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Callus Induction, Artificial Seed Production, and Extraction of Bioactive Compounds from *Stevia rebaudiana* under *In Vitro* Conditions

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ABSTRACT
This study aimed to examine the influence of plant growth regulators on the initiation of callus formation in various parts of the Stevia rebaudiana Bertoni plant, specifically in nodal segments and shoot tips taken from the multiplication stage, under in vitro conditions.The explants were cultured on Murashige and Skoog (MS) medium was supplemented with various combination of naphthalene acetic acid NAA (0.5, 1.0, and 1.5 mg/L) and benzyladenine (BA) at 0.1, 0.2, and 0.3 mg/L. The results revealed that the treatment with 0.3 mg/L BA combined with 1.5 mg/L NAA led to the highest callus induction rate in nodal segments, with a maximum fresh weight of 0.1017 g. Similarly, the same treatment produced the highest fresh weight of callus in shoot tips, reaching 0.1491 g. In this study, the callus segments derived from both nodes and shoot tips were encapsulated using a sodium alginate solution at a concentration of 2% (w/v), then transferred into a 2% (w/v) calcium chloride solution, which led to the formation of a transparent gel matrix around the callus. This encapsulation process achieved a 100% success rate, with complete survival when stored at 4°C for duration of 28 days. Furthermore, callus samples were collected and analyzed for active compounds using high-performance liquid chromatography (HPLC), yielding the highest concentrations of 1704 ppm of stevioside and 98.7 ppm of rebaudioside A, extracted from apical meristem-derived callus. The aim of this study was to optimize callus induction, somatic embryogenesis and artificial seed production in *Stevia rebaudiana* .

تحفيز تكوين الكالس ، وإنتاج البذور الأصطناعية ، واستخلاص المركبات النشطة بيولوجيا من نبات ستيفيا ريبوديانا تحت ظروف المختبر

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الخلاصة
هدفت هذه الدراسة الى التحقيق في تاثير منظمات النمو النباتية على بدء تكوين الكالس في أجزاء مختلفة من نبات ستفيا ريبوديانا وتحديدا في الأجزاء العقدية والقمم النامية والماخوذة من مرحلة التضاعف في ظل ظروف المختبر، زرعت العينات على وسط موراشيك (MS) المدعم بتركيزات مختلفة من NAA (0.5,1.0,1.5) mg/l والبنزيل ادنين (BA) بتركيزات (0.1,0.2,0.3) mg/l . أظهرت النتائج ان المعاملة بتركيز 0.3 mg/l من BA مع 1.5 mg/l من NAA أدت الى أعلى معدل لتحفيز الكالس في الأجزاء العقدية ، اقصى وزن الطازج قدره 0.1017 غرام .وبالمثل ،انتجت نفس المعاملة أعلى وزن طازج للكالس في القمم النامية حيث وصل الى 0.1491 غرام وكذلك في هذه الدراسة، تم تكوين البذور الصناعية عن طريق تغليف أجزاء الكالس الناتجة من العقد والقمم النامية بمحلول الجينات الصوديوم بتركيز 2 % (وزن\حجم) ثم وضعت في محلول الكلوريد الكالسيوم بتركيز 2% (وزن\حجم) ، مما أدى الى تكوين مادة هلامية شفافة حول الكالس.حققت عملية التغليف هذه نسبة نجاح 100%،كما بلغت نسبة البقاء100% عند الحفظ في درجة الحرارة 4 مئوية لمدة 28 يوما. إضافة الى ذلك تم جمع عينات من الكالس وأستخلصت المواد الفعالة باستخدام جهاز HPCL،حيث أظهرت النتائج ان أعلى تركيز مقاس كان 1704 جزءا بالمليون للستيفيوسيد و98.7جزءا بالمليون من رايبوديوسيد -أ في كالس مشتق من القمم النامية.
الكلمات المفتاحية: الكالس ، البذور صناعية ، وسط MS ، HPCL ، ستيفيوسيد ، رايبوديوسيد-أ.

INTRODUCTION

Stevia rebaudiana (commonly known as Stevia) is a perennial shrub native to South America, particularly Paraguay and Brazil (Chang *et al*., 2005) It contains glycosides, which are low-calorie sweeteners approximately 300 times sweeter than sucrose (Ilca *et al*., 2017 ; Lemus-mondaca *et al*., 2012 ; Witono & Chandra., 2020). The leaf extract of Stevia is rich in various phytochemicals, including austromillin, β-carotene, dulcoside, niacin, rebaudiosides, riboflavin, steviol, stevioside, and tannin, which exhibit antimicrobial properties against numerous pathogens (Theophilus, 2015). Stevia extracts are known for their therapeutic properties, including antioxidant effects, as well as antimicrobial and antifungal activities (Lemus-mondaca *et al*., 2012).

