and Steviol Glycosides Accumulation in *Stevia rebaudiana*

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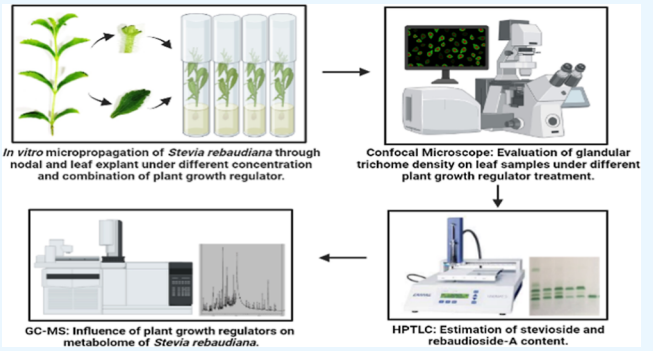
ABSTRACT: With the advent of modern lifestyles, diabetes-related comorbidities attributed the importance of low-caloric natural sweetener plants such as *Stevia rebaudiana*. This plant is the viable source of steviol glycosides (SGs) and other economically important secondary metabolites. Glandular trichomes (GTs) play the role as a reservoir for all secondary products present in the plant species. Therefore, the present study was carried out to evaluate the influence of different plant growth regulators (PGRs) on GT density and its impact on the SG content. The direct shoot regeneration system was developed on Murashige and Skoog (MS) + benzyl aminopurine (BAP) (1.0 mg/L) + naphthaleneacetic acid (NAA) (0.5 mg/L), and MS + BAP (1.5 mg/L) + NAA (0.5 mg/L) from nodal and leaf explants, respectively. Among the combination of PGRs used, MS medium fortified with BAP (1.0 mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5 mg/L) played a significant role in increasing the GT density on leaf and stem tissues of *S. rebaudiana*. Furthermore, high-performance thin-layer chromatography and gas chromatography−mass spectrophotometry data confirmed a notable rise in SGs and other valuable secondary metabolites. Thus, the protocol developed can be used for the propagation of stevia with an improved metabolic profile at a large scale.



1. INTRODUCTION

Stevia rebaudiana (Bertoni) is a valuable medicinal plant known for its low-caloric natural sweetening properties. The leaves of *S. rebaudiana* synthesize steviol glycosides (SGs). These are 300−400 times sweeter than sucrose and low-caloric, non-toxic, and non-mutagenic in nature.[1] Additionally, SGs exhibit a wide range of therapeutic properties such as anti-hyperglycemic,[2,3] antioxidant,[4] anti-microbial,[5] and anti-cancerous.[6] Not only the SGs, but the free sugars, flavonoids, alkaloids, polyphenols, and essential oils are present in the leaf extract. This enriched profile of stevia makes it a promising new industrial crop that meets the demand of low-calorie herbal food ingredients with nutritional and therapeutic properties.[7] By the end of 2024, the global stevia market depicts an incremental opportunity worth US$ 554 million due to the increased demand from the food, beverages, and pharmaceutical industries.[8] Thus, the production of stevia needs to be improved to effectuate the sustainable development goals. The only economically viable source of SGs is the stevia plant. As a result, most of the studies have focused on unraveling its biology in order to increase the production.

Plant trichomes are highly specialized epidermal appendages found on the aerial regions of plants such as stems, leaves, petals, and fruit,[9] which secrete a wide range of chemical substances.[10] Terpenoids,[11] flavonoids,[12−14] methyl ketones, and acyl sugars,[15,16] are among the secondary metabolites synthesized and secreted specifically by glandular trichomes (GTs). Large, small, and GTs were identified on the leaf surface from both the abaxial and adaxial side.[17] A direct relationship was reported between the SG content and GT number on the leaf of *S. rebaudiana*.[18] Recent studies reported the relationship between trichome development in context to the accumulation of SGs, flavonoids, and polyphenols in plants of *S. rebaudiana* under biotic interactions.[19,20] Phytoconstituents secreted by GTs have industrial use; therefore, several strategies have been used to enhance their production.

Plant cell culture is an important technique that ensures the availability of raw material without any seasonal limitation, and bioreactors can be used for mass production.[21] In plant tissue culture techniques, different phytohormones play a critical role in basically every phase of a plant's growth, development, and

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adaptability to its surroundings. Organogenesis-related metabolic pathways, initiated in the presence of externally applied plant growth regulators (PGRs), interacted with a physiological gradient of nutrients and hormones already present in an explant.[22] The role of different phytohormones such as jasmonates, cytokinins, auxins, gibberellins, and brassinosteroids has been extensively proven to modulate epidermal differentiation programs, resulting in a higher density of trichomes.[23] Nguyen et al.[24] reported that PGRs and other constituents of media employed a positive effect on trichome production and artemisinin accumulation in cultures of *Artemisia annua* (Asteraceae family). Another studies suggested the positive effect of benzyl aminopurine (BAP), naphthaleneacetic acid (NAA), proline, methyl jasmonate, and salicylic acid on the amount of stevioside, rebaudioside A, phenols, flavonoids, and antioxidant activity in *S. rebaudiana*.[25−27] However, there are many reports that establish the positive correlation of PGRs with trichome density and secondary metabolite accumulation in different plant species. More research is, however, needed to better define the role played by PGRs in the development of trichomes in *S. reabudiana* with reference to the biosynthesis and accumulation of SGs.[28]

Due to the poor seed germination, conventional multiplication of stevia is limited, necessitating *in vitro* propagation at a large scale. Today's demand will need to be supported by high biomass yielding varieties with improved agronomical traits and higher quantity and quality of secondary products. There is, however, a need to report an efficient propagation method of improved genotype with better metabolite profile to meet the increased economic production rate. We have, therefore, established a rapid method for the mass propagation of *S. rebaudiana* through direct organogenesis of shoots for germplasm conservation and selection of a better yielding variety with higher trichome density, which was positively correlated with the total steviol or non-SG contents. The present study also concluded that PGRs could increase density of GTs on the leaf surface of *S. rebaudiana*, which was related to the enhanced production of important secondary metabolites including SGs.

2. RESULTS

2.1. *In Vitro* Shoot Regeneration and Multiplication. The nodal explants were cultured on full-strength Murashige and Skoog (MS) medium fortified with individual and combinational treatments of cytokinins and auxins (Table 1).

After inoculation of the nodal explant, bud induction was seen during 6−7 days. Afterward, shoot regeneration in percentage and number and length of shoots were recorded. Among various concentrations of BAP, 1.0 mg/L was found optimum for shoot regeneration from the nodal explant with 96.68 ± 0.22^{b} percent shoot regeneration and 2.26 ± 0.03^{de} shoots per explant (5.74 ± 0.03^{c} cm) (Figure 1a).

