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Study the effect of Auxin and Cytokinin on the quantitative concentration of Stevioside and Rebaudioside A in the Stevia rebaudiana In vitro

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Abstract

Stevia rebaudiana, a significant medicinal plant in the Asteraceae family, has attracted global interest for its ability to produce natural, low-calorie sweet compounds, primarily stevioside and rebaudioside A. This study aimed to investigate the effects of benzyl adenine (BA) and indole-3-acetic acid (IAA) on vegetative growth and steviol glycoside accumulation in *Stevia rebaudiana* under tissue culture conditions. The experiment was carried out in the Plant Tissue Culture Laboratory, College of Biotechnology, Al-Nahrain University, during the period from 2025 to 2026. Surface-sterilized seeds were initially cultured on Murashige and Skoog (MS) medium, and the resulting seedlings were then transferred to MS medium supplemented with different concentrations of BA (0.0, 1.0, 2.0, and 3.0 mg/L) and IAA (0.0, 0.50, 0.75, and 1.0 mg/L). To evaluate the treatments, we measured the fresh and dry weight of the aerial parts and determined the levels of stevioside and rebaudioside A using high-performance liquid chromatography (HPLC). Our results revealed clear differences between the tested treatments. Biomass production peaked when 2.0 mg/L of benzyl adenine was combined with 0.50 mg/L of indoleacetic acid, resulting in the highest fresh weight (734.60 mg) and dry weight (61.20 mg), while the control group recorded the lowest growth rate. Although HPLC analysis revealed the presence of both stevioside and rebaudioside A in all tested samples, their accumulation was largely driven by high benzyl adenine levels. Specifically, treatment with 3.0 mg/L of benzyl adenine achieved the highest concentrations of stevioside (310.6 mg/g) and Rebaudioside A (186.3 mg/g). Overall, these effects indicate that moderate levels of BA promote plant biomass; however, higher concentrations are required to initiate the biosynthesis of secondary metabolites in *Stevia rebaudiana*.

Keywords: Tissue culture, Plant growth regulator, HPLC, stevioside, rebaudioside A

Introduction

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In terms of economic significance, *Stevia rebaudiana Bertoni* of the Asteraceae family has drawn significant attention globally. It's among the most beloved compounds because of its natural sweetening constituents, most of all stevioside and rebaudioside A, which have a strong sweetness with almost no calories. Due to this feature, steviol glycosides are widely wanted in food and pharmaceutical industries, which makes them great natural substitutes for sucrose and synthetics. Outside of being sweeteners, new literature also references some biological or pharmaceutical benefits of these glycosides [1, 2].

However, the biosynthesis and accumulation of steviol glycosides in *S. rebaudiana* are intricate processes affected by genetic, environmental, as well as hormonal factors. Because plant tissue culture offers a platform for both propagation and secondary metabolites' enhancement in tightly controlled environments, the processes by which we can amplify these pathways are vastly improved. Operating in an *in vitro* setup enables researchers to precisely manipulate nutrient levels and plant growth regulators, ultimately driving better biomass yield and metabolite production [3,4].

Among these growth regulators, the balance between auxins and cytokinins is fundamental in steering both morphogenesis and secondary metabolism. Indole-3-acetic acid (IAA), a primary natural auxin, is critical for cell elongation, root initiation, and overall vegetative development [5,6]. Conversely, benzyl adenine (BA), a synthetic cytokinin, accelerates cell division, shoot proliferation, and metabolic activity in the cultured tissues. Interestingly, several recent studies highlight that BA treatments can actively stimulate steviol glycoside pathways by regulating the specific genes involved in diterpenoid metabolism [7,8].

Furthermore, recent breakthroughs in molecular and metabolic profiling show that higher accumulations of stevioside and rebaudioside A are closely linked to the upregulation of biosynthetic enzymes and regulatory genes. This underlines how the interplay between auxins and cytokinins can fundamentally alter both biomass production and secondary metabolite accumulation in *in vitro S. rebaudiana* cultures [7,12].

To build on these findings, the present study aimed to evaluate how varying concentrations of BA and IAA, alongside their interactions, affect the vegetative growth and accumulation of stevioside and rebaudioside A in *S. rebaudiana* using HPLC analysis.

