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Qualitative and Quantitative Estimation of Glycosides in *Stevia rebaudiana* Bertoni Callus Cultures under the Influence of Various Growth Regulators

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ABSTRACT

Stevia rebaudiana Bertoni is a novel natural sweetener and hypoglycemic plant. It has many diterpenoid glycosides with no negative effect on the sugar levels of the blood and no calories, although its sweetness is 300 times more than sugar. Traditional propagation methods for *S. rebaudiana* include seed planting and stem cutting but are limited by small seed size, poor seed viability, and limited availability of stem cuttings. The current research assessed the impact of plant growth regulators (PGRs) on the formation and proliferation of callus and quantified glycosides produced in callus tissues, aiming to maximize yields for medicinal, industrial, and commercial applications. Different combinations and concentrations of PGRs: 2,4-dichlorophenoxyacetic acid (2,4-D) with kinetin (Kin), naphthalene acetic acid (NAA) with 6-benzyl adenine (BA), and 0.1, 0.5, and 1.0 mg/L of thidiazuron (TDZ) in combination with 4.0 mg/L NAA and 1.0 mg/L BA, were evaluated to determine the most effective treatment for inducing callus formation from leaf explants. High-performance liquid chromatography was used to analyze the glycosides that were extracted from the tissues of the leaves and calli. The interaction of 2, 4-D and Kin exhibited a weak response in inducing callus formation. In contrast, the combination of 4.0 mg/L NAA and 1.0 mg/L BA was the most effective for callus development and rebaudiosides accumulation. Moreover, supplementation with 1.0 mg/L TDZ alongside 4.0 mg/L NAA and 1.0 mg/L BA resulted in the highest accumulation of stevioside. The study's findings revealed that effective PGR combinations enhanced callus formation and glycoside accumulation in Stevia.

***Keywords*:** Glycosides, Plant Growth Regulator, Callus Induction, High-Performance Liquid Chromatography, *Stevia Rebaudiana*.

Introduction

Stevia (*Stevia rebaudiana Bertoni*), a member of the Asteraceae family, is a plant with natural sweetening properties, attributed to its steviol glycosides (SGs). The leaves possess multiple SGs, like stevioside (Ste), rebaudiosides (Rebs; A–F), steviolbioside, and steviol, which are responsible for the plant's intense sweetness. Stevia is widely grown and used globally as a natural alternative to sugar in food, drinks, and medicinal products. However, the plant is sensitive to salinity levels above 1,200 ppm and drought conditions. It requires a consistently moist environment, though excessive soil moisture can lead to root damage.[1] The byproduct of stevia metabolism is steviol.[2] *Stevia rebaudiana* is used to treat patients suffering from diabetes, skin, digestive, urinary-genital, and other ailments. Perhaps the cultivation of *Stevia rebaudiana* in Uzbekistan is expected to contribute to the development of medications that can be used to treat various ailments.[3] Furthermore, the expansion of medicinal plant cultivation could partially meet the population's demand for herbal remedies and reduce the cost associated with importing raw medicinal materials.[4] Due to its popularity as a dietary supplement, the plant is grown in various locations, including Japan, Taiwan, Malaysia, Hawaii, Philippines, and South America.[5] Natural sweetening chemicals like SGs are regarded as having no calories, making them a healthy alternative to artificial sweeteners, and they also have therapeutic uses. *Stevia rebaudiana* is among the most valuable medicinal plants worldwide due to its exceptional sweetening property and other therapeutic potentials, placing it above other artificial sweeteners like aspartame, xylitol, and saccharin, which have been linked to several negative health effects. Environmental condition, geographic regions, plant physiological state, altitude, nutrient deficiency in the soil, and pathogenic infections are all important variables to determine the extent to which *S. rebaudiana* may produce SGs. Similarly, when it comes to renewable energy, natural factors play a crucial role in influencing productivity and efficiency.[6]