BAP (1.0 mg/L) in the presence of NAA (0.5 mg/L) [MS-C] gave better results than BAP individually [MS-H] in responses to percent shoot regeneration (98.83 ± 0.20^{a}), shoot number (4.08 ± 0.06^{a}), and shoot length (8.22 ± 0.06^{a} cm) from the nodal explant (Figure 1b). Leaf explants were inoculated on MS medium including the combinations of cytokinin (BAP) with auxins (NAA and 2,4-D), as per given in Table 2.

Among those treatments, MS-N (MS + 1.5 mg/L BAP + 0.5 mg/L NAA) showed direct shoot bud regeneration (94.97 ± 0.14^{a} percent) with 4.68 ± 0.23^{a} shoots per leaf explant from the midrib and distal end (Figure 1c). However, MS-R (MS + 1.0 mg/L BAP + 0.5 mg/L 2,4-D) showed shoot organogenesis with an intermediating callus formation on the base of the leaf explant (Figure 1d). 81.77 ± 0.18^{d} percent regeneration with 2.68 ± 0.22^{de} shoots per leaf explant was recorded on MS-R treatment (Table 2). For shoot multiplication, *in vitro* regenerated shoots (∼2−2.5cm) were transferred as an explant on MS medium including 1.0 mg/L BAP with various concentrations of kinetin (Table 3).

Multiple shoots were observed on MS-X treatment (MS + 1.0 mg/L BAP + 1.5 mg/L kinetin). The higher percentage of healthy shoots (99.23 ± 0.14^{a}), better shoot length (6.04 ± 0.33^{a} cm), and maximum number of shoots (10.14 ± 0.09^{a}) per explant were achieved (Figure 1e).

Table 1. Effect of BAP alone and BAP in Combination with NAA on *In Vitro* Shoot Regeneration of *S. rebaudiana* through the Nodal Explant[a]

	PGRs (mg/L)		nodal explant		
MS	BAP	NAA	% shoot regeneration (mean ± SE)	number of shoots per explant (mean ± SE)	length of shoot (cm) (mean ± SE)
A			62.23 ± 0.33^{h}	1.41 ± 0.29^{f}	1.79 ± 0.15^{i}
B	0.5		89.60 ± 0.34^{f}	1.98 ± 0.01^{de}	3.29 ± 0.01^{h}
C	1.0		96.68 ± 0.22^{b}	2.26 ± 0.03^{de}	5.74 ± 0.03^{c}
D	1.5		93.77 ± 0.14^{c}	2.00 ± 0.01^{de}	4.27 ± 0.13^{fg}
E	2.0		93.72 ± 0.23^{c}	1.98 ± 0.01^{de}	4.75 ± 0.02^{e}
F	2.5		92.16 ± 0.21^{d}	1.28 ± 0.01^{f}	4.12 ± 0.06^{g}
G	0.5	0.5	93.92 ± 0.22^{c}	2.29 ± 0.00^{d}	5.20 ± 0.15^{d}
H	1.0	0.5	98.83 ± 0.20^{a}	4.08 ± 0.06^{a}	8.22 ± 0.06^{a}
I	1.5	0.5	92.68 ± 0.22^{d}	3.57 ± 0.01^{b}	6.43 ± 0.21^{b}
J	2.0	0.5	90.97 ± 0.14^{e}	3.03 ± 0.14^{c}	4.50 ± 0.03^{ef}
K	2.5	0.5	80.29 ± 0.35^{g}	1.94 ± 0.02^{e}	3.32 ± 0.04^{h}

[a]MS—Murashige and Skoog medium. Data have been recorded after 4 weeks of culture. Data are in the form of mean ± SEM of three replicates followed by the same letter in uppercase and are not significantly different at a $P < 0.05$ level using Duncan's test.

2.2. *In Vitro* Rooting and Hardening. After proliferation, elongated shoots were inoculated on two strength of MS media (full and half) with different concentrations of IAA (0.5, 1.0, 1.5, and 2.0 mg/L). 1/2MS with IAA gave a better result in comparison to full-strength MS medium. Root induction was noticed from the shoot base after 1 week of culture without any callus formation. After 2 weeks of culture, root proliferation was observed. After 4 weeks of culture, 8.02 ± 0.09^{a} roots/shoot were observed with the highest percent (97.57 ± 0.23^{a}) of rooting response and highest root length (6.33 ± 0.13^{a} cm) on 1/2MS + 1.0 mg/L IAA (Table 4) and (Figure 1f).

We have observed that above 1.0 mg/L of IAA, the rooting percentage was decreased on half-strength MS medium. We have also noticed that the regenerated shoots from MS-R transferred on rooting medium (1/2MS + 1.0 mg/L IAA) were found to be thicker and longer when compared to rooting response of shoots regenerated on MS with other PGR treatment (data not shown). *In vitro* rooted complete plantlets were maintained in the culture room. The healthy plants were transferred under greenhouse conditions and then transplanted to the field with 78% survivability.

2.3. Effect of PGR Treatment on Trichome Density. *In vitro* shoots regenerated on MS-R treatment (MS + 1.0 mg/L

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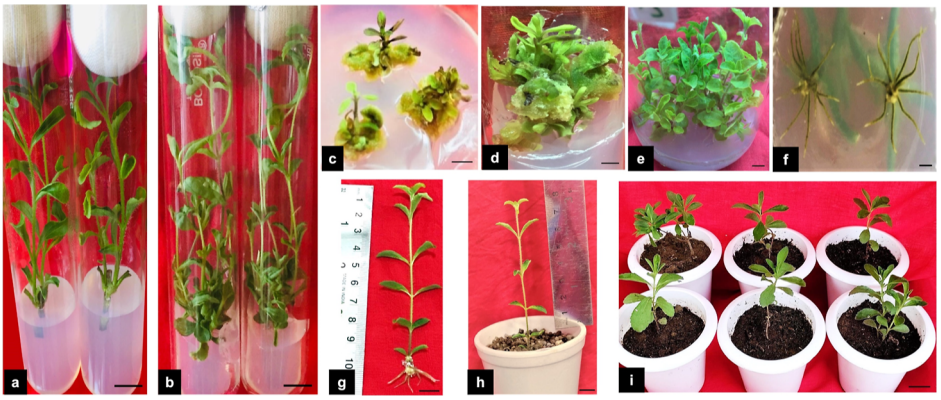

Figure 1. *In vitro* micropropagation of *S. rebaudiana*. (a) Shoot regeneration from the nodal explant on MS + BAP (1.0 mg/L). (b) *In vitro* regenerated shoots on MS + BAP (1.0 mg/L) + NAA (0.5 mg/L) from the nodal explant. (c) Direct shoot organogenesis on MS + BAP (1.5 mg/L) + NAA (0.5 mg/L) from the leaf explant. (d) Indirect shoot organogenesis on MS + BAP (1.0 mg/L) + 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5 mg/L) from the leaf explant. (e) Shoot multiplication on MS + BAP (1.0 mg/L) + kinetin (1.5 mg/L). (f) *In vitro* rooting on 1/2MS + indole acetic acid (IAA) (1.0 mg/L). (g) *In vitro* raised complete plantlet. (h) Hardening and acclimatization in soil rite. (i) Hardened plants in the sand and soil mixture [scale bar (a,b) 1.0 cm; (c−f) 0.5 cm; and (g−i) 1.0 cm].