Materials and Methods

Plant Material and Explant Source

Seeds of *Stevia rebaudiana* were obtained from the Directorate of Seed Testing and Certification at the Iraqi National Herbarium. Prior to experimental use, the collected seed lots were stored under controlled, optimal environmental conditions to maintain viability.

Medium Preparation and Culture Conditions:

The base culture medium was prepared using a plant salt mixture of Murashig and Skoog (MS)[9], purchased from Caisson Laboratories Inc. (USA), at a concentration of 4.91 g/L [10]. 25 g/L of sucrose was added to the medium as a carbon source, and the pH was precisely adjusted to 5.8 using sodium hydroxide or hydrochloric acid before the addition of 8 g/L agar-agar specifically formulated for plant tissue culture. To

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ensure the coagulation agent dissolved completely, the mixture was heated on an electric stove equipped with a magnetic motor. Equal 10 mL portions were then distributed into separate culture tubes. Sterilization was carried out with a steam sterilizer at a temperature of 121 degrees Celsius and a pressure of 15 pounds per square inch for 15 minutes. Culture tubes were then transferred into a laminar flow cabinet and left to cool and solidify at room temperature before inoculation.

Surface sterilization treatments:

Sterile cultures were prepared by sterilization of *Stevia rebaudiana* seeds by a multi-step surface sterilization protocol under strict sterilization conditions inside a laminar flow cabinet. The seeds were initially immersed in a 70% (v/v) ethanol solution for 30 seconds. After that, it was treated with different concentrations of sodium hypochlorite (NaOCl) at concentrations of 10%, 20%, 30% and 40% for different exposure periods (10, 15, 20, and 25 minutes). A few drops of Tween-20 were added to the disinfectant solution as a surfactant to enhance seed wetting. After chemical treatment, the seeds were thoroughly washed three times with sterile distilled water to remove any residue of the disinfectant before being transferred to the germination medium. Details of these experiments are summarized in Table 1.

Table 1: Sodium hypochlorite (NaOCl) concentration and exposure times used for seed surface sterilization.

Treatment	NaOCl	Time
T1	10%	10,15,20,25 min
T2	20%	10,15,20,25 min
T3	30%	10,15,20,25 min
T4	40%	10,15,20,25 min

Hormonal Treatments and In vitro Maintenance:

In the experimental treatments, homogenized, in *vitro*-grown *Stevia rebaudiana* seedlings were transferred to Murashige and Skog base medium supplemented with various concentrations of (BA; 0.0, 1.0, 2.0, and 3.0 mg/L) and (IAA; 0.0, 0.50, 0.75, and 1.0 mg/L) according to a factorial design. Seedlings maintained in hormone-free Murashige and Skog medium (0 mg/L) constituted the control group. All cultures were incubated under the previously described controlled environmental conditions for several weeks to monitor and evaluate the contrasting effects of BA and IAA and their interactions on vegetative biomass and the accumulation of steviol glycosides.

Determining biomass (fresh and dry weight):

To assess vegetative growth, seedlings were harvested, and their fresh weight was recorded directly using a high-precision digital analytical scale. The vegetative tissue was then placed in a hot-air electric oven at 70°C for 24 hours until a constant weight was reached. The dry weight was then recorded using the same analytical balance.

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Sample extraction for glycosidic analysis:

Secondary metabolites were extracted following a modified aqueous protocol based on previously established methods [11]. In short, a 10-gram sample of dried and finely ground leaves was mixed with 25 ml of distilled water and placed in a boiling water bath at 100°C for 30 minutes. After cooling to room temperature, the crude mixture was centrifuged at 2500 rpm for 15 minutes. The supernatant (aqueous phase) was carefully transferred to a 25 mL volumetric flask, and the volume was adjusted to the mark using distilled water. Prior to quantitative chromatographic analysis, the final extract was filtered through a 0.45-micrometer pore membrane filter to remove any particles. The quantitative analysis of stevioside and rebaudioside A was performed using a high-performance liquid chromatography (HPLC) system from Sykam GmbH, Erising, Germany. Chromatographic separation was achieved using a C18-NH2 column (250 mm × 4.6 mm, particle size 5 µm) while maintaining the column temperature at 40°C. Detection was performed using a UV-Vis detector tuned to a wavelength of 210 nm. The mobile phase consisted of a mixture of acetonitrile and sodium phosphate solution at a concentration of 10 mmol/L (pH 2.6) in a ratio of 32:68 (v/v), and was pumped at a constant flow rate of 1 ml/min. The data collection and peak integration process was managed using Clarity chromatography software, and the sample injection volume was set to 100 microliters.

$$Compound\ concentration = \frac{standard\ consentration \times sample\ peak\ area}{standard\ peak\ area} \times \frac{final\ volume}{sample\ weight}$$

Statistical Analysis:

All experimental treatments were arranged in a completely randomized design (CRD) and a factorial experiment design. The pooled data underwent rigorous statistical analysis, and the difference between treatment means was determined using the least significant difference (LSD) test at a confidence level of $P \leq 0.05$ [11].