Traditional methods of propagating *S. rebaudiana* include seed planting and stem cutting.[7,8] However, these techniques are constrained by the small seed size and low germination rate.[9] Furthermore, it is challenging to obtain enough stem cuttings (vegetative parts) for propagation since there are so few healthy mother plants available. In general, market demand is increasing, whereas the production of plant materials is declining.[10] Consequently, the plant tissue culture (PTC) method is a commonly employed procedure for rapidly propagating plants with desirable genetic traits in sterile laboratory settings. The PTC procedure refers to the controlled-environment tissue and organ proliferation, which may then divide and regenerate into new calli or plant parts,[11] and the optimizing large-scale secondary metabolites for commercial applications.[12] Usually, primary metabolism results in the manufacturing of secondary metabolites. The rate of conversion of substrates from primary metabolic pathways determines this, as well as on a range of biological and physical influences. The productivity of metabolites in *in vitro* cultures is determined by the composition of the culture medium (CM), pH, inoculum density, and culture environment.[13] Secondary metabolite formation is highly modulated by the choice of a suitable CM. Also, components of the medium, such as

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macro- and micronutrients, vitamins, carbohydrates (sugars), amino acids, and PGRs, significantly influence metabolite synthesis.[14] The kind and amount of phytohormones applied are crucial for callogenesis.[15]

The current research was carried out to examine the influence of various growth-promoting substances on the formation of callus and proliferation, as well as to quantify the glycosides produced in the callus tissues, with the goal of obtaining the highest possible yield of these compounds for medicinal, industrial, and commercial practices.

Materials and Methods

Source of plant materials

Stevia rebaudiana plants were sourced from Janet Al Nakheel for Plant Tissue Culture Co. Ltd, Iraq. The plants were identified and authenticated by Dr. Sukayna Abbas of the University of Baghdad's College of Science, Department of Biological Sciences. Tender and rapidly developing leaves were chosen for the PTC procedures. Plant leaves were rinsed gently with tap water and then left under tap water for 10-15 minutes to get rid of dust and other attached materials.

Sterilization of leaf explants

Following the placement of the explants (leaves) under a stream of water for 10-15 min, they were transferred to the laminar flow hood. The explants were treated with 70% ethanol for surface sterilization for 30 seconds, followed by immersion in 2% Clorox (NaOCl) surfactant solution prepared by immersing in 3 drops of Tween 20 for 5 minutes under constant agitation to enhance sterilization efficiency. Subsequently, they were thoroughly rinsed thrice in sterile distilled water for 5 minutes to eliminate residual Clorox, then rinsed once more with autoclaved distilled water for 3 minutes.

Callogenesis

Leaf explants were transferred onto Murashige and Skoog (MS) medium enriched at different dosage levels of 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.0, 0.5, 1.0, 2.0, and 4.0 mg/L in combination with kinetin (Kin) at 0.0, 0.1, and 0.2 mg/L. Additionally, different combinations of α-naphthaleneacetic acid (NAA) at 0.0, 1.0, 2.0, 3.0, and 4.0 mg/L and benzyl adenine (BA) at 0.0, 0.5, and 1.0 mg/L were also evaluated. The CM was enriched with thidiazuron (TDZ) at 0.0, 0.1, 0.5, and 1.0 mg/L, as well as with a combination of TDZ (0.0, 0.1, 0.5, and 1.0 mg/L), NAA at 4.0 mg/L, and BA at 1.0 mg/L. Data on callus growth were collected as fresh weight (FW) and dry weight (DW) after two months of culture establishment.

Extraction of steviol glycosides

Steviol glycoside was extracted using a modified technique from the leaves, the whole plant, and the callus culture of *S. rebaudiana*.[16] One gram of fresh callus tissue and mature leaves (used as the control for glycoside estimation) was homogenized in 10 mL of 80% methanol preheated to 80°C using a mortar. Methanol was added to each sample to bring the total volume to fifty milliliters and maintained in a water bath at 80°C for 20 minutes. Centrifugation of the samples was carried out at 6000 rpm for 10 minutes. After being separated, the supernatant was transferred onto Petri plates and dried for a whole day at 40ºC in the oven. By employing high-performance liquid chromatography (HPLC) analysis, the amounts of Ste and Reb in the extracts were estimated both qualitatively and quantitatively.

