ISSN: 2621-4709 E-ISSN: 2621-2528
Journal of Applied Agricultural Science and Technology
Vol. 10 No. 2 (2026): 328-342

Optimization of Culture Media and Elicitation for Enhancing Steviol Glycosides in *Stevia rebaudiana* Callus Culture

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Abstract. *Stevia rebaudiana is a valuable medicinal plant known for producing steviol glycosides, which are widely used as natural sugar substitutes. This study aims to optimize culture media and elicitation treatments to enhance callus induction, growth, and steviol glycoside accumulation in S. rebaudiana callus culture. The experiment was conducted using a Completely Randomized Design (CRD) with four treatments: MS0, DKW0, MS + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3, and DKW + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3, each with five replicates.* The results showed that the highest callus induction percentage (100%) and the fastest callus initiation time (6.33–6.40 days) were achieved on MS and DKW media supplemented with plant growth regulators (2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3). *The highest fresh weight (0.158 g) and dry weight (0.011 g) were obtained on DKW medium supplemented with 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3. The highest stevioside content (0.147 mg/g DW) was recorded in the 4 mg/L GA_3 treatment, whereas the highest rebaudioside A content (0.114 mg/g DW) was observed in the 0.5 mg/L SA treatment. The highest rebaudioside D content (3.256 mg/g DW) was obtained in the 0.25 mg/L SA treatment, while the highest dulcoside content (0.120 mg/g DW) was found in the control treatment. In addition, the highest total phenolic content (19.37 mg GAE/g DW) was observed in the 20 mg/L daminozide treatment. In conclusion, DKW medium supplemented with 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3 was the most effective treatment for promoting callus growth, while elicitation treatments, particularly 4 mg/L GA3, significantly enhanced the accumulation of steviol glycosides in S. rebaudiana callus culture (0.147 mg/L).*
Keywords: *basic media; daminozide; phenolic compounds; salicylic acid; steviol glycosides.*

Type of the Paper: Regular Article.

1. Introduction

Stevia rebaudiana Bertoni is a herbaceous species of the family Asteraceae, commercially valued for its natural non-caloric sweetening properties [1]. The sweet taste of stevia comes from steviol glycosides, diterpenoid compounds that are approximately 300 times sweeter than sucrose. Steviol glycoside, including stevioside, rebaudiosides (Reb A, B, C, D, and E), and dulcoside A, are considered suitable for human consumption due to their natural origin and low caloric value

https://doi.org/10.55043/jaast.v10i2.548
Received December 29, 2025; Received in revised form May 14, 2026; Accepted May 16, 2026; Published May 30, 2026
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[2]. Stevia also contains several essential secondary metabolites, including coumarins, flavonoids, phenols, tannins, and essential oils [3]. Based on its biological activity, stevia exhibits antihypertensive, antidiabetic, anti-obesity, antitumor, anticaries, antioxidant, anti-inflammatory, antimicrobial, and antifungal properties, and can improve kidney function [4].

The demand for stevia as a natural sugar substitute continues to increase commercially [5]. However, the low germination rate remains a major obstacle in stevia production because of seed self-incompatibility. Propagation of stevia through seeds also does not produce a homogeneous population, resulting in variability in plant growth and components, such as sweetener content and composition. In vitro culture techniques provide an alternative approach for plant propagation and secondary metabolite production [6]. Callus suspension–based tissue culture provides optimal conditions for callus growth and is considered an ideal method for the differentiation and biosynthesis of secondary compounds because of enhanced cell proliferation in suspension [7,8].

Callus development is affected by various factors, particularly the composition of the culture medium and the application of plant growth regulators (PGR) [9]. Murashige & Skoog (MS) medium is commonly used for stevia callus induction. Ajijah [10] states that the initial stage of callus induction requires a nutrient-rich basal medium to initiate cell division. *Driver and Kuniyuki Walnut* (DKW) medium contains higher levels of phosphorus (PO_4^-), sulfur (SO_4^-), magnesium (Mg^+), and calcium (Ca^+) compared to MS medium. These elements are essential in cell elongation and division. Studies by Page et al. [11] and Ghannad et al. [12] showed that callus induction of Cannabis sativa using DKW medium resulted in better callus growth compared to MS medium. Additionally, Gati et al. [13] reported that DKW medium promoted more optimal shoot growth compared to MS, AB Mix, Growmore, and Grandasil D media. The addition of PGRs also enhances callus induction and development in stevia. Zayova et al. [14] showed that the treatment with 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L gibberellin induced callus formation in 90% of explants and produced friable callus.