Given these attributes, Stevia holds significant scientific and economic potential, particularly amid rising demand for high-quality raw materials. Notably, its glycoside profile varies substantially depending on genotype, geographic location, plant maturity, environmental conditions, harvest timing, and processing methods (Dyduch-Siemińska *et al*., 2020) . Seed-based propagation is uncommon in commercial plantations due to the plant's cross-pollinating nature, which generates high genetic diversity in seed-derived populations. Instead, in vitro propagation is prioritized, especially in regions lacking access to selected seedlings (Al-Taweel *et al*., 2021).

A mass of undifferentiated (parenchyma) cells, referred to as callus, is produced from plant tissues cultured in vitro on media supplemented with plant growth regulators such as auxins. Numerous callus cells exhibit totipotency, enabling them to regenerate into various plant organs (Abd El-Motaleb *et al*., 2015). Somatic embryogenesis is a highly valuable process in plants and has significant biotechnological applications, including clonal propagation, synthetic seed production, and genetic transformation. In direct somatic embryogenesis, embryos develop from organized cells, whereas in indirect somatic embryogenesis, they form following an intermediate callus phase. These somatic embryos closely resemble zygotic embryos in both anatomical and physiological characteristics (Deo *et al*., 2010).

Although somatic embryogenesis has been documented in over a hundred plant species, only a limited number of reports exist for the Asteraceae family (Nazneen *et al*., 2015).

Synthetic seeds, also known as artificial seeds, are encapsulated somatic embryos or vegetative propagules derived from in vitro cultures, enveloped with nutrient and protective compounds. This innovative approach is particularly advantageous for plant species with limited seed production, as it mimics zygotic seeds by utilizing non-reproductive plant tissues or organs (Ghosh & Haque, 2019). Sodium alginate is extensively utilized for encapsulating somatic embryos in synthetic seed production due to its cost-effectiveness, low toxicity, and stable gel formation. The hardening process of the insoluble calcium alginate matrix around the propagule is facilitated by an ion exchange reaction between sodium ions (Na^+) in the sodium alginate solution and calcium ions (Ca^{2+}) in the calcium chloride solution (Daud *et al*., 2008).

The propagation of the propagule in this manner offers substantial protection and supplies essential nutrients required during the seed regeneration phase. The biodegradable matrix composing the synthetic seed effectively shields the explants from physical damage and external environmental stressors. Additionally, it functions as an artificial endosperm for the somatic embryo, encapsulating nutrients critical for early developmental stages. This method is particularly recommended for the conservation and propagation of endangered species, medicinal plants, and commercially valuable crops (Jang *et al*., 2020).

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. To study callus formation for *Stevia (Stevia rebaudiana)* Bartoni, plant parts (explants) such as nodes and shoot tips were used. The plantlets of *Stevia (Stevia rebaudiana)* were obtained from Al-Jannah Al-Nakhil Company for Tissue Culture in Baghdad, Iraq. The callogenesis experiment for callus induction (callogenesis) was conducted using nodal and shoot tips from the explants obtained from the multiplication stage. The nodal segment and shoot tips were aseptically separated, and their surfaces were gently scarified using asterile scalpel to facilitate, contact with the culture medium and then planted on a medium containing a combination of NAA concentrations (0.5, 1.0, and 1.5 mg/L) and Benzyladenine at concentrations (0.1, 0.2, and 0.3 mg/L). The culture media used was MS (Murashige & Skoog, 1962), basal salt mixture at aconcentration of 4.43 g/L.

Additionally, 6.5 g/L agar and 30 g/L sucrose were added inside a glass container containing 1 liter of distilled water for each concentration on a rotating heater. After boiling and dissolving the agar, the pH was adjusted to 5.75 ± 0.1, and the medium was autoclaved at 121°C for 20 minutes. Aliquots of 40 mL were dispensed into 240 mL culture vessels. Cultures were incubated in a growth chamber at 25 ± 1°C. Data on explant callus induction were recorded after 6 weeks. To produce artificial seeds, callus tissue derived from nodes and shoot tips tissues in vitro was used. The callus was cut into uniform fragments of 2–3 mm^3 in size for encapsulation. A solution of sodium alginate and Calcium Chloride dyhydrate ($CaCl_2 \cdot 2H_2O$) was prepared by dissolving 1 gram of each in 50 mL of distilled water. The pH of both the gel and solution was adjusted to 5.70 ± 0.1 and sterilized by autoclaving at 121°C for 20 minutes. Encapsulation was performed by dropping sodium alginate droplets—each containing a fragment of callus—using a pipette with its tip cut to achieve a diameter of (3-4 mm diameter) into the solution to allow complete polymerization. The capsules were then retrieved, washed with sterile distilled water to remove the $CaCl_2$

residue, and transferred to Petri dishes containing sterile filter paper to remove excess water. All of the above procedures were carried out under sterile laminar airflow conditions.
The capsules were divided into numerically equal portions and subjected to conservation in the dark at two different temperatures (4°C and 25°C) for 7, 12, and 28 days. For storage, the capsules were placed in sterile Petri dishes, with each plate containing 10 capsules. Subsequently, samples corresponding to callus formation in nodes and shoot tips were collected at the optimum concentrations of NAA and BA. The callus 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. Standard samples of Stevioside and Rebaudioside -A were taken from Sigma company.