HPLC analysis

The qualitative and quantitative estimation of Ste and Reb in the extracts was carried out using HPLC analysis, following the method described by Mahmud (2014).[17] The separation conditions for the analysis were as follows: A C-18 column with a 5 µm particle size, 250 mm length, and 4.6 mm internal diameter was used. The eluent included deionized water and acetonitrile in a 66:34 ratio, maintained under isocratic conditions. Detection was performed using UV light at 200 nm. A 20-µl injection volume was applied, and the flow rate was set at 1.0 ml/minute. The analysis was conducted at a constant temperature of 25ºC. Based on these conditions, the concentrations of Ste and Reb in the extracts were calculated.

$$\text{The concentration of compound } (\mu g/g) = \frac{\text{Peak area of compound}}{\text{Peak area of standard}} \times \text{Concentration of standard} \times \text{dilution factor}$$[18]

Statistical analysis

The influence of variables on the variables of interest was determined utilizing the Statistical Analysis System (SAS, 2018) software. The Least Significant Difference test, part of the Analysis of Variance (ANOVA), was employed to make statistically significant comparisons between means at a 0.05 level of significance.[19]

Results and Discussion

Impact of 2,4-D and Kin on fresh and dry weights of induced callus

The influence of varying 2,4-D and Kin concentrations on callogenesis are displayed in Table 1. Administration of 2,4-D alone produced 1.2 mg of callus FW. The synergistic influence of 4.0 mg/L 2,4-D and 0.2 mg/L Kin yielded 0.31 mg of callus, while 0.5 mg/L 2,4-D combined with 0.2 mg/L Kin produced 0.92 mg. The maximum callus FW (237.2 mg) was derived from the exposure enriched with 0.5 mg/L 2,4-D and 0.1 mg/L Kin, showing a significant ($p \leq 0.05$) increase relative to the control. No callus formation was observed in the remaining treatments. As shown in Table 2, supplementation with 2, 4-D alone at 0.5 mg/L resulted in a significant ($p \leq 0.05$) rise in the DW of the callus (0.671 mg). However, an additional rise in Kin concentration induced a considerable increase in the mean callus DW, reaching 0.39 mg at 0.1 mg/L Kin. Regarding the interaction between 2,4-D and Kin, the highest callus DW (1.95 mg) was detected at 0.5 mg/L 2,4-D combined with 0.1 mg/L Kin, which differed significantly ($p \leq 0.05$) from the other combinations that produced a response.

Table 1: Effect of 2,4-D and Kin on the mean fresh weight (mg) of induced callus after two months of culture initiation.

Kin conc. (mg/L)	2,4-D (mg/L)					Mean
	0.0	0.5	1.0	2.0	4.0	
0.0	0.00	0.00	0.00	1.20	0.00	0.240
0.1	0.00	237.20	0.00	0.00	0.00	47.44
0.2	0.00	0.920	0.00	0.00	0.310	0.246
Mean	0.00	79.37	0.00	0.40	0.103	-----
LSD ($P \leq 0.05$)	2,4-D: 7.81*, Kin: 6.05* 2,4-D x Kin: 12.95.					

2,4-D: 2,4-dichlorophenoxyacetic acid; Kin: kinetin; n = 10.

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Table 2: Effect of 2,4-D and Kin on the mean dry weight (mg) of induced callus after two months of culture initiation.