Table 2. Effect of Cytokinins in Combination with Auxins on *In Vitro* Shoot Regeneration in Leaf Explants of *S. rebaudiana*[a]

	PGRs (mg/L)			leaf explant		
MS	BAP	NAA	2,4-D	% regeneration (mean ± SE)	number of shoots (mean ± SE)	callus induction
L	0.5	0.5		88.83 ± 0.16^{b}	3.26 ± 0.19^{bc}	
M	1.0	0.5		85.73 ± 0.21^{c}	2.91 ± 0.05^{cd}	
N	1.5	0.5		94.97 ± 0.14^{a}	4.68 ± 0.23^{a}	
O	2.0	0.5		81.67 ± 0.24^{d}	3.58 ± 0.22^{b}	+
P	2.5	0.5				+++
Q	0.5		0.5	79.57 ± 0.26^{e}	2.27 ± 0.14^{ef}	++
R	1.0		0.5	81.77 ± 0.18^{d}	2.68 ± 0.22^{de}	+
S	1.5		0.5	75.67 ± 0.24^{f}	1.97 ± 0.02^{f}	++
T	2.0		0.5			+++
U	2.5		0.5			++++

[a]Data have been recorded after 4 weeks of culture. Data are in the form of Mean ± SEM of three replicates followed by the same letter in uppercase and are not significantly different at a $P < 0.05$ level using Duncan's test.

Table 3. Effect of BAP with Different Concentrations of Kinetin on the Shoot Multiplication of *S. rebaudiana*[a]

	PGRs (mg/L)		*in vitro* regenerated shoots as an explant		
MS	BAP	kinetin	% regeneration of shoots (mean ± SE)	number of shoots per explant (mean ± SE)	length of shoot (cm) (mean ± SE)
V	1.0	0.5	96.23 ± 0.33^{cd}	7.75 ± 0.03^{c}	4.38 ± 0.30^{c}
W	1.0	1.0	98.07 ± 0.29^{b}	8.52 ± 0.04^{b}	5.33 ± 0.24^{ab}
X	1.0	1.5	99.23 ± 0.14^{a}	10.14 ± 0.09^{a}	6.04 ± 0.33^{a}
Y	1.0	2.0	95.23 ± 0.50^{d}	6.69 ± 0.09^{d}	3.10 ± 0.20^{d}
Z	1.0	2.5	96.67 ± 0.35^{c}	4.80 ± 0.06^{e}	4.83 ± 0.20^{bc}

[a]Data have been recorded after 4 weeks of culture. Data are in the form of mean ± SEM of three replicates followed by the same letter in uppercase and are not significantly different at a $P < 0.05$ level using Duncan's test.

BAP + 0.5 mg/L 2,4-D) showed different growth characteristics. A noticeable higher density of trichomes were found on stem tissues of the cultured shoots (Figure 2).

A confocal microscopic study of leaf samples from all MS treatments was performed, which revealed that *in vitro* plant regenerated on BAP (MS-C) did not exhibit any major difference in GTs in comparison to control plants regenerated on MS medium free from PGR (MS-A). However, BAP with kinetin (MS-X) and NAA (MS-H, N) deliberated a positive effect on the density of GTs and non-GTs (Figure 3).

Leaf area covered by GTs in percentage was calculated to analyze the overall impact of various PGR treatments on the density of GTs (Figure 4).

The highest impact on the number and the size of GTs on the leaf were found in the MS-R treatment. Therefore, for authentication of active correlation between the concentration of 2,4-D and GT formation, the regenerated *in vitro* shoots were further cultured on MS medium supplied with BAP (1.0 mg/L) + 2,4-D (0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/L). Correspondingly, the enhanced concentration of 2,4-D was associated with the increased density of GTs on shoots (Figure 5).

However, augmentation of 2,4-D at 3.0 mg/L or above showed morphological alterations such as epinasty in leaves and deformed shoots with trichome bunches in culture.

2.4. Quantitative Analysis of the SG Content. High-performance thin-layer chromatography (HPTLC) analysis unveiled a higher content of stevioside and rebaudioside-A with respect to increased GT density on leaf and stem tissues of *in vitro* plants treated with different PGRs when compared to *in vitro* raised control plants along with *in vivo* mother plants (Figures 6 and 7).

In vitro plants cultured on MS-A (without any PGR) and MS-C (1.0 mg/L BAP) showed a similar content of stevioside (leaf: 102.5 and 103.6 mg/g; stem: 28.5 and 28.8 mg/g) and rebaudioside-A (leaf: 30.2 and 30.8 mg/g; stem: 7.8 and 7.6 mg/g). On the other hand, BAP in combination with NAA (MS-H or MS-N) and kinetin (MS-X) influences the SG content, when compared to MS-A or MS-C. The increased content of stevioside and rebaudioside-A was observed in MS-X treatment (123.8 and 46.1 mg/g) in comparison to MS-H (120.2 and 40.5 mg/g) but not in MS-N (132 and 49.5 mg/g). However, in MS-X treatment, lower GT covered leaf area was calculated against MS-H and MS-N also. Furthermore, the highest stevioside and rebaudioside-A content (165.3 and 57.8

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Table 4. Effect of MS Medium with IAA on *In Vitro* Rooting of Elongated Shoots of *S. rebaudiana*[a]

rooting media	IAA (mg/L)	percentage regeneration (mean ± SE)	number of roots per shoot (mean ± SE)	length of roots (cm) (mean ± SE)
half-strength MS medium	0.5	96.90 ± 0.05[b]	7.18 ± 0.04[b]	4.17 ± 0.08[d]
	1.0	97.57 ± 0.23[a]	8.02 ± 0.09[a]	6.33 ± 0.13[a]
	1.5	96.69 ± 0.20[b]	6.58 ± 0.11[c]	5.12 ± 0.07[c]
	2.0	91.67 ± 0.22[c]	4.64 ± 0.14[e]	3.35 ± 0.07[e]
full-strength MS medium	0.5	85.94 ± 0.06[e]	5.22 ± 0.06[d]	4.13 ± 0.06[d]
	1.0	87.73 ± 0.17[d]	4.74 ± 0.12[e]	6.14 ± 0.07[a]
	1.5	84.87 ± 0.13[f]	3.13 ± 0.67[g]	4.11 ± 0.05[d]
	2.0	83.72 ± 0.14[g]	4.03 ± 0.12[f]	5.74 ± 0.04[b]

[a]Data have been recorded after 4 weeks of culture. Data are in the form of mean ± SEM of three replicates followed by the same letter in uppercase and are not significantly different at a $P < 0.05$ level using Duncan's test.