Experimental design and statistical analysis

A Completely Randomized Design (CRD) with ten replicates per treatment was employed, and means were compared using Duncan's multiple range tests at a 5% significance level (Al-Rawy & Khalaf Allah 1980). Statistical analyses were performed using SAS software (Littell *et al*., 2002).

Sample Extraction:

10 gm of dried callus were extracted with 25 mL of water in a boiling water bath for 30 minutes (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.

HPLC

A high-performance liquid chromatography (HPLC) model SYKAM (German method) was performed, according to the analysis of stevioside and rebaudioside. Chromatographic separation was carried out on a C18-NH_2 (250 mm: 4.6 mm: 5 µm) column with temperature control at 40°C and a UV-Vis detector set to a wavelength of 210 nm. The mobile phase was a 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffers (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:

Data from Table 1 indicate that after 6 weeks of cultivating Stevia nodal segments on MS medium supplemented with varying concentrations of benzyl adenine (BA) and naphthalene acetic acid (NAA), the treatment with 0.3 mg/L BA and 1.5 mg/L NAA exhibited the highest callus formation response (80%). In contrast, no callus formation response was observed with the combination of 0.3 mg/L BA and 0.5 mg/L NAA. The lowest response rate (20%) was recorded for the treatment containing 0.2 mg/L BA and 0.5 mg/L NAA.
Statistical analysis further revealed that the 0.3 mg/L BA + 1.5 mg/L NAA treatment yielded the highest fresh weight (0.1017 g) and active growth (denoted as +++) of callus. The callus produced under this treatment displayed a distinct whitish-brown color. The lowest callus formation weight and growth were observed under the treatment with a combination of 0.2

mg/L BA and 0.5 mg/L NAA, where the fresh weight was 0.0068 g, growth was minimal (+), and the callus exhibited a whitish-brown coloration.
These symbols indicate the following: No callus (-), weak growth (smaller than a lentil seed) (+), medium growth (equivalent to the size of a lentil seed) (++), vigorous growth (equivalent to the size of a chickpea) (+++), and vigorous growth (larger than the size of a chickpea) (++++). (Kamal, 2024)

Table (1) The Effect of Combined Concentrations of Benzyl Adenine (BA) and Naphthalene Acetic Acid (NAA) on Callus induction from nodal explants of *Stevia rebaudiana*

BA (mg·L⁻¹)	NAA (mg·L⁻¹)	Response (%)	Fresh Weight (g)	Growth	Color
0.1	**0.5**	40	0.0576 ab	+	brown
	1.0	30	0.0804 ab	+	Whitish brown
	1.5	40	0.0131 b	+	Whitish brown
0.2	**0.5**	20	0.0068 b	+	Whitish brown
	1.0	60	0.0363 ab	+	Whitish brown
	1.5	40	0.0156 b	+	Light green
0.3	**0.5**	0.0	0.0 b	–	–
	1.0	60	0.0431 ab	++	Whitish brown
	1.5	80	0.1017 a	+++	Whitish brown

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

Data from Table 2 indicates that after 6 weeks of cultivating Stevia nodal segments on MS medium supplemented with varying concentrations of benzyl adenine (BA) and naphthalene acetic acid (NAA), the treatment with 0.1 mg/L BA and 1.0 mg/L NAA exhibited the highest callus formation response (100%). In contrast, no callus formation response was observed with the combination of 0.1 mg/L BA + 1.5 mg/L NAA, 0.2 mg/L BA + 0.5 mg/L NAA, and 0.2 mg/L BA + 1.0 mg/L NAA. The lowest response was recorded for the combination of 0.2 mg/L BA + 1.5 mg/L NAA, and 0.3 mg/L BA + 0.5 mg/L NAA. Statistical analysis further revealed that the 0.1 mg/L BA + 0.5 mg/L NAA treatment yielded the highest fresh weight (0.1491 g) and active growth (denoted as (+++) of callus. The callus produced under this treatment displayed a distinct whitish-brown color. The lowest callus formation weight and growth were observed under the treatment with a combination of 0.2 mg/L BA and 1.5 mg/L NAA, where the fresh weight was 0.0005 g, growth was minimal (+), and the callus exhibited a whitish-brown coloration.
These symbols indicate that:

No callus (-), weak growth (smaller than a lentil seed) (+), medium growth (equivalent to the size of a lentil seed) (++), vigorous growth (equivalent to the size of a chickpea) (+++), vigorous growth (larger than the size of a chickpea) (++++). (Kamal, 2024).