Previous research indicates that chemical treatments can induce the accumulation of secondary metabolites in plants. Salicylic acid (SA), a phenolic elicitor, is commonly used to enhance metabolite synthesis and accumulation [15]. Exogenous application of SA can promote plant physiological processes by increasing enzyme activity and antioxidant levels [16]. SA has been proven effective to increase steviol glycoside content in in vitro stevia cultures [17,18]. Furthermore, gibberellin (GA_3) application effectively stimulates the production and accumulation of steviol glycosides, as reported in previous studies [19,20]. This relationship is important because GA_3 and steviol glycosides are produced through the same MEP metabolic pathway, arising from a shared precursor, kaurenoic acid [2]. In addition to SA and GA_3, the use of GA_3 inhibitors, such as daminozide, in in vitro stevia cultures is potentially effective in increasing steviol glycoside accumulation. Daminozide inhibits the activity of 2-oxoglutarate-dependent dioxygenases (2OGDDs) involved in the inhibition of gibberellin biosynthesis in the downstream pathway, without affecting steviol glycoside biosynthesis [21]. It blocks the conversion of ent-kaurenoic acid to gibberellin, thereby increasing steviol glycoside accumulation [22,23]. Therefore, this study aims to evaluate the effect of culture media on callus induction and the effectiveness of elicitation on secondary metabolites production in callus cultures of *Stevia rebaudiana* Bertoni. The findings of this study provide important implications for plant biotechnology development, particularly in optimizing in vitro culture systems to enhance production of valuable secondary metabolites. This research also supports the development of natural low-calorie sweeteners that may contribute to healthier dietary alternatives and potentially reduce the risk of metabolic disorders associated with excessive sugar consumption.

2. Materials and Methods

2.1. Materials

Callus induction was performed using explants derived from in vitro stock cultures. The explants used were internode segments approximately 1 cm in length.

2.2. Methods

2.2.1. Evaluation of Media Type on Callus Induction

This study was conducted experimentally using a Completely Randomized Design (CRD). The treatments consisted of the use of different media types supplemented with plant growth regulators (PGRs). The experiment included four treatments (MS0, DKW0, MS + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3, and DKW + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3) with five replicates. The MS medium was prepared at 4.43 g/L, and the DKW medium at 5.32 g/L. The culture media contained 3% sucrose and 3 g of Gelrite. The pH was adjusted to 5.79–5.81, and the media was sterilized at 121°C for 20 minutes [24].

2.2.2. Callus Induction

The internodes were wounded and induced in a Petri dish with 30 mL of induction medium, then incubated in dark conditions at 25±2°C for 4 weeks [25]. Each Petri dish contained eight explant segments.

2.2.3. Effect of Media Type on Callus Induction

After four weeks, callus development was evaluated based on callus induction percentage (%), initiation time (days), fresh weight (g), dry weight (%), as well as callus texture and color. Callus texture was categorized as friable, compact, or intermediate. Callus color was determined using the RHS (Royal Horticultural Society) Color Chart, Sixth Edition, 2015 [26].

2.2.4. Callus Multiplication

Callus from the medium producing friable callus was initially subcultured onto a semi-solid medium containing the same treatment and PGR for two weeks. Subsequently, 1.5 to 2 grams of callus were transferred to 50 mL of a liquid medium. Subculturing was performed three times at 10-day intervals. The cultures were incubated on a rotary shaker at 100 rpm under dark conditions at 25 ± 2°C [27].

2.2.5. Evaluation of the Effect of Elicitation

Callus (2 g) was inoculated into 30 mL of elicitation medium with different treatments, including control, SA (0.25–0.5 mg/L), GA_3 (2–4 mg/L), and daminozide (10–20 mg/L). The cultures were incubated on a rotary shaker at 100 rpm in dark condition at 25±2°C for 6 days [27].