Figure 2. Increased density of GTs at the base of the *in vitro* regenerated shoots (arrow head) from the leaf explant through indirect organogenesis in *S. rebaudiana* on MS + BAP (1.0 mg/L) + 2,4-D (0.5 mg/L). Scale bar: 0.5 cm.

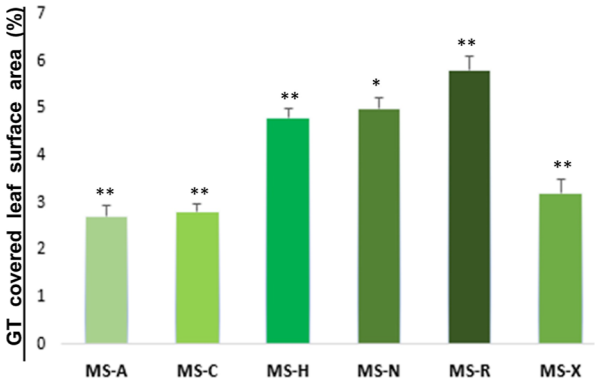

Figure 4. Effect of MS medium containing different PGRs on GT density in leaf samples of *S. rebaudiana*. **MS-A**: MS's medium without any PGR (control), **MS-C**: MS + BAP (1.0 mg/L); **MS-H**: MS + BAP (1.0 mg/L) + NAA (0.5 mg/L); **MS-N**: MS + BAP (1.5 mg/L) + NAA (0.5 mg/L); **MS-R**: MS + BAP (1.0 mg/L) + 2,4-D (0.5 mg/L); and **MS-X**: MS + BAP (1.0 mg/L) + kinetin (1.5 mg/L). Error bars indicate ±SE ($n \geq 3$) (*$P \leq 0.05$ and **$P \leq 0.01$).

mg/g) was observed with the use of BAP + 2,4-D (MS-R) compared to *in vitro* raised control plants (MS-A) along with *in vivo* mother plant (120 mg/g and 40 mg/g). A similar trend was observed in stem tissues also. From overall scenario of the study, we found that auxin (2,4-D more than NAA) played a critical role in the enhancement of SG contents.

2.5. Qualitative Analysis of Steviol and Other Important Secondary Metabolites through Gas Chromatography−Mass Spectrophotometry. The metabolome profiling approach was applied to assess the chemical composition of stevia leaf and stem extracts through gas chromatography−mass spectrophotometry (GC−MS) analysis. The study focused on the changes in secondary metabolite accumulation in response to increased trichome density, under various concentrations of PGRs. Total 58 and 47 compounds were found out in the methanolic extract of *in vitro* derived leaf and stem tissues of stevia, respectively, which were categorized as terpenoids, polyphenols, sugars, and steviol compounds. Hierarchically clustered heatmap generated to correlate the various metabolites of leaf and stem tissues of *in vitro* plants raised on MS-A, C, H, N, R, and X (Figure 8a). The partial least squares-discriminant (PLS-DA) analysis determined relative concentrations of the correspondent metabolites in different samples (Figure 8b,c).

An improved metabolite profile was found in the *in vitro* plants regenerated on MS medium with 2,4-D due to increased trichome density. D-Allose, isosteviol, stevioside, *ent*-kaurene, phytol, *β*-caryophyllene, isomenthol, and flavone glucosides in

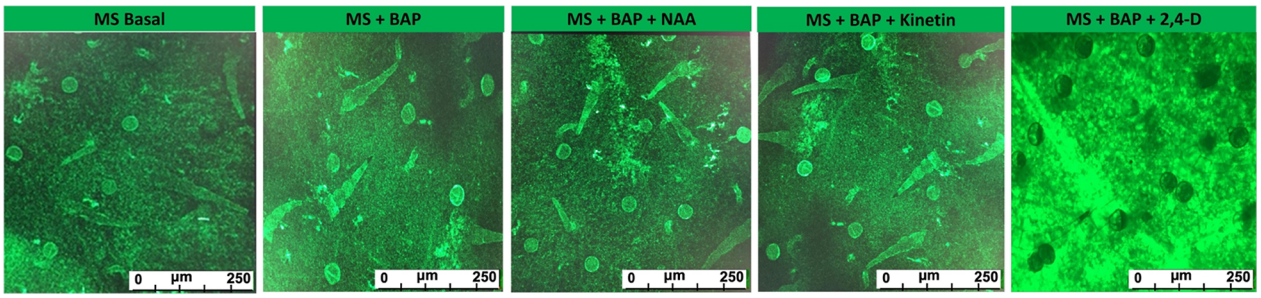

Figure 3. GT density on *in vitro* leaves of *S. rebaudiana* cultured on different combinations of PGRs under a confocal microscope. Scale bars: 250 *µ*m.

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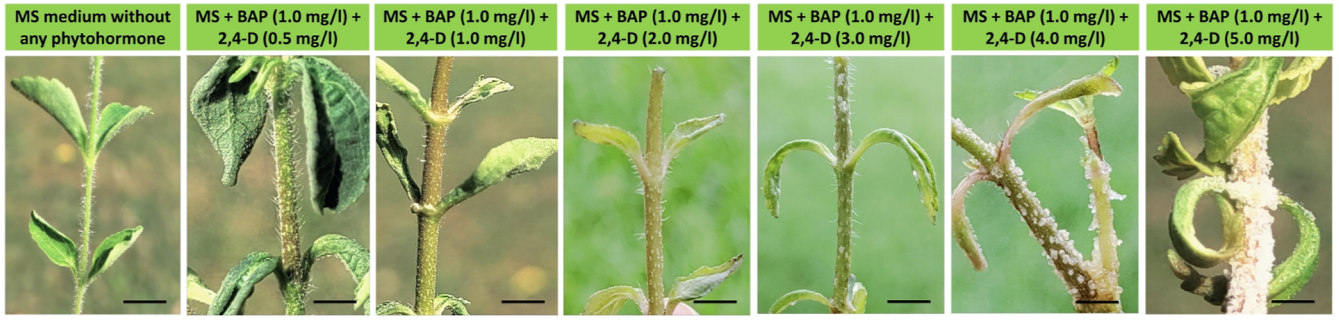

Figure 5. Increased density of GTs on *in vitro* regenerated shoots of *S. rebaudiana* cultured on MS medium containing an increased concentration of 2,4-D. Scale bars: 0.5 cm.