Table (2) The Effect of Combined Concentrations of Benzyl Adenine (BA) and Naphthalene Acetic Acid (NAA) on Callus induction from Shoot Tips of *Stevia rebaudiana*

BA ($mg \cdot L^{-1}$)	NAA ($mg \cdot L^{-1}$)	Response (%)	Fresh Weight (g)	Growth	Color
0.1	**0.5**	60	0.1491 a	++++	Whitish brown
	1.0	100	0.0692 b	+++	Whitish brown
	1.5	0.0	0.0 b	–	–
0.2	**0.5**	0.0	0.0 b	–	–
	1.0	0.0	0.0 b	–	–
	1.5	10	0.0005 c	+	Whitish brown
0.3	**0.5**	10	0.0025 c	+	brown
	1.0	40	0.0359 cb	++	Whitish brown
	1.5	20	0.0151 cb	+	Whitish brown

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

The results presented in Tables 1 and 2 indicate that callus induction in in vitro cultures primarily occurs at the incision sites formed during explant isolation or through direct exposure of the explant surface to a suitable culture medium. Specific growth regulators, particularly balanced ratios of auxins and cytokinins, are critical for inducing callus formation (Grafi & Barak, 2014; Bairu *et al*., 2011; Cao & Hammerschlag, 2000 ; Shah *et al*., 2015; Khalid & Kassab, 2018). Recent research has highlighted the influence of plant growth regulators in inducing diverse pigmentation patterns in callus cultures, including light green, dark green and yellowish-white hues, alongside variations in texture, such as friable or compact callus structures (Mahmud *et al*., 2014). The application of specific concentrations of auxins, cytokinins, and their synergistic interactions significantly enhances callogenesis by promoting cellular metabolic activity, mitotic division, and biosynthesis of critical growth components (Hedden & Stephen, 2006). These regulators further facilitate cell wall expansion, elevate protein synthesis via RNA transcription mechanisms, and drive cytokinesis. Notably, cytokinins are pivotal in modulating protein and sucrose biosynthesis to accelerate cell proliferation (Mahesh, 2008). As in the following results (AL-Hasany, 2021 ; Blinstrubienė *et al*., 2020 ; Röck-Okuyucu *et al*., 2016 ; Sharma *et al*., 2015 ; Zayova *et al*., 2020 ; Keshvari *et al*., 2018 and Abdulraheem *et al*., 2024). The

results presented in Table 3 demonstrate the effects of sodium, calcium chloride, and storage method on the encapsulation success rate of somatic embryos derived from callus for artificial-seed production. The findings revealed that a transparent gel matrix formed around the callus fragments at 100%. Moreover, storage in a refrigerator at 4 °C for 28 days resulted in a 100% survival rate, as did storage in a growth chamber at (25 ± 1 °C) for seven days; however, storage for 14 days yielded an 80% survival rate.

Table (3) Artificial Seed Production from Callus Derived from Nodal Segments of *Stevia rebaudiana*

Seed Conversion Rate (%)	**Temperature**	**Storage Duration**	**Survival Rate (%)**
100	25 °C	7 day	100
100	25 °C	12 day	80
100	4 °C	28 day	100

The results presented in Table (4) demonstrate the effects of sodium, calcium chloride, and storage method on the encapsulation success rate of somatic embryos derived from callus for Artificial-seed production. The findings revealed that a transparent gel matrix formed around the callus fragments at 100%. Moreover, storage in a refrigerator at 4°C for 28 days resulted in a 100% survival rate, as did storage in a growth chamber at 24°C for seven days; however, storage for 14 days yielded a 90% survival rate.

Table (4) Artificial Seed Production from Callus Derived from Shoot Tips of *Stevia rebaudiana*

Seed Conversion Rate (%)	Temperature	Storage Duration	Survival Rate (%)
100	25 °C	7 day	100
100	25 °C	12 day	90
100	4 °C	28 day	100

The results shown in Tables 3 and 4 can be interpreted as follows: the use of a sodium-alginate–calcium-chloride solution formed a transparent, hydrated gel matrix around the somatic body, thereby protecting it from injury and desiccation. Moreover, storage at 4 °C in complete darkness provided the longest preservation period for the artificial seeds, as it minimized moisture loss—thus preventing the surrounding gel mass from drying out—and also inhibited premature germination. These findings are consistent with previous reports. (Shaafi *et al*., 2021 ; Subrahmanyeswari *et al*., 2023 ; Yücesan *et al*., 2016 ; Ali, 2024; Khalid & Kassab, 2018)