Kin conc. (mg/L)	2,4-D (mg/L)					Mean
	0.0	0.5	1.0	2.0	4.0	
0.0	0.00	0.00	0.00	0.10	0.00	0.02
0.1	0.00	1.95	0.00	0.00	0.00	0.39
0.2	0.00	0.063	0.00	0.00	0.023	0.017
Mean	0.00	0.671	0.00	0.00	0.007	---
LSD	2,4-D: 0.347*, Kin: 0.219*					
($P \leq 0.05$)	2,4-D x Kin: 0.511*					

2, 4-D: 2, 4-dichlorophenoxyacetic acid; Kin: kinetin; n = 10.

Influence of NAA and BA on fresh weight of induced callus

The results (Table 3) show that the control group demonstrated no callus induction. All of the CM that included phytohormones of various NAA/BA combinations supported callogenesis. Additionally, the results showed that callus formation triggered in CM containing NAA independently reached 61.76 mg mean FW. However, the NAA/BA combinations had a significantly ($p \leq 0.05$) greater influence on callogenesis, with the greatest quantity of callus noted in the growth medium fortified with 4.0 mg/L NAA and 1.0 mg/L BA. Under this combination, the FW of the callus reached 4154 mg.

Impact of NAA and BA on the dry weight of callus

The impacts of varying NAA and BA concentrations on callus DW are displayed in Table 4. A significant ($p \leq 0.05$) rise in callus DW was noted at 2.0, 3.0, and 4.0 mg/L NAA, with mean values of 55.56, 117.60, and 226 mg, respectively, relative to the control (hormone-free medium) and the 1.0 mg/L NAA treatment. Furthermore, the addition of BA resulted in a significant ($p \leq 0.05$) rise in callus DW, with the highest mean value of 43.72 mg recorded at a concentration of 0.5 mg/L BA. The interaction between NAA and BA led to a significant ($p \leq 0.05$) increase in callus DW. The highest mean DW (559.50 mg) was obtained at 4.0 mg/L NAA combined with 1.0 mg/L BA, relative to the control treatment, which recorded a mean DW of 19.90 mg.

The 2,4-D/Kin-supplemented medium produced calli that were smaller than average, more compact, and exhibited a yellowish-cream coloration (Figure 1a–c). In contrast, calli cultured on MS medium enriched with NAA/BA were bigger, more delicate in structure, and displayed a pale green color (Figure 1d–f). These observations are consistent with previous findings,[20] which reported that calli induced on media enriched with 2,4-D/Kin or NAA/BA were exhibiting a broad form, tender consistency, and faint green appearance, exhibiting prolonged growth and irregular dispersion in MS medium.

Table 3: Effect of NAA and BA on the fresh weight (mg) of induced callus after two months of culture initiation.

BA conc. (mg/L)	NAA (mg/L)					Mean
	0.0	1.0	2.0	3.0	4.0	
0.0	0.00	147.60	0.00	0.00	161.20	61.76
0.5	0.00	0.00	1207.00	210.40	1108.00	505.08
1.0	0.00	778.00	764.40	2159.00	4154.00	1571.08
Mean	0.00	308.53	657.13	789.80	1807.73	---
LSD	NAA: 71.26*, BA: 59.84*					
($P \leq 0.05$)	NAA x BA: 116:94					

NAA: naphthalene acetic acid; BA: 6-benzyl adenine; n = 10.

Table 4: Effect of NAA and BA on the mean dry weight (mg) of induced callus after two months of culture initiation.

BA conc. (mg/L)	NAA (mg/L)					Mean
	0.0	1.0	2.0	3.0	4.0	
0.0	0.00	18.30	0.00	0.00	19.90	7.64
0.5	0.00	0.00	96.20	32.80	98.70	43.74
1.0	0.00	71.40	70.50	329.00	559.50	20.08
Mean	0.00	29.90	55.56	117.60	226.03	---
LSD	NAA: 45.39*, BA: 38:66*					
($P \leq 0.05$)	NAA x BA: 72.06*					

NAA: naphthalene acetic acid; BA: 6-benzyl adenine; n = 10.