Results and Discussion

Sterilization results:

The efficiency of establishing sterile cultures of *Stevia rebaudiana* seeds was significantly affected by both the sodium hypochlorite (NaOCl) concentration and the exposure time (Table 2). Our results showed that treating the seeds with a 20% sodium hypochlorite solution for 15 minutes was the optimal protocol, achieving a 100% seed survival rate with effective elimination of microbial contamination. Although similar survival rates (90%) were observed with a 10% sodium hypochlorite solution for 20 minutes, and also with a 20% sodium hypochlorite solution for 20 minutes, these formulations were not generally effective in sterilization, allowing for high subsequent contamination rates during the early stages of cultivation. In contrast, reducing the sodium hypochlorite concentration to 10% for shorter periods was insufficient to eliminate surface pathogens. Conversely, increasing the concentration to 30% or 40% especially when combined with prolonged exposure—resulted in a sharp decline in seed viability to 10–30%. This sharp decrease is closely related to the phytotoxicity of concentrated chlorine compounds, which can penetrate the seed coat and damage embryonic tissues. Statistical analysis confirmed these clear differences between treatments (LSD = 19.4 at $P \leq 0.05$), confirming that treatment with a 20% sodium

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hypochlorite solution for 15 minutes is the most balanced framework for initiating the cultivation of healthy *S. rebaudiana* plants.

Table (2): Sodium hypochlorite concentration and sterilization duration effect on the percentage of survival of Stevia seeds for 10 days. n=10

Treatments	sodium hypochlorite	Time				Mean
		10	**15**	**20**	**25**	
T1	10%	30	60	90	30	52
T2	20%	60	100	90	30	67
T3	30%	50	40	60	10	37
T4	40%	20	30	10	10	22
L.S.D 0.05			38.9			19.4
Mean		40	57	62	20	
L.S.D 0.05			19.4			

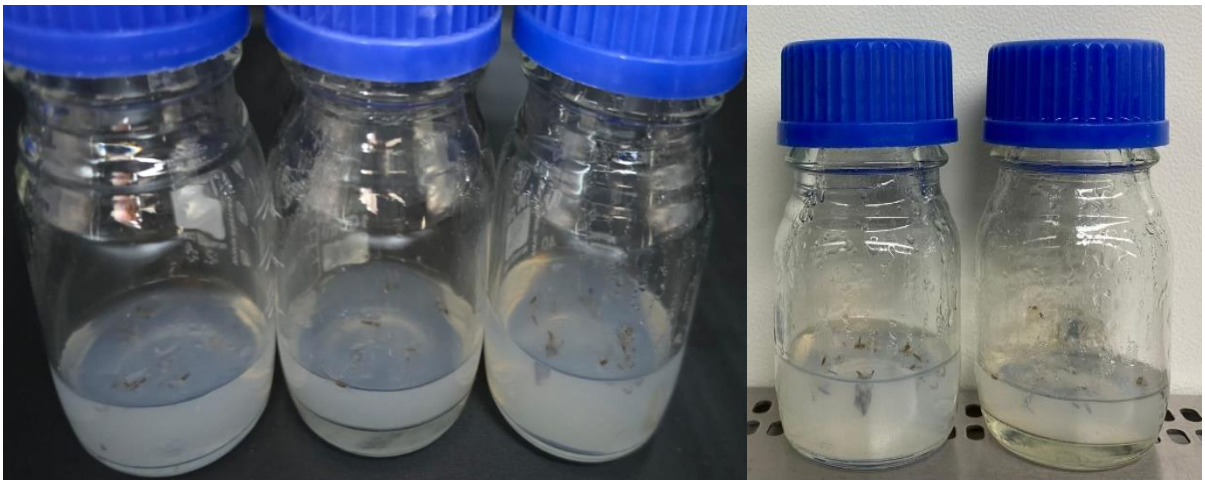

Figure 1: Sterilization of the seed in a concentration 20%

Fresh dry weight:

The morphological development of *S. rebaudiana* responded variably to different levels of benzyl adenine (BA), indole-3-acetic acid (IAA), and their combination (Table 3). A clear synergistic effect was observed. The interaction between 2.0 mg/L BA and 0.50 mg/L IAA maximized vegetative growth, registering the highest fresh weight (734.60 mg), while the hormone-free control group produced the lowest growth (184.40 mg). Considering individual effects, a moderate dose of 2.0 mg/L (BA) proved superior, producing an average fresh weight of 568.50 mg compared to the control group (485.70 mg).

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Table (3) Effect of BA and IAA concentrations and their interaction on the average fresh weight (g) of the vegetative parts of Stevia grown on MS medium for 30 days.

BA	IAA				Mean
	0.0	0.50	0.70	1.0	
0	206.40	706.40	623.40	406.80	485.70
1	194.80	611.40	577.40	535.80	479.90
2	243.80	734.60	691.40	604.20	568.50
3	184.40	711.80	689.60	403.60	497.40
L.S.D 0.05		17.33			8.66
Mean	207.30	691.05	645.50	487.60	
L.S.D 0.05		8.66			

In auxin experiments, a concentration of 0.50 mg/L indoleacetic acid (IAA) emerged as the most effective, with an average fresh weight of 691.05 mg, which then gradually decreased at a concentration of 0.70 mg/L (645.50 mg), while the average fresh weight in the control group was 207.30 mg. This marked increase in fresh biomass confirms the classic interaction between auxins and cytokinins; BA stimulates rapid bud proliferation and cell division in meristematic centers, while indoleacetic acid facilitates cell elongation and enhances nutrient uptake pathways. These observations are consistent with recent micropropagation studies on *S. rebaudiana*, which confirm that balancing the ratios of endogenous and exogenous hormones is essential for improving biomass in vitro [15,16].

Effect of BA and IAA Interaction on Dry Weight of Aerobic Parts:

Consistent with fresh weight trends, dry matter accumulation was significantly affected by the combination of exogenous hormones (Table 4). The highest dry weight (61.20 mg) was achieved using a combination of 2.0 mg/L BA and 0.50 mg/L IAA, which contrasts sharply with the minimum (14.00 mg) observed in the control group. Separately, the 2.0 mg/L BA concentration resulted in the highest mean dry weight (37.50 mg), while the mean dry weight in the control group was limited to 27.30 mg. For indole acetic acid (IAA), the 0.50 mg/L concentration achieved the highest mean among treatments at 45.80 mg, while the concentration in the control group remained at 18.27 mg. The parallelism between fresh and dry weight indicates that these specific hormonal formulations not only retain water but also actively stimulate tissue biosynthesis and cell division. Moderate levels of benzyl adenine and indole acetic acid work together to regulate the metabolic and physiological pathways necessary for structural carbon fixation. This response is consistent with the findings of Ghazal et al. (2024), who confirmed that precisely adjusting the ratios of

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growth regulators significantly increases biomass production and metabolic rate in *S. rebaudiana* systems [14].

Table (4) BA and IAA concentrations and their interaction effect on the average Dray weight (g) of the vegetative parts of Stevia grown on MS medium for 30 days.

BA	IAA				Mean
	0.0	**0.50**	**0.70**	**1.0**	
0	90.40	50.40	40.30	24.40	33.62
1	18.60	52.80	37.60	28.80	34.45
2	21.10	61.20	38.40	29.40	37.50
3	14.00	54.80	20.00	20.40	27.30
L.S.D 0.05		4.05			2.02
Mean	18.27	45.80	34.10	25.75	
L.S.D 0.05		2.02			

Figure 2: *Stevia rebaudiana*

Quantification of steviol glycosides (stevioside and rebaudioside A):

Stevia rebaudiana leaf extracts for HPLC analysis confirmed the presence of steviol glycosides, including stevioside and rebaudioside A, in all treatments.100µl injection of each individual standard compound was given to ascertain the retention time for each analyte. The results revealed a peak on the retention time of 4.8 min. represent the standard compound stevioside. And another peak at a retention time of 6.0 min. represent the standard compound rebaudioside A. at shown in the Figures (3,4) below.