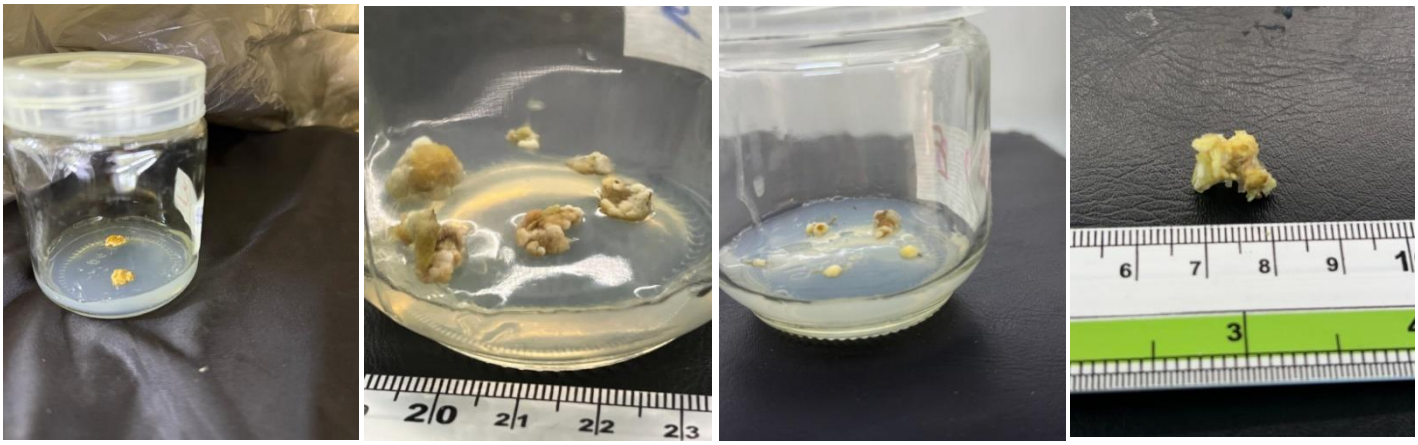
The results presented in Tabal 5 the Effect of BA and NAA Treatments on the Accumulation of Steviol Glycosides in the Stevia Plant (*Stevia rebaudiana*). The analysis revealed significant variations in the concentrations of stevioside and rebaudioside among the different treatments. Treatment with callus from shoot tips containing a combination of NAA concentration and benzyladenine at concentration BA notably increased both compounds, reaching 1704 ppm for stevioside and 98.7 ppm for rebaudioside. It was less than the callus from nodal segment to 174.6 ppm for stevioside and 102.6 ppm for rebaudioside, respectively.

Table (5) The Effect of NAA and BA on the Accumulation of Steviol Glycosides (Rebaudioside A / Stevioside) in Callus Cultures from Nodal Segment and Shoot Tip Explants.

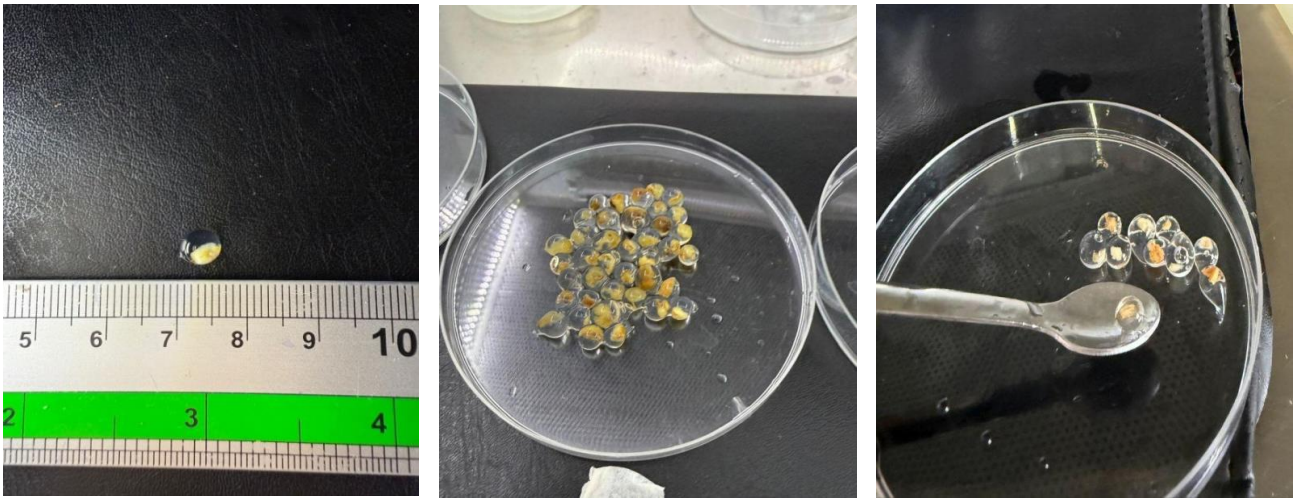
Callus	**Stevioside (ppm)**	**Rebaudioside (ppm)**
Callus from nodal segment	174.6	102.6
Callus from shoot tips	1704	98.7

