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Micropropagation of *Stevia rebaudiana* plant *in vitro*

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ABSTRACT

Stevia rebaudiana is a valuable natural sweetener, but its mass propagation faces challenges. This study aimed to develop an efficient micropropagation protocol by optimizing surface sterilization and evaluating cytokinin effects. Nodal explants were sterilized with sodium hypochlorite (NaOCl) and cultured on MS medium with different concentrations of benzyladenine (BA) or kinetin (Kin). Sterilization with 4% NaOCl for 6 minutes was most effective. During Initiation, BA at 1.5 mg L^{-1} achieved the highest response rate (100%) and longest shoot length, while BA at 1.0 mg L^{-1} produced the maximum shoot number. In multiplication, BA at 1.5 mg L^{-1} yielded the highest shoot and leaf production. For Kin, 2.0 mg L^{-1} was optimal in multiplication. Importantly, BA treatment also resulted in the highest accumulation of stevioside (199.8 ppm) and rebaudioside-A (125.9 ppm), confirming its dual role in enhancing both biomass and metabolite yield.

***Keywords*:** *Stevia rebaudiana*, MS medium, HPLC, stevioside, rebaudioside-A.

INTRODUCTION

Stevia rebaudiana (Bertoni), a perennial plant of the Asteraceae family, has garnered considerable interest as a natural, zero-calorie sugar alternative due to its elevated levels of steviol glycosides (SGs), particularly stevioside and rebaudioside A [1]. These glycosides exhibit a sweetness intensity about 300 times greater than sucrose, making them commercially valuable as sugar substitutes in food, beverages, and pharmaceutical products [2]. Beyond their sweetening properties, these compounds demonstrate significant biological activities. Notably, Stevia rebaudiana and its major glycosides, stevioside and rebaudioside A, exhibit promising anti-inflammatory activity through mechanisms involving the suppression of the NF-κB signaling pathway and modulation of oxidative stress [3].The rising global demand for S. rebaudiana products is driven by increasing health concerns related to sugar consumption [4]. However, meeting this demand through conventional agriculture faces significant challenges. Consequently, in vitro micropropagation has emerged as an essential technique for the rapid and efficient production of uniform, disease-free planting material, leveraging the totipotent nature of plant cells [5]. The success of this approach critically depends on optimized culture media supplemented with appropriate plant growth regulators (PGRs). Among PGRs, cytokinins such as benzyladenine (BA) are particularly effective for stimulating shoot formation and proliferation [6], and have been successfully applied for the mass propagation of S. rebaudiana [7]. Notably, plant growth responses in vitro are concentration-dependent for PGRs [8] [9]. Additionally, genetic variability across species leads to distinct in vitro growth performances [10][11]. Previous research has demonstrated that regenerated Stevia plants exhibit reduced steviol glycosides (SGs) content and compositional diversity compared to field-grown counterparts[12]. To address this limitation, diverse strategies have been implemented to generate homogeneous Stevia populations with elevated SG yields using tissue culture methodologies. These approaches encompass the modulation of tissue and organ culture parameters, including explant selection [13], basal medium composition and concentration, plant growth regulators (PGRs) [14] [15] [16], carbon sources, and nutrient optimization [17] [18][19][20]. A major challenge in *in vitro* cultures derived from field-grown plants is microbial contamination, which can lead to complete batch failures rather than minor losses. Effective sterilization is critical, as bacteria and fungi can infiltrate cultures and pose persistent risks throughout cultivation. Producing sterile and viable in vitro plantlets is essential for improving clonal propagation efficiency in plant tissue culture [21]. Thus, employing accurate and reliable sterilization methods is vital for optimizing time and resource utilization in tissue culture practices [22].

Materials and Methods

This study was conducted between April 2024 and May 2025 at the Plant Cell and Tissue Culture Laboratory of the Horticulture and Landscape Design Department, College of Agriculture, University of Kirkuk, Iraq. The research focused on the micropropagation of *Stevia rebaudiana*, using nodal explants obtained from Al-Jannah Al-Nakhil Company for Tissue Culture in Baghdad, Iraq.