2.2.6. Callus Extraction and Analysis of Steviol Glycosides Content

The callus was dried in an oven at 40°C for 24 hours. For each treatment, 100 mg of dry callus powder was extracted with 5 mL of 60% ethanol. The solution was sonicated for 15 minutes at 40°C and then filtered using filter paper. The resulting extraction residue was re-extracted using 5 mL of 60% ethanol. This step was repeated three times. The resulting filtrate was adjusted to 20 mL using the same solvent and filtered using a 0.45 µm syringe filter [28]. The extract was then used for HPLC analysis of stevioside, rebaudioside A, rebaudioside D, and Dulcoside A.

HPLC analysis was conducted by injecting 20 µL of callus extract into the HPLC system. The stationary phase consisted of a Eurosphere C-18 column (250 × 4.6 mm, 5 µm). The mobile phase comprised water and methanol in a ratio of 90:10 (v/v), with the pH adjusted to 3 using phosphoric acid. Additionally, acetonitrile and trifluoroacetic acid (TFA) were included in the mobile phase in a ratio of 65:35:0.01 (v/v/v). The mobile phase was homogenized by sonication for 30 minutes. The column temperature was maintained at 30°C, and the mobile phase flow rate was 0.3 mL/min. Detection was performed using a UV detector at 210 nm [28].

2.2.7. Callus Extraction and Analysis of Total Phenolic Content

For each treatment, 200 mg of dry callus powder was extracted with 10 mL of 70% ethanol. The solution was sonicated at 25°C for 60 minutes and then centrifuged at 5600 rpm for 10 minutes. The supernatant was stored at -20°C [29]. The standards used were gallic acid solutions (0, 20, 40, 60, 80 mg/L). A mixture of 0.1 mL of sample extract, 1.5 mL of aquabides, and 0.1 mL of Folin-Ciocalteu was homogenized using a vortex and incubated for 8 minutes. Next, 0.3 mL of 20% sodium carbonate was added, and homogenized again using a vortex. The solution was then incubated for 2 hours in a dark room. Absorbance was measured using a UV-Vis spectrophotometer at 765 nm. Total phenolic content was quantified as Gallic Acid Equivalent (GAE) (mg/g dry weight) [30].

2.2.8. Data Analysis

Quantitative data, including callus formation time, callus formation percentage, fresh weight, dry weight, and callus area percentage, were statistically analyzed using Analysis of Variance (ANOVA). If the results were significantly different, Duncan's New Multiple Range Test (DNMRT) at the 5% level ($p \leq 0.05$) was performed. Meanwhile, qualitative parameters (callus texture and color) were analyzed descriptively [31].

3. Results and Discussion

2.3. Effect of Media Type on Callus Induction

MS and DKW media with PGR increased the percentage of callus formation to 100% and accelerated callus formation time (6.33 and 6.40 days, respectively) compared to treatments without PGRs. No significant differences were observed between MS and DKW basal media, either in the control treatment or in media supplemented with PGRs, for these two parameters (Table 1). Callus formation in treatments without PGRs occurred only as a response to wounding, characterized by protrusions at the wound site and slow proliferation activity. Conversely, explants cultured on media supplemented with PGRs exhibited faster proliferation activity [32,33]. This indicates that PGR supports callus cell development activity.

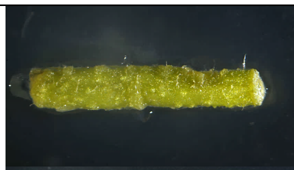
Table 1. Percentage of callus induction (%) and initiation time (days) in stevia with different media treatments

Media type	Percentage of callus induction (%)	Initiation time (day)
A. MS0	77.8	19.82 ± 0.95 b
B. DKW0	80.2	20.18 ± 2.24 b
C. MS + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	100	6.33 ± 0.28 a
D. DKW + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	100	6.40 ± 0.24 a

Note: Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$)

The medium supplemented with auxin and cytokinin acts synergistically to regulate cell division, proliferation, and callus growth [34]. Explants initiated in auxin-containing medium increased dedifferentiation and proliferation in xylem pore periclym cells. Auxin activates Lateral Organ Boundaries Domain (LBD) and Wuschel-Related Homeobox (WOX) proteins in the auxin response pathway (ARF), thereby promoting cell proliferation and cell wall relaxation [35]. Cytokinin enhances auxin activity by regulating gene expression in the ARF pathway and upregulating auxin-responsive genes, including LBD genes, which are crucial for callus formation [36]. Zayova et al. [14] also reported that MS medium enriched with 2 mg/L 2,4-D, 0.5 mg/L BAP, and 0.5 mg/L GA_3 showed the highest callus formation percentage (80%) in stevia internode explants, compared to other PGR combinations or treatments without PGR.