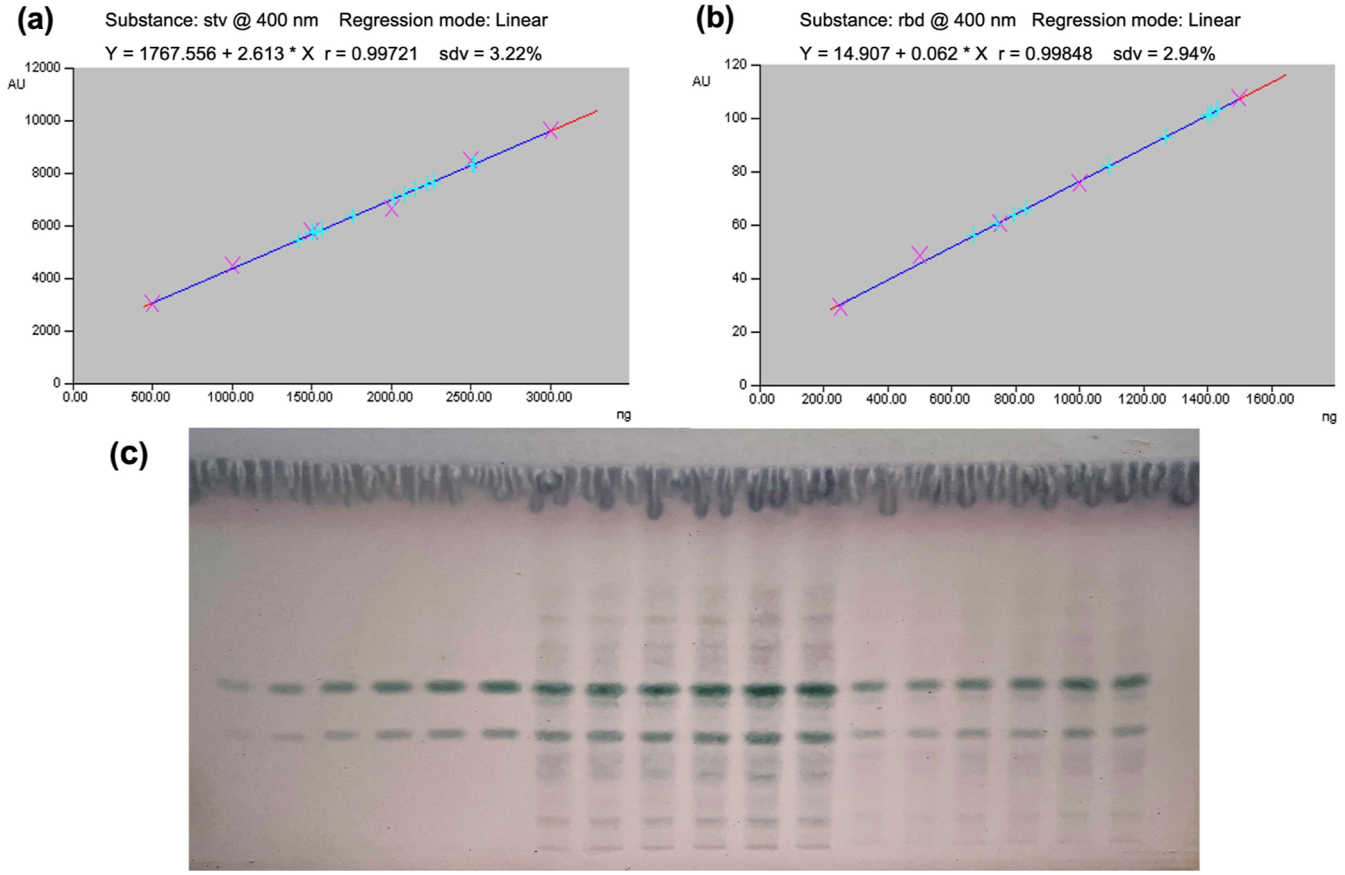

Figure 6. Calibration curve for standard stevioside (a) and rebaudioside-A (b). HPTLC chromatoplate (c) showing tracks 1−6 are of stevioside and rebaudioside-A standard mixture, 7−12 and 13−18 represent *in vitro* samples of leaf and stem tissues, respectively.

leaf samples, whereas lupeol, isosteviol, nerolidol, 1-octadecanol, and methyl abietate in stem samples of *in vitro* plants regenerated on MS-R showed a remarkable increase against *in vitro* control plants cultured on basal MS medium (MS-A).

3. DISCUSSION

Prevalence of diabetes-related metabolic disorders accredited the importance of natural sweetening compounds such as SGs. The increased global market demand bringing all the attention of the researchers toward the enhancement of the SG content. Different conventional and biotechnological approaches facilitate the improved production of SGs, in which the plant tissue culture technique provides large-scale production in any seasonal and regional condition without safety and quality-related public issues over other approaches.

3.1. *In Vitro* Micropropagation of *S. rebaudiana* through Direct and Indirect Shoot Organogenesis. In the present study, we find out the impact of various PGRs on shoot organogenesis and GT density with reference to the enhancement of SG contents. The direct and indirect shoot regeneration system was established from nodal and leaf explants. MS amended with BAP was found best for shoot induction from nodal explants. It was observed that the frequency of shoot bud induction was reduced after increasing the concentration of BAP above 1.0 mg/L. A similar study was also reported by Thiyagarajan and Venkatachalam,[29] Aman et al.,[30] and Ahmad et al.[31] in *S. rebaudiana* and in *Tylophora indica* by Faisal and Anis.[32] The combination of BAP + NAA was found to be better than BAP alone in response to number of shoots regenerated from the nodal explant. Studies unveiled

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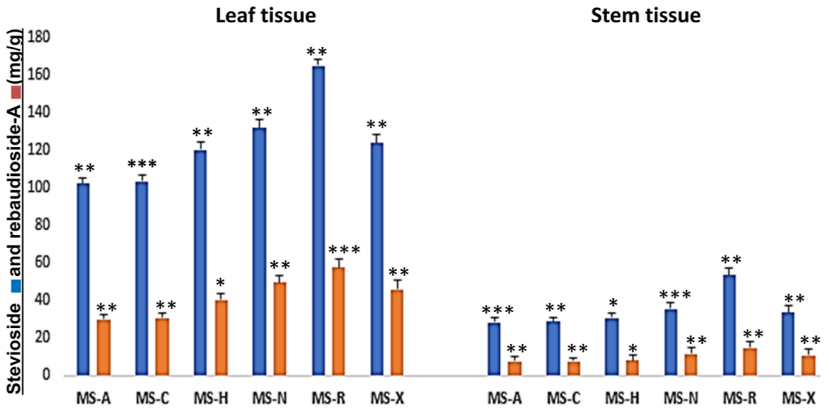

Figure 7. Effect of MS medium containing different PGRs on content of stevioside and rebaudioside-A in leaf and stem tissues of *S. rebaudiana*. **MS-A**: MS's medium without any PGR (control), **MS-C**: MS + BAP (1.0 mg/L); **MS-H**: MS + BAP (1.0 mg/L) + NAA (0.5 mg/L); **MS-N**: MS + BAP (1.5 mg/L) + NAA (0.5 mg/L); **MS-R**: MS + BAP (1.0 mg/L) + 2,4-D (0.5 mg/L); and **MS-X**: MS + BAP (1.0 mg/L) + kinetin (1.5 mg/L). Error bars indicate ±SE ($n \geq 3$) ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$).