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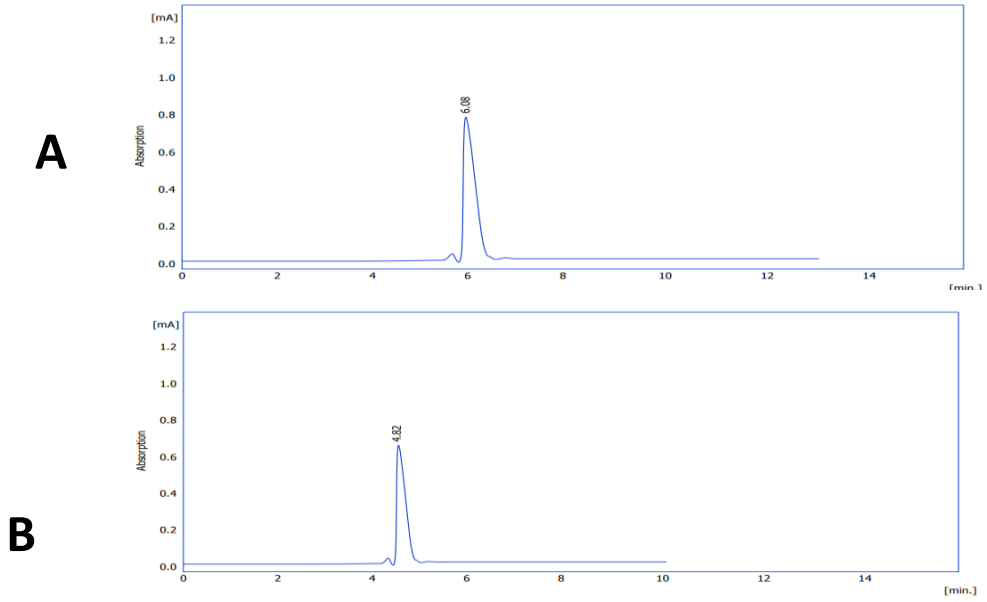