Table 2. Color and texture of callus with different types of media treatment

Treatment	Callus texture	Callus color	Fig.
A. MS0	Compact	Strong yellow green (N144C)	
B. DKW0	Compact	Strong yellowish green (N144A)	
C. MS + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	Friable	Brilliant yellow green (150C)	
D. DKW + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	Compact	Brilliant yellow green (154C)	

The type of medium and the addition of PGR showed different effects on callus color and texture in stevia. In MS medium with PGR addition, the resulting callus had a friable texture. In contrast, callus formed in both MS medium without PGR and DKW medium, with or without PGR, exhibited a compact texture (Table 2). The friable callus formed in MS medium with PGR addition indicates that the callus cell walls have not undergone lignification [37]. Meanwhile, the compact callus formed in DKW medium with PGR addition indicates a denser and harder cell structure due to lignification. The callus color in all treatments falls within the yellow-green color group. The callus color in all treatments indicates that the callus has entered the final phase of cell division [38].

Variations in callus texture and color are influenced by several factors, particularly the composition of the basal medium. The DKW medium has higher levels of calcium (Ca) and zinc (Zn) than MS medium [39]. Calcium contributes significantly in enhancing cell turgidity, thereby strengthening cell walls and preserving membrane stability in plant cells. It also acts synergistically with boron, which enhances calcium metabolism in cell walls and contributes to cell wall lignification. Low calcium content has been shown to weaken cell walls. Furthermore, zinc in the medium also functions in strengthening callus cell walls [40].

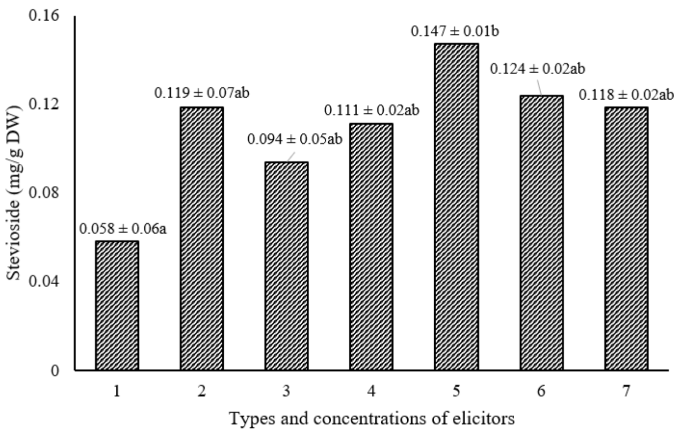
Ghannad et al. [12] reported that pistachio (*Pistacia vera* L.) explants induced on DKW medium produced compact callus. Meanwhile, explants initiated on MS medium produced friable callus. Similar results were reported by Sidorov et al. [41], who showed that the induction of quinoa (*Chenopodium quinoa*) callus induced on DKW also exhibited a compact texture.

Table 3. Fresh and dry weight of callus with different types of media treatment

Media type	Fresh weight (g)	Dry weight (g)
A. MS0	0.065 ± 0.00 a	0.005 ± 0.00 ab
B. DKW0	0.055 ± 0.01 a	0.003 ± 0.00 a
C. MS + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	0.116 ± 0.03 b	0.007 ± 0.00 ab
D. DKW + 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3	0.158 ± 0.06 b	0.011 ± 0.00 b

Note: Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$)

The highest callus fresh and dry weights were obtained on DKW medium supplemented with 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3, reaching 0.158 g and 0.011 g, respectively (Table 3). DKW medium contains lower ammonium levels and higher concentrations of macronutrients, such as phosphate, sulfate, calcium, and magnesium, along with micronutrients including iron, manganese, molybdenum, zinc, and copper, compared to MS medium. This result suggests that DKW medium offers better nutrient availability, supporting plant growth and increasing biomass production [42]. These results align with Page et al. [11], who reported that *Cannabis sativa* callus induced on DKW medium resulted in significantly higher callus weight compared to MS medium. Furthermore, Rudiyanto et al. [43] found that *Moringa oleifera* shoot cultures grown on DKW medium exhibited the highest fresh and dry weights compared to those grown on MS, WPM, NN, and B5 media.