Explant surfaces were cleaned under running tap water. After rinsing with sterile distilled water, Surface sterilization was then performed in a laminar flow hood by immersing explants in commercial 6% sodium hypochlorite (NaOCl) at concentrations of 2%, 4%, and 6% for varying durations (2, 4, and 6 minutes). Followed by three rinses in sterile distilled water. Contamination rates and survival percentages were recorded 30 days later. For cultivation, nodal segments (1–1.5 cm) were cultured on MS medium supplemented with plant growth regulators .Tow independent cytokinin experiments Benzyladenine (BA) at concentrations of 0.0, 0.5, 1.0, 1.5, and 2.0 mg L^{-1} and Kinetin (Kin) at 0.0, 2.0, 4.0, 6.0, and 8.0 mg L^{-1}. The medium was prepared by dissolving 4.43 g L^{-1} MS basal salts and 30 g L^{-1} sucrose in distilled water. Subsequently, 6.5 g L^{-1} agar was added, and the pH was adjusted to 5.7 ± 0.1 before autoclaving.The cultures were incubated in a growth chamber at 25 ± 1°C under a 16-h photoperiod (3000 lux illumination using white fluorescent lamps). Data on explant survival and regeneration were recorded after 4 and 8 weeks of culture . A Completely Randomized Design (CRD) was employed with ten replicates per treatment, and means were compared using Duncan's multiple range test at the 5% probability level [23]. Statistical analyses were performed using SAS software [24].

2.1.Sample Preparation for Glycoside Analysas

Subsequently, samples corresponding to the optimum concentrations of both BA and kinetin-as well as the untreated control-were collected. The plant materials were dried in an electric oven at 30°C for four hours and then ground into a fine powder for the extraction of rebaudioside A and stevioside from the samples.Compounds Standard samples of Stevioside and Rebaudioside -A were taken from Sigma company.

2.2.Sample Extraction:

10 gm of dried *Stevia rebaudiana* plant were extracted with 25 mL of water in a boiling water bath for 30 min (100°C). Extracts were cooled to room temperature and centrifuged at 2500 rpm for 15 minutes. The aqueous phases were transferred to a 25 mL volumetric flask and filled to capacity. The solution was filtered through a 0.45 µm membrane filter before HPLC analysis.

2.3.HPLC

A high-performance liquid chromatography model SYKAM - German method was performed, according to analysis of stevioside and rebaudioside, Chromatographic method was carried out on C18 -NH2 (250 mm: 4.6 mm: 5 µm) column SAPARETION with temperature control 40 c with UV-Vis detector set to a wavelength of 210 nm. Mobile phase was a 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6) at a flow rate of 1 mL/min. The sample injection volume was 100 µL and analysis was performed with Clarity software.

Results and Discussions

3.1.Surface Sterilization

The results (Table 1) indicated that sodium hypochlorite (NaOCl) concentration and sterilization duration had a significantly interactive effect on explant survival rates, interaction analysis revealed that the highest survival rate (100%) was achieved with 4% NaOCl for 6 minutes, while the lowest survival rate (50%) occurred with 2% NaOCl for 4 minutes, 4% NaOCl for 4 minutes, and 6% NaOCl for 2 minutes. as shown in Figure (2).

Table 1. Effect of sodium hypochlorite (NaOCl) concentration and sterilization duration on contamination and survival rates of *Stevia rebaudiana* explants.

Concentration (%) Duration (min)	2	4	6	Duration effect (minute)
2	70 ab	70 ab	50 b	63 a
4	50 b	50 b	70 ab	56 a
6	70	100	60	76

	ab	a	ab	a
Concentration effect (%)	63 a	73 a	60 a	

Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test at the 5% probability level.