Quantitative Analysis of the SG Content. High-performance Liquid chromatography (HPLC) analysis revealed a higher content of stevioside and rebaudioside-A in callus derived from shoot tip tissues of *in vitro* plants treated with different PGRs, compared to callus from nodal segments *in vitro*. These results are consistent with previous research findings reported by. (Gunasena & Senarath, 2023 ; Javad *et al*., 2016 ; Kumari & Chandra, 2015 ; Gunasena & Senarath, 2024 ; Mahdi & Jasim, 2023)

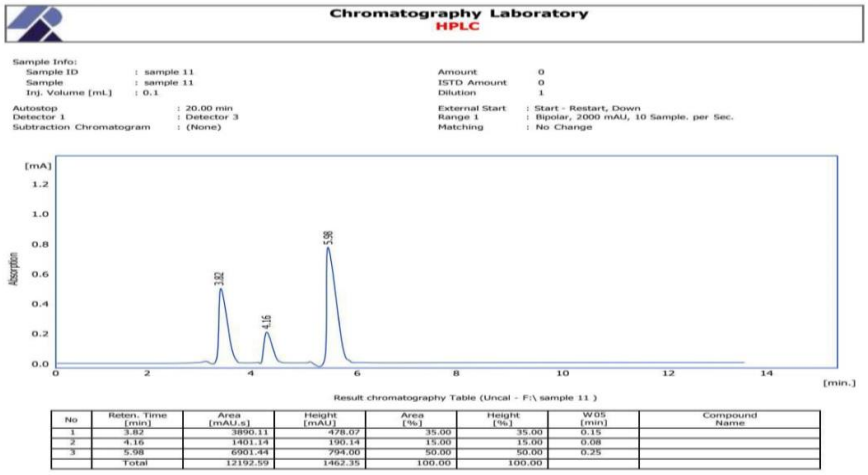
Picture (1): Plant Explants Were Taken From the Multiplication Stage of Stevia (*Stevia Rebaudiana*)



Picture (2): Formation of Different Callus Morphologies Derived From Nodal and Apical Explants of *Stevia Rebaudiana*.

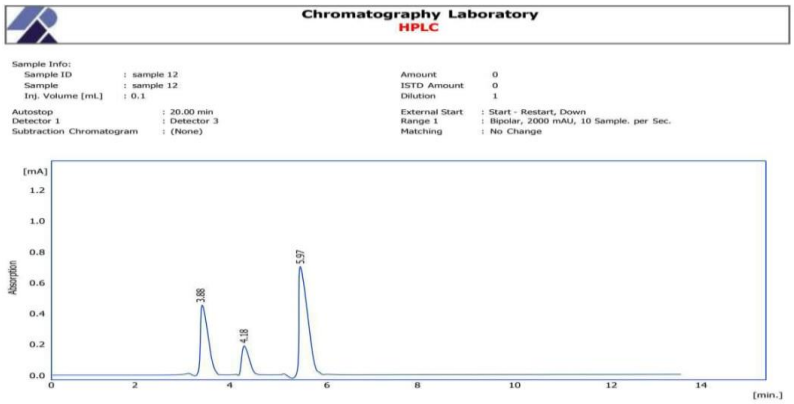


Picture (3): Illustrating the Encapsulated Artificial Seeds



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.82	3890.11	478.07	35.00	35.00	0.15	
2	4.16	1401.14	190.14	15.00	15.00	0.08	
3	5.98	6901.44	794.00	50.00	50.00	0.25	
	Total	12192.59	1462.35	100.00	100.00		

Figure 1.HPLC chromatogram of Callus extracts derived from the nadal segment of *Stevia rebaudiana*



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.88	3260.14	413.65	35.00	35.00	0.15	
2	4.18	1350.11	175.77	15.00	15.00	0.08	
3	5.97	6233.56	656.09	50.00	50.00	0.25	
	Total	10843.65	1245.74	100.00	100.00		

Figure 2. HPLC chromatogram of Callus extracts derived from the shoot tips of *Stevia rebaudiana*

CONCLUSION

In conclusion, this study demonstrated that Benzyl Adenine (BA) and Naphthalene Acetic Acid (NAA) on Callus Formation in Nodal and shoot tips of *Stevia rebaudiana*. Significant callus formation from nodal explants was achieved with a combination of 0.3 mg/ L BA and 1.5 mg/L NAA. For shoot tip explants, optimal callus formation occurred with 0.1 mg/L BA and 0.5 mg/L NAA. Successful production of synthetic seeds from Stevia callus was accomplished, with effective preservation at 4°C, yielding the highest concentration of Stevioside in callus Cultures derived from shoot Tip Explants.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

REFERENCES

Abd El-Motaleb, M., Abd El-Hameid, A. S., Elnaggar, H. M. N., & Abdel-Hady, M. (2015). Callus induction and regeneration of Stevia rebaudiana Bertoni. International Journal of Chemical Technology Research, 8, 868–877.

AL-Hasany, Z. H. A. R. (2021). The potential of increasing sweetening compounds and studying molecular markers in EMS-mutated tissue cultures of Stevia rebaudiana Bertoni plants Doctoral dissertation]. University of Baghdad.