Description:
1. Control
2. Salicylic acid 0.25 mg/L
3. Salicylic acid 0.5 mg/L
4. GA_3 2 mg/L
5. GA_3 4 mg/L
6. Daminozide 10 mg/L
7. Daminozide 20 mg/L

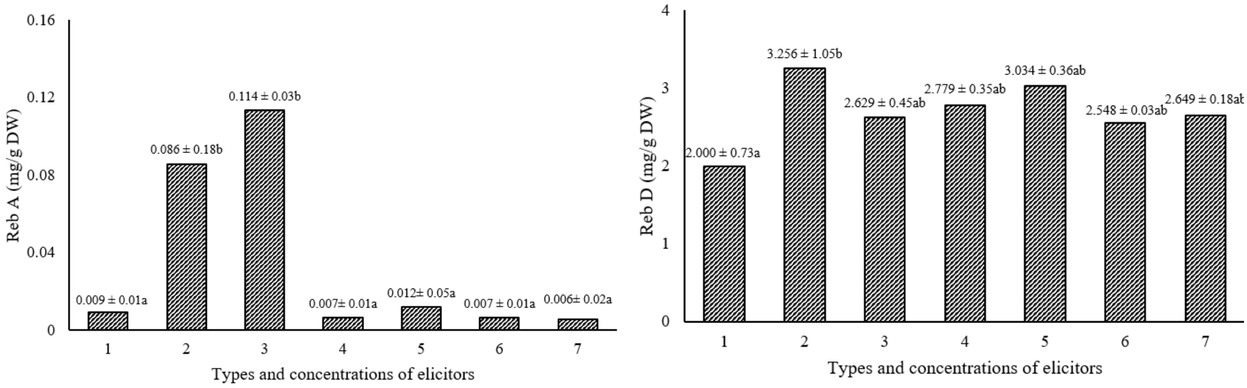
Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$).

Fig. 1. The effect of elicitation treatment on stevioside content in stevia suspension cultures.

2.4. Effect of Elicitation on Secondary Metabolites Production

2.4.1. Steviol Glycosides Content

Differences in elicitor type and concentration result in varying levels of stevioside accumulation (Fig. 1). The stevioside content in suspension cultures treated with elicitors was significantly higher than that in control. Specifically, GA_3 treatment at 4 mg/L produced the greatest accumulation of stevioside, reaching 0.147 mg/g DW. Yoneda et al. [21] reported similar findings, showing that GA_3 treatment increased stevioside content. This increase can be attributed to the enhanced regulation of the UGT74G1 and UGT85C2 genes. These genes encode enzymes required for steviolbioside glycosylation and the conversion of steviol to steviolmonoside,, respectively. GA_3 and steviol glycosides share the same biosynthetic pathways, using kaurenoic acid as a precursor [21].



(a) **(b)**

Description:
1. Control
2. Salicylic acid 0.25 mg/L
3. Salicylic acid 0.5 mg/L
4. GA_3 2 mg/L
5. GA_3 4 mg/L
6. Daminozide 10 mg/L
7. Daminozide 20 mg/L

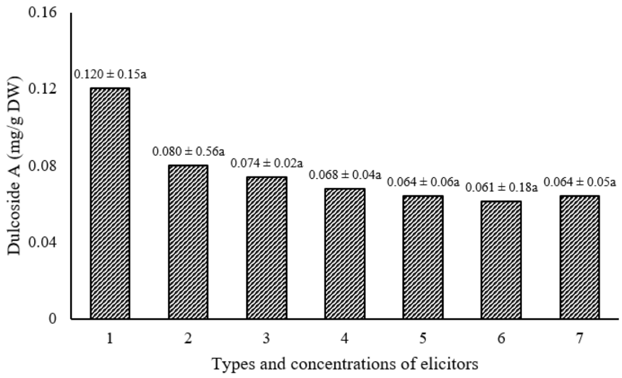
Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$).

Fig. 2. The effect of elicitation treatment on rebaudioside content in stevia suspension cultures; (a) Reb A and (b) Reb D.

Differences in elicitor type and concentration have different effects on the accumulation of reb A and reb D compounds (Fig. 2). Stevia suspension cultures treated with 0.5 mg/L SA showed significantly higher accumulation of reb A compounds than the control, reaching 0.114 mg/g DW. Furthermore, HPLC analysis of stevia suspension cultures showed that reb D exhibited the highest accumulation among the steviol glycosides tested, ranging from 2.000 to 3.256 mg/g DW. Elicitor treatment resulted in a significant increase in rebaudioside D accumulation compared to the control, with the highest level being recorded in cultures treated with 0.25 mg/L SA.