The survival of uncontaminated (living) plant parts and the absence of microbial contamination (e.g., bacteria and fungi) can be attributed to the toxicity of sodium hypochlorite (NaOCl) against microorganisms and the differential tolerance of plant tissues to the applied concentrations and exposure times during sterilization. Researchers have highlighted that residual commercial NaOCl solutions can effectively be removed during the sterilization process by repeatedly washing the explants. This is facilitated by the decomposition of NaOCl into less toxic compounds at low concentrations, making it easier to eliminate through successive rinsing [25] The 4% for 6-minute treatment likely achieved this balance, whereas incompatible concentrations or durations resulted in either incomplete sterilization (e.g., 2% for 4 min) or excessive phytotoxicity (e.g., 6% for 2 min). Comparable findings were reported in studies by [26] [27][28][29][30][31][32].

3.2.Effect of Benzyladenine (BA) on Initiation and Multiplication Stages of Stevia Nodal Explants

The data presented in Table 2 demonstrates that after 4 weeks of culturing Stevia Nodal explants on MS media supplemented with benzyladenine (BA), the highest explant response rate (100%) was recorded for media containing (0.5 and 1.5 mg L^{-1} BA). Statistical analysis revealed that the addition of 1.0 mg L^{-1} BA to the culture medium produced the highest number of shoots (9.20 shoots explant^{-1}), significantly outperforming the control treatment (2.10 shoots explant^{-1}), while no significant differences were observed among other BA concentrations. Regarding shoot elongation, the 1.5 mg L^{-1} BA treatment yielded the longest shoots (7.15 cm), significantly exceeding the control (1.65 cm). In terms of leaf production, MS medium containing 1.0 mg L^{-1} BA generated the highest number of leaves (58.30 leaves explant^{-1}), which was substantially greater than the control (16.00 leaves explant^{-1}), with no significant variation among other treatments.

After 8 weeks of culture, the 1.5 mg L^{-1} BA treatment maintained a 100% response rate. This concentration also produced the maximum number of shoots (33.70 shoots explant^{-1}), showing significant improvement over the control (1.10 shoots explant^{-1}) and other treatments. Interestingly, the optimal shoot length at this stage was achieved with 0.5 mg L^{-1} BA (5.77 cm), significantly greater than the control (0.90 cm). For leaf production, the 1.5 mg L^{-1} BA treatment again showed superior performance, generating 139.40 leaves explant compared to just 7.00 leaves explant^{-1} in the control, with statistically significant differences among all treatments.

Table (2): Effect of BA on initiation and multiplication stages of Stevia explants (nodes) after 4 and 8 weeks of cultivation

BA mg.L^{-1}	After 4 weeks (Initiation)				After 8 weeks (multiplication)		
	Response %	NO. of shoots	Length of the longest shoot (cm)	Total NO. of leaves	NO. of shoots	Length of longest shoot (cm)	Total NO. of leaves
0.0	70	2.10 b	1.65 c	16.00 b	1.100 d	0.90 b	7.00 d
0.5	100	6.80 a	5.37 ab	57.90 a	21.10 b	5.77 a	111.50 ab
1.0	90	9.20 a	3.72 bc	58.30 a	11.90 bc	2.15 b	70.30 bc
1.5	100	5.30 ab	7.15 a	47.30 a	33.70 a	5.17 a	139.40 a
2.0	80	7.80 a	2.15 c	51.20 a	8.700 c d	1.42 b	47.90 dc

Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test at the 5% probability level.

3.3.Effect of Kinetin (Kin) on Initiation and Multiplication Stages of Stevia Nodal Explants

Data from Table 3 indicate that after 4 weeks of culturing Stevia Nodal explants on MS media supplemented with kinetin (Kin), treatments with 4.0 and 8.0 mg L^{-1} Kin showed a 100% response rate. Statistical analysis revealed that the addition of 8.0 mg L^{-1} Kin to the MS medium produced the highest number of shoots (4.50 shoots $explant^{-1}$), significantly exceeding both other concentrations and the control treatment (2.10 shoots $explant^{-1}$). Regarding shoot elongation, the 4.0 mg L^{-1} Kin treatment yielded the longest shoots (5.02 cm), demonstrating significant superiority over both the control (1.65 cm) and other concentrations. For leaf production, MS medium containing 8.0 mg L^{-1} Kin generated the highest number of leaves (33.80 leaves $explant^{-1}$), which was significantly greater than the control (16.00 leaves $explant^{-1}$), with all treatments showing statistically significant differences.