Abdulraheem, Z. H., Noori, A. M., Husni, H. S., Lateef, M. A., & Alsaffar, M. F. (2024). Digitalis purpurea L. Propagated in Vitro with Various Hormones. IOP Conf. Series: Earth and Environmental Science, 1371 (4), 042452. https://doi.org/10.1088/1755-1315/1371/4/042050

Ali, H. H. (2024) In vitro propagation of pomegranate (punica granatum L.) Saimi cultivar [Master's thesis, Kirkuk University] . College of Agriculture, Kirkuk University.

Al-Rawy, K. M., & Khalaf Allah, A. A. M. (1980). Design and analysis of agricultural experiments. Dar Al Kutub Printing Press.

Al-Taweel, S. K., Azzam, C. R., Khaled, K. A., & Abdel-Aziz, R. M. (2021). Improvement of stevia (Stevia rebaudiana Bertoni) and steviol glycoside through traditional breeding and biotechnological approaches. SABRAO Journal of Breeding and Genetics, 53(1), 88–111.

Bairu, M. W., Aremu, A. O., & Van Staden, J. (2011). Somaclonal variation in plants: Causes and detection methods. Plant Growth Regulation, 63, 147–173. https://doi.org/10.1007/s10725-010-9554.

Blinstrubienė, A., Burbulis, N., Juškevičiūtė, N., Vaitkevičienė, N., & Žūkienė, R. (2020). Effect of growth regulators on Stevia rebaudiana Bertoni callus genesis and influence of auxin and proline to steviol glycosides, phenols, flavonoids accumulation, and antioxidant activity in vitro. Molecules, 25(12), 2759.

Cao, X., & Hammerschlag, F. A. (2000). Improved shoot organogenesis from leaf explants of highbush blueberry. HortScience, 35(5), 945–947. https://doi.org/10.21273/HORTSCI.35.5.945.

Chang, J. −C., Wu, M. C., Liu, I. −M., & Cheng, J. −T. (2005). Increase of insulin sensitivity by stevioside in fructose−rich chow−fed rats. Hormone and Metabolic Research, 37, 610–616.

Daud, N., Taha, R. M., & Hasbullah, N. A. (2008). Artificial seed production from encapsulated micro shoots of Saintpaulia ionantha Wendl. (African violet). Journal of Applied Sciences, 8(24), 4662–4667.

Deo, P. C., Tyagi, A. P., Taylor, M., Harding, R., & Becker, D. (2010). Factors affecting somatic embryogenesis and transformation in modern plant breeding. South Pacific Journal of Natural and Applied Sciences, 28, 27–40.

Dyduch-Siemińska, M., Najda, A., Gawroński, J., Balant, S., Świca, K., & Żaba, A. (2020). Stevia rebaudiana Bertoni, a source of high-potency natural sweetener-biochemical and genetic characterization. Molecules, 25(4), 767. https:// doi.org/ 10.3390 /molecules 25040767.

Ghosh, B., & Haque, S. M. (2019). Synthetic seeds: An alternative approach for clonal propagation to avoiding the heterozygosity problem of natural botanical seeds. In M. Faisal & A. A. Alatar (Eds.), Synthetic Seeds (pp. 77–112). Springer.

Grafi, G., & Barak, S. (2014). Stress induces cell dedifferentiation in plants. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1839(4), 345–348. https://doi.org/10.1016/j.bbagrm.2014.01.004.

Gunasena, M. D. K. M., & Senarath, W. T. P. S. K. (2023). Phytochemical analysis of Stevia rebaudiana using HPLC and GC-MS.

Gunasena, M. D. K. M., & Senarath, W. T. P. S. K. (2024). Comparison of Phytochemicals in naturally grown plants, tissue cultured plants and calli of Stevia rebaudiana using HPLC and GC-MS.

Hedden, P., & Stephen, G. (2006). Plant hormone signaling. Blackwell Publishing Ltd.

Ilca, V., Vukmirovica, S., Stilinovic, N., Capo, I., Arsenovi, M., & Milija, B. (2017). Insight into antidiabetic effect of low dose of stevioside. Biomedicine & Pharmacotherapy, 90, 216–221. https://doi.org/10.1016/j.biopha.2017.03.045.

Jang, B. K., Cho, J. S., & Lee, C. H. (2020). Synthetic seed technology development and production studies for storage, transport, and industrialization of bracken spores. Plants, 9(9), 1079.

Javad, S., Naz, S., Ilyas, S., & Aftab, A. (2016). Production of stevioside from callus and cell suspension cultures of Stevia rebaudiana (Bert.). Journal of Animal & Plant Sciences, 26(5).

Kamal, T. F. (2024). Effect of plant growth regulators on micropropagation of petunia and the production of synthetic seeds [Master's thesis]. University of Kirkuk.