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Figure 1: *In vitro* callus induction from leaf explants from *Stevia rebaudiana.*

a: The control treatment is free of plant growth regulator; b: The highest fresh weight from 2,4-D/Kin treatments; c: The lowest fresh weight from 2,4-D/Kin; d: Treatment of 2.0 mg/L NAA and 1.0mg/L BA; e: Treatment of 3.0 mg/L NAA and 1.0mg/L BA; f: Treatment of 4.0 mg/L NAA and 1.0 mg/L BA; d, e, and f are the highest fresh weights obtained from callus; g, h, i, and j are the best fresh weights from the TDZ media supplementation; 2,4-D: 2,4-dichlorophenoxyacetic acid; Kin: kinetin; NAA: naphthalene acetic acid; BA: 6-benzyl adenine; TDZ: thidiazuron.

Conversely, calli induced from 2,4-D/Kin or 2,4-D/BA combinations were smaller, round in shape, and more compact, indicating that callus morphology is significantly affected by the hormone composition of the CM. The results indicated that all the tested hormones, NAA, BA, 2,4-D, and Kin, triggered callogenesis from *Stevia rebaudiana* leaf tissues maintained on MS CM. Increasing the concentration of NAA proved more effective for callus induction, particularly when combined with BA. This enhanced response can be attributed to the complementary functions of auxins and cytokinins: auxins (such as NAA) promote cell elongation, apical dominance, and root initiation, while cytokinins stimulate cell division and tissue proliferation. [21] In leaf explant cultures treated with 2,4-D, the calli exhibited the lowest fresh and DWs.[22] Nevertheless, other studies have observed the effective function of 2,4-D in stimulating callogenesis in *Stevia rebaudiana*.[23] Enhanced doses of 2,4-D suppress callus growth, while lower concentrations allow morphogenesis to occur. Hence, CM enriched with 0.5 mg/L 2,4-D with 0.1 mg/L Kin was found optimum for callus induction from *S. rebaudiana* leaves.[24]

Influence of TDZ on the induction of callus

The results presented in Table 5 show a significant ($p \leq 0.05$) difference in callus weight among treatments. The mean FW of callus was 1,180.00 mg, and the mean DW was 98.70 mg at 1.0 mg/L TDZ, compared to the control treatment (4.0 mg/L NAA and 1.0 mg/L BA), which recorded higher mean FW and DW of 1,703.00 mg and 252.40 mg, respectively. The calli grown in TDZ-rich CM are denser and yellower than those grown in TDZ-free media, as shown in Figure 1g-j. The results indicated that all the tested hormones, NAA, BA, 2,4-D, and Kin, were capable of inducing callogenesis from *Stevia rebaudiana* leaf tissues maintained on MS CM. Increasing the concentration of NAA was particularly effective in promoting callus formation, especially in combination with BA. This synergistic effect can be attributed to the complementary roles of auxins and cytokinins; auxins (such as NAA) stimulate cell elongation, apical dominance, and root initiation, while cytokinins enhance cell division and tissue proliferation.[21] In leaf explant cultures treated with 2,4-D, the calli exhibited the lowest FW and DW.[22] Nevertheless, other studies have documented the effective function of 2,4-D in callogenesis in *Stevia rebaudiana.*[23] It has been observed that elevated levels of 2,4-D suppress callus multiplication, whereas lower concentrations promote morphogenesis. Accordingly, a CM enriched with 0.5 mg/L 2,4-D and 0.1 mg/L Kin was found to be optimal for callogenesis from *S. rebaudiana* leaf explants.[24]

Table 5: Effect of different concentrations of TDZ with 4.0 mg/L NAA and 1.0 mg/L BA on the mean fresh and dry weights (mg) of induced callus after two months of culture initiation

TDZ conc. (mg/L)	Fresh weight	Dry weight
0	1703.00	252.40
0.1	838.00	74.50
0.5	823.00	77.30
1.0	1180.00	98.70
LSD value	94.25*	21.664*

TDZ: thidiazuron; NAA: naphthalene acetic acid; BA: 6-benzyl adenine; *: $P \leq 0.05$; $n = 10$.