After 8 weeks of culture, treatments with 4.0 and 8.0 mg L^{-1} Kin maintained a 100% response rate. Interestingly, the optimal shoot production at this stage was achieved with 2.0 mg L^{-1} Kin (6.50 shoots $explant^{-1}$), showing significant improvement over the control. In terms of shoot length, the 2.0 mg L^{-1} Kin treatment produced the longest shoots (7.60 cm), substantially exceeding both the control (0.90 cm) and other concentrations. For leaf development, MS medium supplemented with 2.0 mg L^{-1} Kin resulted in the highest leaf count (48.00 leaves $explant^{-1}$), which was significantly greater than the control (7.90 leaves $explant^{-1}$), with all treatments demonstrating statistically significant differences.

Table (3): Effect of Kin on initiation and multiplication stages of Stevia explants (Nodal) for after 4 and 8 weeks of cultivation

Kin mg.L-1	After 4 weeks (Initiation)				After 8 weeks (multiplication)		
	Response %	NO. of shoots	Length of the longest shoot (cm)	Total NO. of leaves	NO. of shoots	Length of longest shoot (cm)	Total NO. of leaves
0.0	70	2.10 b	1.65 c	16.00 b	1.10 b	0.90 b	7.90 b
2.0	90	2.70 b	3.92 ab	25.60 ab	6.50 a	7.60 a	48.00 a
4.0	100	2.50 b	5.02 a	24.90 ab	5.90 a	7.35 a	40.90 a
6.0	70	2.50 b	2.20 bc	19.90 b	2.00 b	0.65 b	11.10 b
8.0	100	4.50 a	4.00 ab	33.80 a	1.70 b	0.37 b	8.70 b

Values followed by the same letter are not significantly different according to Duncan's Multiple Range Test at the 5% probability level.

The results presented in Tables 2 and 3 demonstrate that cytokinins exert their most pronounced effects when applied at low concentrations to explants [33]. Both benzyladenine and kinetin, which share similar chemical structures, belong to the cytokinin family [34]. These plant growth regulators enhance nutrient uptake capacity and stimulate cell division in plant tissues [35]. Cytokinins influence numerous plant species through their multifaceted roles in growth and developmental processes. Their physiological impacts extend to multiple biological functions including: dormancy release, apical dominance regulation, shoot initiation and development, phyllotaxis determination, vascular differentiation, leaf senescence delay, shooting promotion, nodulation stimulation, seed germination enhancement, nutrient mobilization, and mediation of responses to both biotic and abiotic stress factors [36].

The study findings revealed that while cytokinins are essential for plant growth regulation, they demonstrate inhibitory effects when concentrations exceed optimal levels. Notably, benzyladenine (BA) showed superior performance

compared to kinetin (Kin) at their respective optimal concentrations in explant culture systems. This enhanced activity of BA can be attributed to its distinctive molecular structure containing reactive double bonds, which facilitate more efficient stimulation of cell division and expansion processes. These structural advantages translate to more pronounced effects on overall plant growth and development, explaining BA's widespread preference as the cytokinin of choice for micropropagation across various plant species [37]. The higher efficacy of BA compared to Kin is due to structural differences: BA contains a benzene ring with three double bonds, whereas Kin has only two. This increased number of double bonds enhances biological activit [38]. These results are consistent with previous research findings reported by [39][27][40][41][42][43][44][45][46][47][48] [49]. Overall, BA at 1.5 mg L^{-1} demonstrated the highest efficacy in shoot multiplication and leaf production across both stages, whereas Kin required higher concentrations for optimal response, aligning with its known role as a less potent cytokinin in Stevia micropropagation [33, 37].