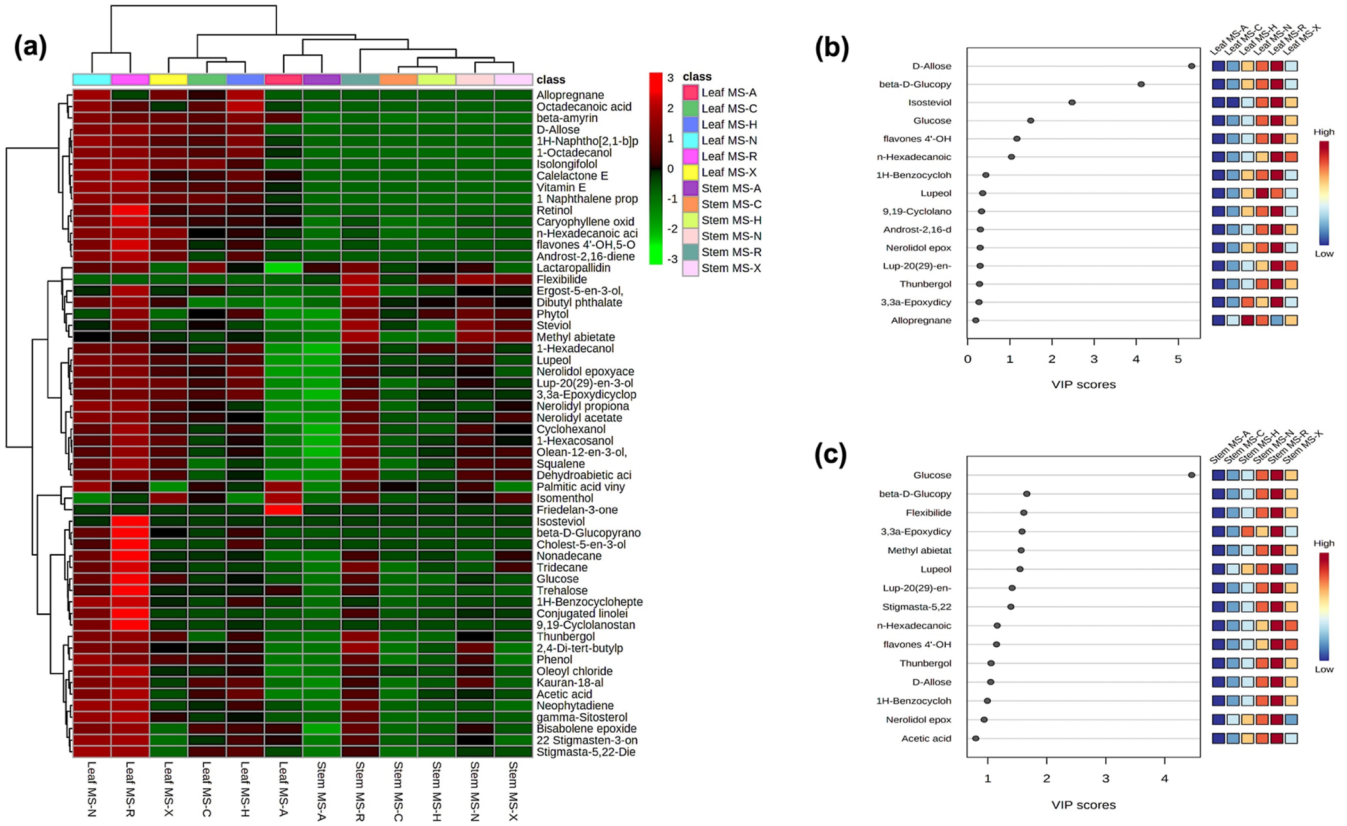

Figure 8. (a) Hierarchically clustered heatmap showing correlation between different metabolites. PLS-DA analysis identified the relative concentrations of the corresponding metabolites under different PGR treatments in leaf (b) and stem (c) samples of *S. rebaudiana*.

that augmentation of BAP with the low concentration of auxin showed better responses with multiple shoot induction. There are various reports supporting this statement, for example, combination of BAP with lower concentrations of NAA and IAA found better for multiple shoot formation in *S. rebaudiana*.[29,33−35] In contrast to this, Röck-Okuyucu et al.[36] reported that BAP (0.5 mg/L) with higher concentration of NAA (1.0 mg/L) showed eight shoots per nodal explant in *S. rebaudiana*.

Direct shoot organogenesis from the leaf explant was observed from the midrib and distal ends on different concentrations of BAP + NAA. In contrary, the leaf explants were failed to exhibit induction of shoots in the presence of BAP + NAA.[34] We reported better results than previous studies of direct shoot regeneration from the leaf explant in *S. rebaudiana*.[5,7,35,37] Moreover, it was observed that BAP + 2,4-D showed indirect shoot organogenesis with an intervening callus on the base of the leaf explant. 2,4-D is known to have cell proliferation, elongation, and callus induction properties at low doses. The role of 2,4-D in callus formation was reported earlier in *S. rebaudiana*.[38] However, 2,4-D in the presence of BAP decreased the number of shoots originated from the leaf explant in contrast to NAA.[30,31,38] We also observed that BAP + NAA treatment showed better results with higher number of

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shoots through direct regeneration, whereas BAP + 2,4-D treatment produced indirect organogenesis system with lesser number of shoots.

Cytokinin are known to play an important role in shoot proliferation; therefore, we cultured the *in vitro* regenerated shoots as an explant on MS medium supplied with BAP + kinetin. Consequently, a better shoot multiplication response was observed. Our results were supported by other studies where adding additional cytokinin to the MS medium containing BAP showed a positive impact on shoot organogenesis.[31,37] Aman et al.[30] also reported that BAP alone (2.0 and 4.0 mg/L) or with kinetin (1.0 mg/L) produced around 15 shoots per explant in *S. rebaudiana*. Similar observations by other researchers favored the combinational treatment of BAP + kinetin for shoot proliferation from nodal explants.[35,39] For the *in vitro* rooting, better results were achieved with IAA on 1/2MS rather than full MS. Ahmed et al.,[40] Soliman et al.,[41] and Nower et al.[42] found the similar observations. Contrarily, Thiyagarajan and Venkatachalam[29] found that rooting respond well on NAA. After shoot and root organogenesis, *in vitro* raised complete plantlets were shifted to the field with 78% survivability after hardening and acclimatization.