The effectiveness of TDZ in callogenesis was comparable to that of the NAA/BA combination at 4.0 mg/L NAA and 1.0 mg/L BA, as shown in Table 5. Interestingly, it was reported that leaf tissues maintained on MS medium enriched with 2.0 mg/L NAA exhibited the maximum callus induction frequency and mass increase.[25] Calli originating from leaf explants likewise exhibited the maximum amount of SGs. Furthermore, callus characteristics, such as color and morphology vary according to the nature and level of growth regulators applied, as previously reported.[26] The response of plant cuttings largely depends on the internal concentration and rate of absorption of growth regulators, which must reach an optimal level to support the initiation and development of callus tissue.[27] Some cells undergo a change in polarity, leading to a loss of differentiation that enables continuous division and proliferation into callus tissue, one of the reasons leaf explants are highly valued in PTC. Variations in callus FW and DW are influenced by the composition of the nutrient medium, which is primarily determined by the nature and level of PGRs incorporated.[28]

HPLC assessment of stevioside and rebaudiosides from Stevia rebaudiana extracts

Based on the results of callus FW and DW, the highest mean FW (4,154.00 mg) was obtained from the medium enriched with 4.0 mg/L NAA and 1.0 mg/L BA, while a mean weight of 1,180.00 mg was recorded from the medium fortified with 4.0 mg/L NAA, 1.0 mg/L BA, and 1.0 mg/L TDZ. These calli were selected for HPLC evaluation to estimate the concentrations of glycosides (Ste and Reb) and to compare them with those found in the intact leaves of the plant, which served as the control. The detection of glycosides in the samples was performed by comparing their retention times (RTs) and peak areas (PAs) with those of standard compounds, as presented in Table 6. Rebaudioside was detected at a RT of 3.249 minutes with a PA of 667,135 µV (Figure 2a), while Ste appeared at a RT of 3.922 minutes with a PA of 842,628 µV (Figure 2b). In contrast, HPLC analysis of the intact *Stevia rebaudiana* leaves revealed Reb at a RT of 3.214 minutes exhibiting a PA of 151,881 µV and Ste at a RT of 3.950 minutes with a PA of 9,592 µV (Figure 2c). Rebaudioside and Ste were detected at RTs of 3.250 and 4.029 minutes, respectively, with corresponding PAs of 730,868 µV and 244,638 µV in the *Stevia rebaudiana* callus extract obtained from the treatment supplemented with 4.0 mg/L NAA and 1.0 mg/L BA (Figure 2d). Rebaudioside and Ste were detected at RTs of 3.203 and 3.950 minutes, respectively, with corresponding PAs of 313,347 µV and 354,193 µV in the *Stevia rebaudiana* callus extract obtained from the treatment containing 4.0 mg/L NAA, 1.0 mg/L BA, and 1.0 mg/L TDZ (Figure 2e). The concentrations of Reb and Ste in the extracts of all tested samples are illustrated in Table 6. The maximum glycoside content was documented in callus induced from the treatment with 4.0 mg/L NAA and 1.0 mg/L BA, containing 109.5 µg/g of Reb and 29 µg/g of Ste. The lowest concentrations of glycosides were recorded in the extracts from intact plant leaves, which contained 45.5 µg/g of Reb and 2.27 µg/g of Ste, as determined from the RT and PA measurements after eight weeks of culture.

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Table 6: Concentration of rebaudiosides and stevioside (µg/g) in intact plant leaves and tissue culture extracts of *Stevia rebaudiana* Bertoni after eight weeks of culture initiation.