3.5. Effect of BA and Kin Treatments on the Accumulation of Steviol Glycosides in the Stevia Plant (*Stevia rebaudiana*)

HPLC analysis was conducted to quantify stevioside and rebaudioside in explant derived from the optimal treatments identified in the multiplication stage. The results, presented in Table 4, show significant variation among treatments.The control sample (0.0) showed baseline levels of 160.5 ppm stevioside and 92.5 ppm rebaudioside. The treatment with BA notably increased both compounds, reaching 199.8 ppm for stevioside and 125.9 ppm for rebaudioside. Similarly, the Kin treatment elevated the levels to 183.5 ppm for stevioside and 110.3 ppm for rebaudioside, respectively.

Table (4): Quantitative Assessment of Steviol Glycosides (Stevioside and Rebaudioside) Content in Different SG Samples .

Treatments	Stevioside (ppm)	Rebaudioside (ppm)
Control	160.5	92.5
BA	199.8	125.9
Kin	183.5	110.3

The Analysis revealed that the application of cytokinins, particularly BA, not only enhanced shoot proliferation (Tables 2 and 3) but also positively influenced the biosynthesis of steviol glycosides. The highest accumulation of both target compounds was recorded in the BA-treated explant. This increase in secondary metabolite content correlates with the observed enhancement in vegetative growth parameters (shoot and leaf number). The results suggest that optimized in vitro conditions with appropriate cytokinin supplementation can stimulate pathways involved in steviol glycoside production, beyond merely increasing biomass. These findings align with previous reports on the positive effect of optimized tissue culture protocols on steviol glycoside yield in S. rebaudiana [50], [51],[52]and[3].

Conclusion

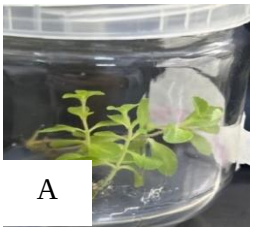
This study presents an optimized and integrated protocol for the micropropagation of stevia (Stevia rebaudiana Bertoni) applicable at the tissue culture level. The results demonstrated that sterilizing nodal segments with 4% sodium hypochlorite for 6 minutes is optimal for ensuring the highest contamination-free survival rate. Regarding the stimulation of vegetative growth, benzyladenine (BA) at a concentration of 1.5 mg L^{-1} emerged as a superior agent during the multiplication stage, achieving the highest productivity in terms of shoot count (33.70 shoots/explant) and leaf number (139.40 leaves/explant). Most importantly, this same treatment led to the highest quantitative accumulation of the economically active compounds, stevioside and rebaudioside-A, confirming a dual advantage that extends beyond mere biomass increase to enhancing the qualitative value of the produced plants

Based on these findings, it is recommended to adopt the aforementioned sterilization protocol and use MS medium supplemented with 1.5 mg L^{-1} BA to obtain the highest propagation rate and quality in the resulting plants. For future research, we suggest validating these results on a larger scale using temporary immersion bioreactors, testing the effect of other plant growth regulator combinations (such as thidiazuron) or biotic/abiotic stresses as elicitors to enhance glycoside production, and studying the genetic stability of the propagated plants using molecular techniques to ensure the homogeneity of the produced line.

Figure (1): Stevia (*Stevia rebaudiana*) plants were obtained from Al-Jannah Al-Nakhil Company for Tissue Culture in Baghdad, Iraq.

Figure (1): Stevia (*Stevia rebaudiana*) plants were obtained from Al-Jannah Al-Nakhil Company for Tissue Culture in Baghdad, Iraq.

Figure (2): Germination of nodal segments in *Stevia rebaudiana* (A) and contamination of nodal segments (B).