3.2. Influence of PGR Treatment on GT Density. Interestingly, our study observed that *in vitro* shoots regenerated through indirect organogenesis with BAP + 2,4-D showed a distinguishable increased GT density. For further confirmation of 2,4-D influencing the GT density, its concentration in the medium was increased, and inevitably an active correlation was found between them. These results thus suggest the role of 2,4-D in gland cell differentiation. In cultures of *Passiflora foetida*, medium consisting 4.0 mg/L 2,4-D had more than three times the density of trichomes per leaf than those grown on a medium with 3.0 mg/L 2,4-D. However, the concentration of 2,4-D above 5.0 mg/L caused the deleterious effect.[43] Rodríguez-Serrano et al.[44] reported that 2,4-D promotes growth and developmental processes at low doses but at higher concentration causes growth retardation. Subsequently, GT density evaluation of leaf samples revealed the impact of all PGR treatment used in the present study. PGRs are known to play a critical role in regulation of trichome differentiation processes.[9] In *Lavandula dentata*, auxin showed a positive effect on GT formation rather than BAP.[45] In contrast, BAP and GA3 positively affected the trichome number and size in *A. annua*.[23] In our observations, BAP with kinetin and NAA deliberated a positive effect on the density of GTs and non-GTs in *S. rebaudiana*. However, BAP + 2,4-D combination highly influenced the GT density on *in vitro* leaf samples. To the best of our knowledge, this is the first report on the positive impact of PGRs on the GT density in the *in vitro* culture of *S. rebaudiana*. Kim et al.[46] previously reported higher frequency of GT formation in the zygotic embryos of *Tilia amurensis* cultures on medium containing 1.0 mg/L of 2,4-D. Furthermore, Zhang et al.[47] reported the critical role of auxin signal transduction in the formation of multicellular GTs in tomato. Based on these studies, we also speculate that auxin signaling can play a critical role in differentiation of GTs in *S. rebaudiana*. Thus, it was interesting to discover a positive correlation between auxins and density of GTs. However, a detailed analysis of the regulatory mechanism involved in trichome development under the influence of auxin is yet to be elucidated.

3.3. Increased SGs and Other Secondary Metabolite Accumulation. PGRs are an important abiotic factor that can be used to change the amount of stevioside and rebaudioside-A, as well as the SG ratio in the culture of *S. rebaudiana*.[26] The influence on active principles was compared between *in vivo* donor plants and *in vitro* raised plants with reference to the use of different growth regulators, previously reported by Singh et al.[39] and Sivaram and Mukundan.[34] We observed somewhat similar contents in leaf and stem tissues of *in vitro* plants cultured on basal MS and MS with BAP. Similar observations were made by Röck-Okuyucu et al.[36] and Sivaram and Mukundan.[34] In *A. annua* also, BAP does not stimulate artemisinin biosynthesis within the glands, regardless of their positive effect on the number and size of GTs.[23] In contrast, recent study reported the BAP treatment as a foliar application for the enhancement of the SG content in *S. rebaudiana*. On the other hand, BAP in combination with NAA and kinetin influences the stevioside and rebaudioside-A content. Similarly, Röck-Okuyucu et al.[36] reported increased stevioside production under BAP + NAA treatment. Mohammad et al.[48] observed enhancement in the artemisinin content under the supplementation of BAP + NAA in callus culture of *A. annua*. However, Aman et al.[30] reported BAP + kinetin as the best combination for the increased SG production in cultures of *S. rebaudiana*. In our study, we also observed higher content of SGs in treatment of BAP + kinetin. Furthermore, the highest stevioside and rebaudioside-A contents were observed with the use of BAP + 2,4-D against *in vitro* raised control plants along with *in vivo* mother plant. BAP + 2,4-D showed a higher content in leaves of shoots regenerated through a callus-mediated phase rather than directly regenerated shoots on BAP + NAA treatment. In the same manner, Kumari and Chandra[5] reported higher quantity of SGs in callus regenerated *in vitro* shoots in comparison to direct leaf regenerated shoots and *in vivo* shoots of *S. rebaudiana*. Consequently, 2,4-D influenced the density of trichomes on leaf and stem tissues in such a manner, which helped drive the increased rate of synthesis and accumulation of SGs. Thus, this study provides a new insight into the selection of suitable combination of PGRs with reference to the improved metabolic profile in *S. rebaudiana*. However, the detailed mechanism of phytohormonal regulation on the secondary metabolism is not well understood.[34]

GTs are known as the biochemical factories for the mass production of secondary metabolites.[49] Consequently, a significant change was noticed in the concentration of various metabolites along with SGs. Complete metabolome analysis determined a positive correlation between auxin and density of GTs with numerous secondary metabolites. Our data revealed the elevated concentration of total alkaloids, terpenoids, glycosides, and flavonoids concerned with higher GT density. Similarly, GC−MS profiling was used to analyze various secondary metabolites,[50] phytochemical components,[51] and free amino acid[52] accumulation in different plant species.

4. CONCLUSIONS

The developed protocol can be used for micropropagation in *S. rebaudiana* through direct and indirect shoot regeneration from nodal and leaf segments with higher yield of not only SGs but also other numerous valuable secondary metabolites. This study reported the significant role of PGRs, specially auxin, on density of GTs with an improved metabolic profile. We believe that our results could be used to improve the *in vitro* mass production in *S. rebaudiana* with higher yield of stevioside and rebaudioside-A due to the increased number of GT and secondary metabolite accumulation.

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5. METHOD

In the present study, the direct and indirect shoot regeneration system was developed through nodal and leaf explants with different PGR treatments. Interestingly, GTs were increased on stem tissues under combination of BAP + 2,4-D. Furthermore, those shoots were cultured for a prolonged period with an increased concentration of 2,4-D for validating the role of 2,4-D only. From the first study, only best concentrations were counted for further analysis of GT density in leaf samples through a confocal microscopic study. Afterward, leaf and stem samples with the same treatments were processed for HPTLC and GC−MS analyses.

5.1. *In Vitro* Propagation of *S. rebaudiana*. *5.1.1. Explant Collection, Surface Sterilization, and Inoculation.* *S. rebaudiana* cultivar CIM-Mithi (registered variety from CIMAP, Pant Nagar, India) was cultivated and maintained at botanical garden of Jamia Hamdard campus, New Delhi, India. Juvenile nodal and leaf explants were collected from healthy mother plants and thoroughly washed. 5% TWEEN-20 (v/v) was used for 10 min and subsequently treated for 5 min with 0.002% bavistin (w/v) for removal of most external contamination. Thereafter, surface sterilization was carried out in the laminar air flow cabinet using 0.1% $HgCl_2$ for 2 min and then finally rinsed three times with autoclaved water. These explants were then blot dried and inoculated in culture vessels supplemented with MS medium (MS, 1962)[53] consisting 3% sucrose (w/v) and solidified by adding 0.7% agar (w/v). The pH of the media was maintained at 5.8 ± 0.2 before adding agar. Prepared media was sterilized at 15 psi for 21 min in an autoclave. All plant tissue culture grade chemicals used in the study were acquired from Himedia, India. Culture room conditions were regulated at 25 ± 2 °C, 3000 lux of light intensity with 16 (light)/8 (dark) h of photoperiod, and 75% relative humidity.