Keshvari, T., Najaphy, A., Kahrizi, D., & Zebarjadi, A. (2018). Callus induction and somatic embryogenesis in Stevia rebaudiana Bertoni as a medicinal plant. Cellular and Molecular Biology, 64(2), 46–49.

Khalid, W. K., & Kassab, A. Z. A. (2018). Production of synthetic seeds in almond plan Prunus amygdalus (local type) In Vitro. Kirkuk University Journal For Agricultural Sciences, 9(4), 88-101.

Kumari, M., & Chandra, S. (2015). Stevioside glycosides from in vitro cultures of Stevia rebaudiana and antimicrobial assay. Brazilian Journal of Botany, 38, 761–770. https://doi.org/10.1007/s40415-015-0182-6.

Lemus-mondaca, R., Vega-gálvez, A., Zura-bravo, L., & Ah-hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chemistry, 132(3), 1121–1132. https://doi.org/10.1016/j.foodchem.2011.11.140.

Littell, R., Stroup, W. W., Freund, R. (2002) SAS for linear models. John Wiley & Son.

Mahdi, S. A., & Jasim Alsaedi, N. J. (2023). Improving Steviol Glycosides Production in Stevia Rebaudian Bertoni by Plant Tissue Culture Technology (Revi. Journal of Kirkuk University for Agricultural Sciences, 14(3).

Mahesh, S. (2008). Plant molecular biotechnology (1st ed.). New Age International Publishers.

Mahmud, S., Akter, S., Jahan, I. A., Khan, S., Khaleque, A., & Islam, S. (2014). Comparative analyses of stevioside between fresh leaves and in-vitro derived callus tissue from Stevia rebaudiana Bert. using HPLC. Bangladesh Journal of Scientific and Industrial Research, 49(4), 199–204.

Nazneen, H., Sridhar, S., & Reddy, S. K. (2015). Studies on somatic embryogenesis of Stevia rebaudiana (Bertoni) by scanning electron microscope (SEM). World Journal of Pharmaceutical Research, 4, 918–924.

Röck-Okuyucu, B., Bayraktar, M., Akgun, I. H., & Gurel, A. (2016). Plant growth regulator effects on in vitro propagation and stevioside production in Stevia rebaudiana Bertoni. HortScience, 51(12), 1573–1580.

Shaafi, B., Mosavi, S. S., Abdollahi, M. R., & Sarikhani, H. (2021). The optimized protocols for production, adaptation and keeping of the produced artificial seeds from encapsulated lateral buds in Stevia rebaudiana (Bertoni). Agrotechniques in Industrial Crops, 1(1), 24-35.

Shah, S. H., Ali, S., Jan, S. A., Din, J., & Ali, G. M. (2015). Callus induction, in vitro shoot regeneration and hairy root formation by the assessment of various plant growth regulators in tomato (Solanum lycopersicum Mill.). Journal of Animal & Plant Sciences, 25(2).

Sharma, N., Gauchan, D. P., Dhakal, A., Luitel, A., Shakya, S., & Shakya, R. (2015). Establishment of regenerative callus, cell suspension system and molecular characterization of Stevia rebaudiana Bertoni for the production of stevioside in

vitro. International Journal of Research in Applied Science and Engineering Technology, 3(3), 133–148.

Subrahmanyeswari, T., Laha, S., Kamble, S. N., Singh, S., Bhattacharyya, S., & Gantait, S. (2023). Alginate encapsulation of shoot tips and their regeneration for enhanced mass propagation and germplasm exchange of genetically stable Stevia rebaudiana Bert. Sugar Tech, 25(3), 542–551.

Theophilus, P. A. S., Victoria, M. J., Socarras, K. M., Filush, K. R., Gupta, K., Luecke, D. F., & Sapi, E. (2015). Effectiveness of Stevia rebaudiana whole leaf extract against the various morphological forms of Borrelia burgdorferi in vitro. European Journal of Microbiology and Immunology, 5, 268–280. https://doi.org/10.1556/1886.2015.00031.

Witono, J. R., & Chandra, A. (2020). The study on the method for maximizing steviol glycoside extract from Stevia leaves. International Conference on Chemical Engineering UNPAR, 012048. https://doi.org/10.1088/1757-899X/742/1/012048 .

Yücesan, B., Mohammed, A., Büyükgöçmen, R., Altuğ, C., Kavas, Ö., Gürel, S., & Gürel, E. (2016). In vitro and ex vitro propagation of Stevia rebaudiana Bertoni with high Rebaudioside-A content—A commercial scale application. Scientia Horticulturae, 203, 20–28.

Zayova, E., Nedev, T., Petrova, D., Zhiponova, M., Kapchina, V., & Chaneva, G. (2020). Tissue culture applications of Artemisia annua L. callus for indirect organogenesis and production of phytochemicals. Plant Tissue Culture and Biotechnology, 30(1), 97–106.