Figure 3: The standard peaks of (A)rebaudioside A and (B) stevioside

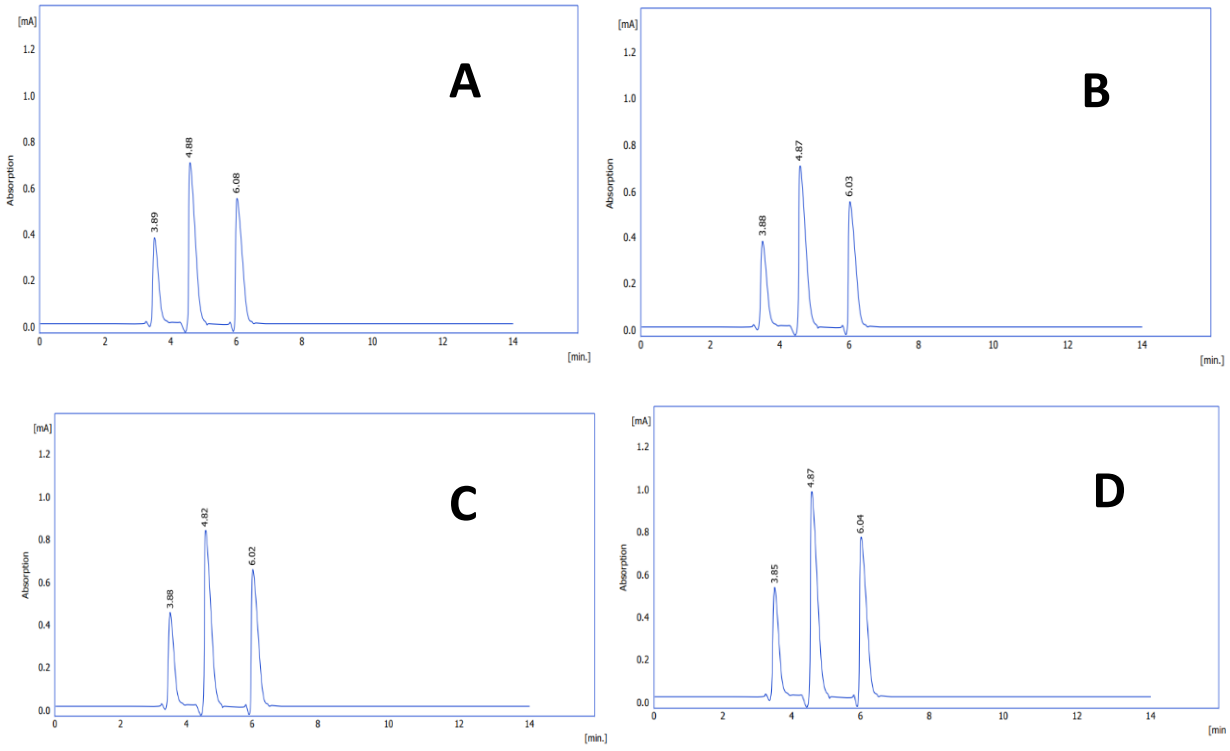

Figure 4: peaks of four samples showed rebaudioside A and stevioside (A) sample 1,(B)sample 2, (C)sample 3, and (D) sample 4.

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Chromatographic histograms of the experimental samples consistently revealed three prominent peaks. The first one, which appears at 3.88- A time of 3.89 minutes, indicates the presence of an unidentified secondary metabolite. The second and third peaks closely matched the retention curves of the parameters, confirming the presence of stevioside at 4.82–4.88 minutes and rebaudioside A at 6.02–6.08 minutes.

HPLC sample selection: Due to the multiple combinations of the sixteen factors, four baseline treatments were selected for HPLC analysis to map metabolic shifts during the experiment. These samples represent the control group T1(0.0 mg/ L), the moderate treatment that promotes growth, and the higher BA levels (up to 3.0 mg/L) designed to test for metabolic shift toward secondary metabolites. Integration of these chromatograms showed a progressive increase in peak areas with increasing BA concentration. The baseline control sample exhibited the smallest overall peak area, while the 2.0 mg/L benzyl adenine treatment significantly increased the peak area to 27,300.14, demonstrating a direct correlation between cytokinin utilization and glycoside synthesis.

Table 5: HPLC analysis of stevioside and rebaudioside A content.

No (mg/gm)	Stevioside	Rebaudioside
T1(0.0 mg/ L)	254.6	145.9
T2(1.0 mg/ L)	274.0	161.5
T3(2.0 mg/ L)	298.0	177.4
T4(3.0 mg/ L)	320.6	191.3

Our quantitative data in Table 5 clearly illustrate this upward trend. The T1(0.0 mg/ L) control group yielded the lowest baseline values for both targets. In contrast, T4, which was exposed to the highest concentration of benzyl adenine (3.0 mg/L), reached peak concentrations for both compounds, accumulating 310.6 mg/g of stevioside and 186.3 mg/g of rebaudioside A

Physiologically, this dramatic, significant increase in glycoside production points to the catalytic role of benzyl adenine in upregulating key transcription factors and enzymes (such as those in the MEP and diterpenoid pathways) responsible for steviol glycoside structures [7,12]. However, an interesting biological trade-off was observed: while the use of a 3.0 mg/L benzyl adenine concentration maximized the synthesis of secondary metabolites (Table 5), it resulted in a marked decrease in the fresh and dry weight of the plants compared to the use of a 2.0 mg/L benzyl adenine concentration. This apparent shift indicates a metabolic change of the plant under high cytokinin stress, which shifts its energy reserves away from skeletal biomass building and towards defense and secondary metabolic pathways [13,14]

Conclusions

This study demonstrated that increasing the BA concentration from 2 to 3 mg/L resulted in a significant reduction in vegetative growth, with the highest biomass observed at 2 mg/L and the lowest at 3 mg/L.

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Conversely, the accumulation of steviol glycosides, such as stevioside and rebaudioside A, was significantly increased at 3 mg/L. This inverse correlation implies that higher BA levels might disrupt the equilibrium between cell division and differentiation, resulting in restricted vegetative growth. At the same time, increased hormone levels seem to activate the biosynthetic pathways for secondary metabolites. This change indicates that in response to higher BA concentrations, *Stevia rebaudiana* will redirect its metabolic energy away from vegetative growth to the formation of steviol glycosides.

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Author's Declaration

- There is no conflict of interest.
- The study received no external funding; the author is responsible for all aspects of the study, including experimental work, data analysis, and manuscript preparation

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