SA at 0.5 mg/L increased rebaudioside A accumulation, whereas 0.25 mg/L SA increased rebaudioside D accumulation in stevia suspension cultures. SA treatment at optimal concentrations upregulates UGT76G1, encoding an enzyme responsible for the glycosylation of stevioside to rebaudioside A, thereby enhancing biosynthesis of the target compound [44]. According to Zhou et al. [45], UGT76G1 gene plays a role in the glycosylation of reb A and in many other steviol glycoside biosynthesis processes, including reb D formation. The UGT76G1 gene is involved in the glycosylation of reb E into reb D. This finding aligns with Golkar et al. [17], who reported that treatment with 0.25 mg/L SA for 6 days in stevia callus cultures increased reb A content to 5.73 mg/g DW.

The application of various types and concentrations of elicitors to stevia suspension cultures did not produce significant differences in dulcoside A content compared with the control (Fig. 3). The use of elicitors reduced dulcoside A content compared to the control, indicating that the elicitation treatments were ineffective in promoting the accumulation of dulcoside A in stevia suspension cultures. Moreover, variations in elicitor treatments resulted in different levels of steviol glycosides, indicating that elicitors specifically influence the production of these compounds. Alcalde et al. [46] reported similar findings, indicating that elicitors are essential for initiating and enhancing the biosynthesis of certain compounds. Additionally, elicitors activate specific transcription factors involved in plant metabolic pathways [47].



Description:
1. Control
2. Salicylic acid 0.25 mg/L
3. Salicylic acid 0.5 mg/L
4. GA_3 2 mg/L
5. GA_3 4 mg/L
6. Daminozide 10 mg/L
7. Daminozide 20 mg/L

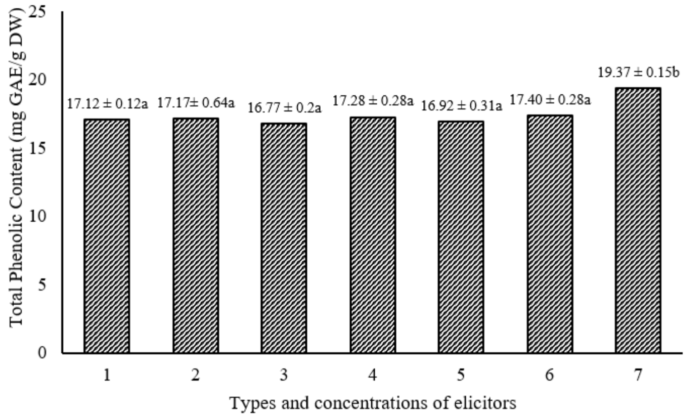
Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$).

Fig. 3. The effect of elicitation treatment on dulcoside A content in stevia suspension cultures.

The low steviol glycosides observed in this study is likely influenced by the type of explant used for callus induction. Steviol glycosides are typically abundant in stevia leaves, with concentration ranging from 7% to 15% [48]. In contrast, steviol glycosides content in stems is 12 to 13 times lower than that in leaves [49]. Similar findings were reported by Blinstrubienė et al. [29], who showed that the accumulation of stevioside and reb A in callus derived from leaf explants was significantly higher than in callus derived from stem explants.

2.4.2. Total Phenolics Content

Treatment with 20 mg/L daminozide had a significant effect compared to other treatments on the total phenolic content in stevia suspension cultures (Fig. 4). Application of 20 mg/L daminozide produced the highest total phenolic content (19.37 mg/GAEg DW). Meanwhile, treatments with SA, GA_3, and daminozide at a concentration of 10 mg/L did not differ significantly from the control. Phenolic compounds can act as antioxidants that neutralize free radicals in plants [50]. Their accumulation increases as a defense mechanism against oxidative stress triggered by increased free radical production under stressful environmental conditions [51]. The elevated total phenolic content observed in callus cells treated with 20 mg/L daminozide is considered a physiological response of suspension cultures to compound-induced stress.