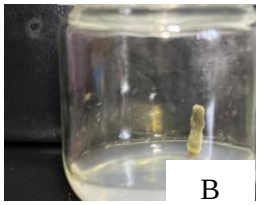


Figure (3): Multiplication stages with different (BA) concentrations (mg.L^{-1})

0.0

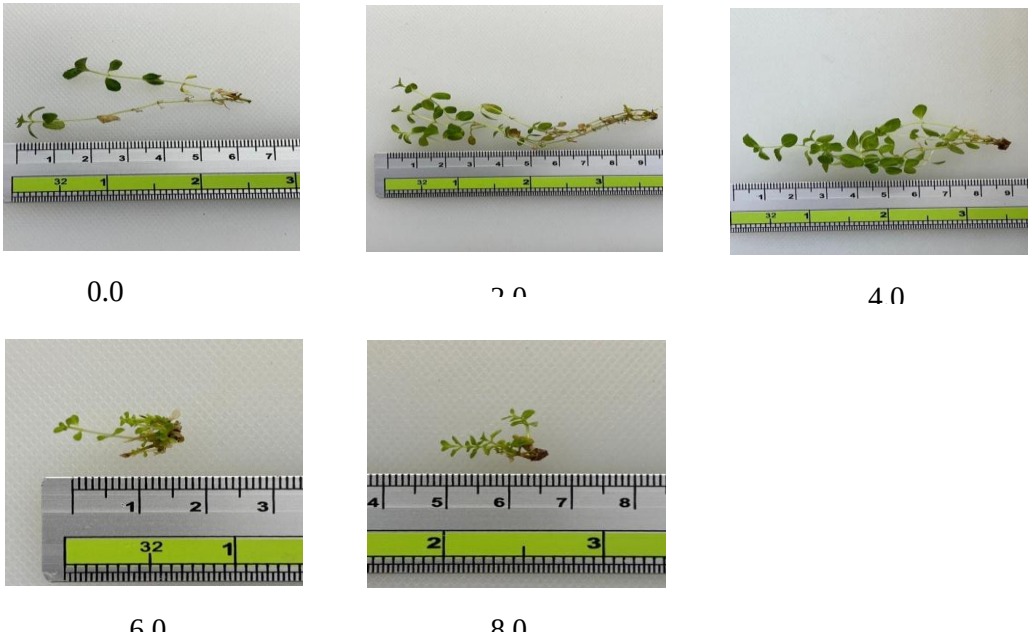
0.5

1.0

1.5

2.0

Figure (4): Multiplication stages with different (Kin) concentrations (mg.L^{-1})