Sample	Rebaudiosides			Stevioside		
	RT (min)	PA (µvolet)	Conc. (µg/g)	RT (min)	PA (µvolet)	Conc. (µg/g)
Intact plant leaves	3.214	151881	45.5	3.950	9592	2.27
Induced callus						
4.0 mg/L NAA+1.0 mg/L BA	3.250	730868	109.5	4.029	244638	29
4.0 mg/L NAA+1.0 mg/L BA+1.0 mg/L TZD	3.203	313347	46.9	3.950	354193	42

RT: retention time; PA: Peak area

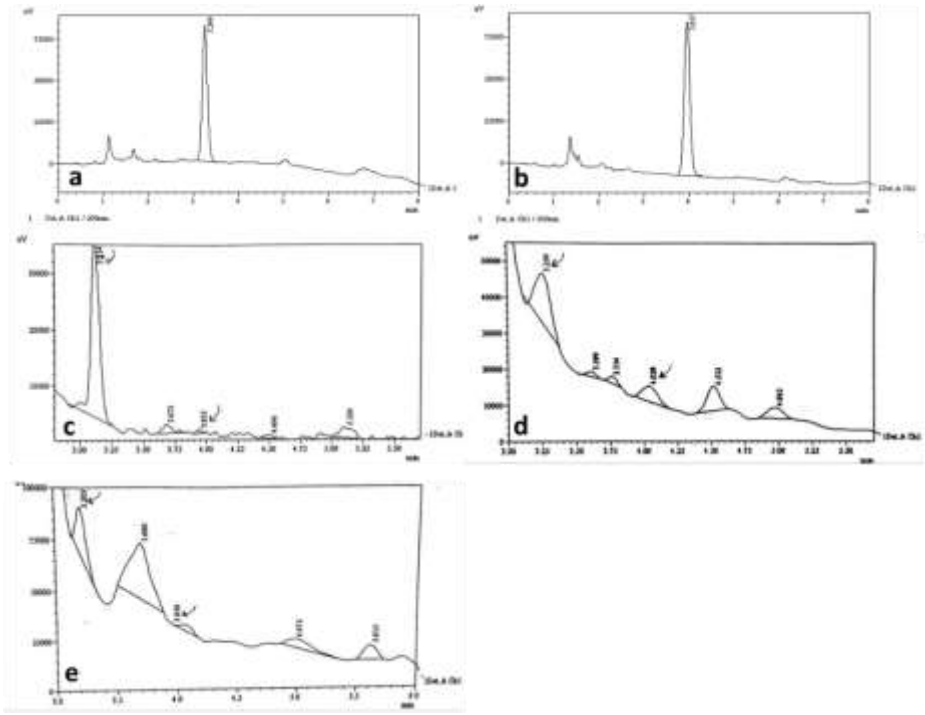

Figure 2: High performance liquid chromatography analysis of glycosides from *Stevia rebaudiana*. a: rebaudiosides standard; b: Stevioside standard; c: extract of intact plant leaves; d: extract of callus at a treatment of 4.0 mg/L NAA + 1.0 mg/L BA; e: extract of callus at a treatment of 4.0 mg/L NAA + 1.0 mg/L BA + 1.0 mg/L TDZ.

The results requiring confirmation by molecular techniques have been recommended in several biological studies.[29-32] This may be attributed to the slow-growing nature of callus cultures, which allows the accumulation of secondary metabolites within the tissue.[33] The accumulation of such secondary metabolic compounds in callus tissues is affected by various factors, like the concentration and type of growth regulators, exposure duration, nutrient composition, culture age, and environmental conditions.[34]Additionally, PTC media influence the metabolic processes within cells by supplying the vital nutrients, growth regulators, and supplements needed for cell division and growth. Creating a CM that promotes maximum cell biomass accumulation is essential to achieving the best possible secondary metabolite production.

Conclusion

The findings showed that 2, 4-D and Kin reduced callogenesis. The highest average FW and DW of callus were from leaf tissues grown on 4.0 mg/L NAA and 1.0 mg/L BA media. Furthermore, the addition of varying concentrations of TDZ to media fortified with 4.0 mg/L NAA and 1.0 mg/L BA resulted in lower callus weights compared to the treatment without TDZ. The concentration of glycosides in the callus was about double that seen in the whole leaves.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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