Description:
1. Control
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3. Salicylic acid 0.5 mg/L
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6. Daminozide 10 mg/L
7. Daminozide 20 mg/L

Means followed by the same letter were not significantly different from each other (DNMRT, $p \leq 0.05$).

Fig. 4. The effect of elicitation treatment on total phenolic content in stevia suspension cultures.

According to Karimi et al. [52], various types of growth retardants can improve plant tolerance to abiotic stress. However, the use of daminozide in combination with auxin may induce physiological stress in plants. Additionally, because daminozide inhibits the gibberellin biosynthesis pathway, it may activate secondary biosynthesis pathways, including phenolic compound biosynthesis [53]. The stress induced may also be associated with the high concentration of daminozide applied to the medium, as high concentrations of plant hormones inhibits plant metabolism [54]. According to Srinivasan et al. [55], daminozide treatments at 1000, 2000, and 3000 mg/L in field-grown *Celosia* spp. significantly increased higher total phenolic content to 11.50, 17.00, and 20.00 mg/g, respectively, compared with the control (1.05 mg/g).

The present study demonstrates the novelty of integrating DKW medium optimization with elicitation strategies to enhance both callus growth and steviol glycoside accumulation in *Stevia rebaudiana* callus culture. These findings contribute to the advancement of plant tissue culture and metabolic engineering approaches for the sustainable production of natural sweeteners and bioactive compounds. In addition, the optimized protocol shows strong potential for industrial-scale applications through bioreactor-based production systems, supporting the growing demand for low-calorie natural sweeteners in the food and pharmaceutical industries.

4. Conclusions

In conclusion, MS and DKW media supplemented with 2 mg/L 2,4-D + 0.5 mg/L BAP + 0.5 mg/L GA_3 were the most effective treatments for promoting callus induction in *Stevia rebaudiana* callus culture, as indicated by 100% callus induction and the fastest callus initiation time (6.33–6.40 days). Meanwhile, DKW medium supplemented with the same combination of plant growth regulators produced the highest fresh and dry biomass (0.158 g and 0.011 g, respectively), demonstrating its effectiveness in supporting callus growth. Elicitation treatments also significantly influenced steviol glycoside accumulation. Treatment with 4 mg/L GA_3 resulted in the highest stevioside content (0.147 mg/g DW), while 0.5 mg/L SA enhanced rebaudioside A accumulation (0.114 mg/g DW), and 0.25 mg/L SA produced the highest rebaudioside D content (3.256 mg/g DW). In addition, the highest total phenolic content (19.37 mg GAE/g DW) was observed in cultures treated with 20 mg/L daminozide. These findings demonstrate the potential of optimized culture media and elicitation strategies for improving the in vitro production of steviol glycosides and phenolic compounds as valuable natural sweeteners and bioactive compounds. Furthermore, this study provides practical implications for the stevia industry by supporting the development of sustainable and controlled production systems for high-value steviol glycosides independent of environmental and seasonal limitations. The optimized callus culture system also shows strong potential for future large-scale production through bioreactor-based culture systems, which may further enhance biomass productivity and metabolite accumulation for commercial applications.

Abbreviations

MS	*Murashige and Skoog*
DKW	*Driver and Kuniyuki Walnut*
BAP	*benzylaminopurine*
GA_3	*gibberellin*
PGR	*plant growth regulators*
SA	Salicylic acid
2,4-D	*Dichlorophenoxyacetic acid*

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Zozy Aneloi Noli: Validated the data, finalized the manuscript draft, and performed review and editing. **Betalini Widhi Hapsari**: Designed the experimental treatments and conceptual framework, validated the data, and acquired funding. **Rezka Sutrianti**: Conducted the experiments, including treatment setup, observations, data compilation, and data analysis; wrote the manuscript. **Erwin Al Hafiidz**: Provided resources and maintained the plant culture materials used in the study. **Deritha Ellfy Rantau**: Prepared the treatment media. **Evan Maulana**: Performed sample extraction.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Declaration of Use of AI in the Writing Process

The authors used chatgpt during the preparation of this manuscript to improve grammar. After using the tool/service, the authors carefully reviewed and edited the content and take full responsibility for the content of the publication.

Acknowledgement

Authors would like to acknowledge the funding support provided by the "Rumah Program" 3 Theme 2 Research Organization Life Sciences and Environment BRIN and also PT. Tapanuli Investasi Agro which has supported the in initial plant materials

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