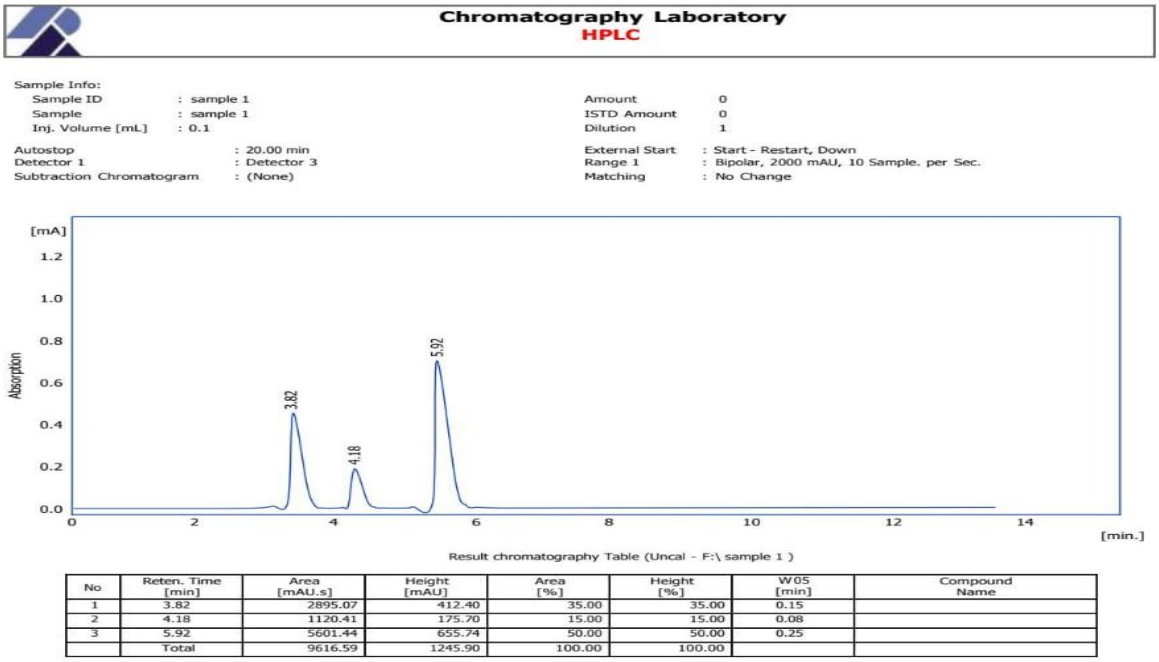
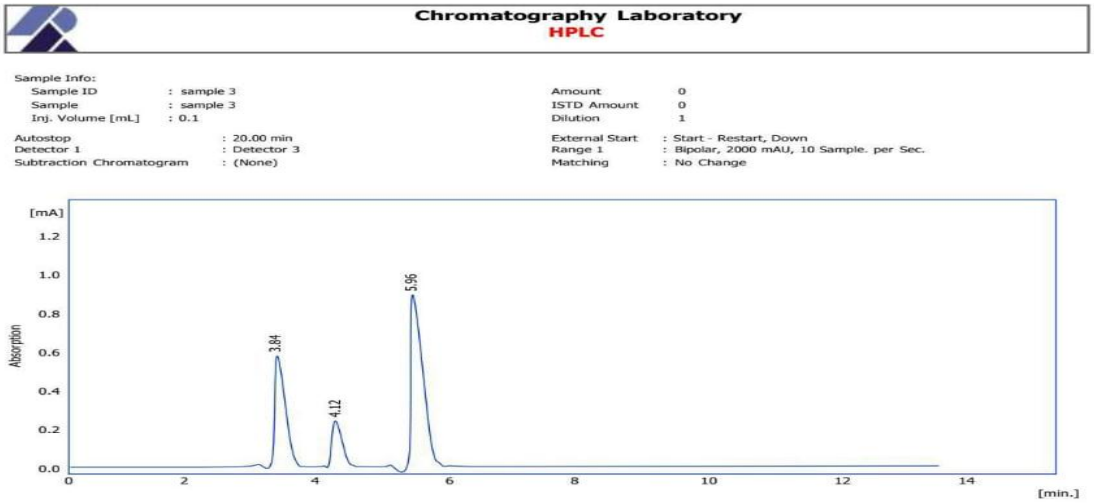


Figure (5) HPLC chromatogram of the extracts from tissue-cultured *Stevia rebaudiana* control plants.