5.1.2. Shoot Regeneration and Multiplication. The nodal and leaf explants were inoculated on MS medium supplemented with cytokinin and auxins individually and in combination, such as BAP at 0.5, 1.0, 1.5, 2.0, and 2.5 mg/L individually or with 0.5 mg/L of NAA and 2,4-D for induction and regeneration of shoots, as given in Tables 1 and 2. While for shoot multiplication, different concentrations of kinetin were used with 1.0 mg/L of BAP, as given in Table 3. After 4 weeks of culture, all the parameters such as regeneration percentage of shoots and number and length of shoots per explant were measured.

5.1.3. In Vitro Rooting. The regenerated shoots (∼4.0 cm) were cultured onto half-strength and full-strength MS media supplemented with various concentrations of IAA for root induction and proliferation, as given in Table 4. After 4 weeks of shoot transferring, number of roots per plantlet, the mean length, and rooting percentage were measured.

5.1.4. Hardening and Acclimatization. Rooted plants were carefully excised from culture jars, cleaned through distilled water to remove agar, and planted in sterilized soil rite packed in plastic cups. To keep the plants moist, plastic bags were used to cover them for humidity control. Up to 3 weeks, these plants were kept under culture room conditions for hardening. Thereafter, these plants were placed under greenhouse conditions and transferred into pots containing an equal amount of sterilized soil rite and sand for acclimatization.

5.1.5. Statistical Analysis. All experiments were performed in triplicates. The data values were denoted as means ($n = 3$) ± SE. At weekly intervals, cultures were observed regularly, and different parameters such as regeneration percentage of shooting and rooting and number and length (cm) of shoots and roots were measured by visual observations. SPSS software was used for one-way ANOVA analysis. At a 5% significance level, the differences between means were determined using Duncan's test.

5.2. Glandular Trichome Density Analysis. Leaves were excised from 28 days old healthy plants cultured on MS medium with varied treatments of PGRs. Thereafter, each leaf sample was analyzed under a confocal microscope (Leica TCS SP5 Microsystems) in triplicates. The leaf area and number of GTs were evaluated using ImageJ software (http://rsb.info.nih.gov/ij). Leaf surface area covered by GTs was calculated for each sample.[54]

$$\%\ \text{GTs covered leaf area} = [(\text{GTs number} \times \text{GT size}\ (\text{mm}^2)/\text{total leaf area in image}\ (\text{mm}^2)] \times 100$$

5.3. Targeted and Non-Targeted Metabolome Analyses. The leaf and stem tissues from *in vitro* raised plants were used for the analysis of targeted and non-targeted metabolites using HPTLC and GC−MS-based protocols developed in our laboratory.[55,56]

5.3.1. Extraction Procedure. Air-dried samples of leaf and stem tissues were crushed with a pestle and mortar separately into fine powder. 1 gm of each sample was extracted with 5 mL of methanol in triplicates at room temperature (minimum 3 h to maximum overnight). After extraction, samples were sonicated for 10 min and centrifuged. The supernatant was thereafter collected and concentrated up to 1 mL using a vacuum evaporator. The concentrated supernatants were then filtered using syringe filters (0.45 μm) for HPTLC and Whatman filter paper no. 1 for GC−MS analysis to evaluate targeted and non-targeted metabolites, respectively.

5.3.2. Quantification of Targeted Compounds (Stevioside and Rebaudioside-A) Using HPTLC. The standards of stevioside and rebaudioside-A were procured from Sigma Ltd., USA (HPLC Grade, 95% purity). 1 mg of standards was dissolved in 1 mL of methanol to prepare stock solutions. Stock solutions of stevioside and rebaudioside-A were mixed in 2:1 volume, and further dilutions were made for the calibration curve by plotting concentration against peak area. The HPTLC plate of aluminum (20 × 10 cm) pre-coated with silica gel (60F254, Merck) was activated at 110 °C in a hot air oven for 30 min. After activation of the plate, all the sample solutions with standard were applied as a band of 6.0 mm width with a 100 μL syringe fitted in a Camag Linomat 5 applicator (Switzerland). The twin trough CAMAG chamber was pre-saturated with the mobile phase consisting chloroform/methanol/water (60:32:4 v/v/v). The TLC plate was air dried for 5−10 min after the run of mobile phase up to 90 mm. Spraying reagent consisting a mixture of glacial acetic acid/H_2SO_4/anisaldehyde, 50:1:0.5 (v/v/v), was used for band visualization followed by heating at 110 °C for 5 min. After post-chromatographic derivatization, the plate was scanned at 400 nm in the absorption reflection mode using Camag TLC Scanner 3. Quantification of stevioside and rebaudioside-A was assessed through comparative peak analysis from densitogram of the chromatoplate comprising standard, control, and samples (Figures S1−S3).

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5.3.3. Screening of Non-Targeted Secondary Metabolites Using GC−MS. Methanolic extracts of stevia leaf and stem tissues were analyzed through a GC−MS QP-2010 from Shimadzu (Japan). The MetaboAnalyst tool (http://www.MetaboAnalyst.ca/) was used for the identification of different important metabolites, and heatmaps were generated to visualize the comparative difference in the concentration of metabolites present in the samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02957.

Typical densitogram of the mixture of rebaudioside-A and stevioside standards obtained at λ_{400}; typical densitogram of leaf samples from the plants cultured on MS-A: MS medium without any PGR (control) and MS-R: MS + BAP (1.0 mg/L) + 2,4-D (0.5 mg/L) in track 7 and 11, respectively; and typical densitogram of stem samples from the plants cultured on MS-A: MS medium without any PGR (control) and MS-R: MS + BAP (1.0 mg/L) + 2,4-D (0.5 mg/L) in track 13 and 17, respectively (PDF)

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Author Contributions

K.A.: methodology, validation, culture's photography, and writing—original draft. M.Z.A.: designing the research and approving the manuscript. S.I.: manuscript preparation and GC−MS analysis. M.S.: manuscript preparation. S.K.: HPTLC data analysis. F.Q. and A.M.: tissue culture preparation. S.N.Q.: confocal microscopic studies.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SGs, steviol glycosides
GTs, glandular trichomes
PGRs, plant growth regulators
BAP, benzyl aminopurine
NAA, naphthaleneacetic acid
2,4-D, 2,4-dichlorophenoxyacetic acid
IAA, indole acetic acid
MS, Murashige and Skoog's medium (1962)
GC−MS, gas chromatography−mass spectrophotometry
HPTLC, high-performance thin-layer chromatography

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