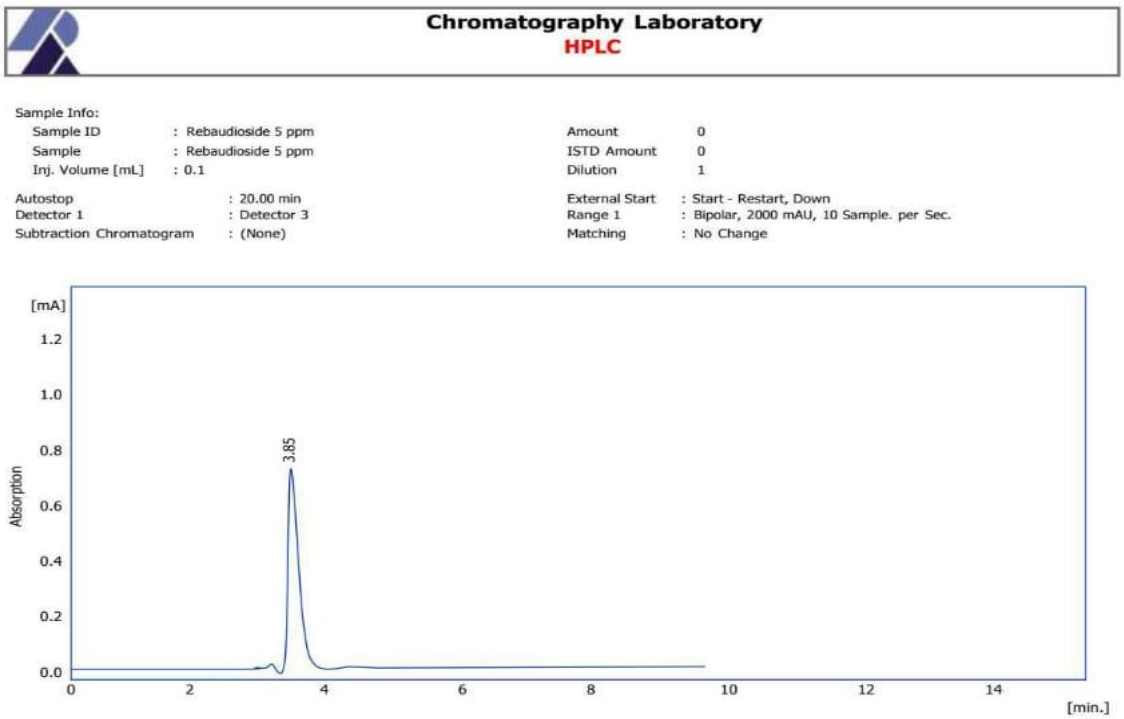
Figure (6)HPLC chromatogram of plant extracts resulting from treatment with benzyladenine in the tissue culture of *Stevia rebaudiana*.



Result chromatography Table (Uncal - F:\ sample 3)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.84	5901.44	572.65	35.00	35.00	0.15	
2	4.12	2922.65	210.11	15.00	15.00	0.08	
3	5.96	10114.80	825.80	50.00	50.00	0.25	
	Total	18932.65	1607.70	100.00	100.00		

Figure (7)HPLC chromatogram of plant extracts resulting from treatment with kinetin in the tissue culture of *Stevia rebaudiana*



Result chromatography Table (Uncal - F:\ Rebaudioside 5 ppm)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.85	2014.99	774.09	100.00	100.00	0.25	
	Total	2014.99	774.09	100.00	100.00		

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اكثار نبات الستيفيا ريبوديانا خارج الجسم الحي

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الخلاصة
يُعد نبات الستيفيا مصدراً طبيعياً مهماً للتحلية، لكن إكثاره على نطاق واسع يواجه تحديات. هدفت هذه الدراسة إلى تطوير بروتوكول فعال للإكثار الدقيق من خلال تحسين التعقيم السطحي وتقييم تأثير السيتوكينينات. عُقمت القطع العقدية باستخدام هايبوكلورات الصوديوم وزرعت على وسط ***MS*** بتراكيز مختلفة من البنزل أدينين (***BA***) أو الكينيتين (***Kin***). كان التعقيم بتركيز 4% لمدة 6 دقائق الأكثر فعالية. في مرحلة النشوء، حقق ***BA*** بتركيز 1.5 ملغم لتر$^{-1}$ أعلى نسبة استجابة (100%) وأطول طول برعم، بينما أنتج ***BA*** بتركيز 1.0 ملغم لتر$^{-1}$ أعلى عدد للفروع. في مرحلة التضاعف، حقق ***BA*** بتركيز 1.5 ملغم·لتر$^{-1}$ أعلى إنتاج للفروع والأوراق. بالنسبة للكينيتين، كان التركيز 2.0 ملغم لتر$^{-1}$ هو الأمثل في مرحلة التضاعف. والأهم من ذلك، أدت معاملة ***BA*** أيضاً إلى أعلى تراكم للستيفيوسيد (199.8 جزء في المليون) والريباوديوسيد-أ (125.9 جزء في المليون)، مما يؤكد دورها المزدوج في تعزيز كل من الكتلة الحيوية ومحتوى المركبات النشطة.

الكلمات المفتاحية: ستيفيا ريبوديانا ، وسط MS ،HPLC ، ستيفيوسيد ، ريباوديوسيد-أ