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Steviol Glycoside Production and Growth in *Stevia rebaudiana* Shoot Culture after Methyl Jasmonate Elicitation

(Penghasilan Steviol Glikosida dan Pertumbuhan Kultur Pucuk *Stevia rebaudiana* selepas Elisitasi Metil Jasmonat)

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ABSTRACT

Steviol glycosides are a group of compounds derived from the *Stevia rebaudiana* metabolites which are commonly used as sugar replacements. Methyl jasmonate (MeJA) elicitation is one strategy to induce high steviol glycoside content. MeJA can enhance the synthesis of secondary metabolites, however, it can also negatively impact the growth of *Stevia*. The objective of this study was to establish the concentration and exposure duration of MeJA elicitation to obtain the highest level of steviol glycoside production while achieving the best growth for *Stevia* shoot cultures. The shoot cultures were elicited using MeJA at concentrations of 10 µM, 50 µM, and 100 µM for 24, 48, and 72 h. The cultures were then transferred into the medium without MeJA for 7 days, and then growth characteristics were measured. Analysis of stevioside and rebaudioside A levels was performed using HPLC from samples harvested directly after treatment and 7 days post-elicitation. MeJA was found to suppress growth in terms of height, adventitious shoot formation, and leaf size across all concentrations. However, the number of leaves, fresh weight, number of axillary buds, and number of nodes showed no significant difference. The optimal condition for stevioside synthesis was achieved at 50 µM MeJA for 48 h (0.73 mg/g), while the optimal condition for rebaudioside A synthesis was achieved with 100 µM MeJA 24 h treatment (0.63 mg/g). Analysis of the plant growth and secondary metabolite production suggested that the treatment using 50 µM MeJA for 48 h was found to be the best conditions which gave high secondary metabolite production while plant growth was also high. This finding can serve as a fundamental approach for enhancing the production of steviol glycosides in the industry.

Keywords: Growth; *in vitro* culture; rebaudioside A; *Stevia*; stevioside

ABSTRAK

Steviol glikosida adalah sekumpulan sebatian terbitan daripada metabolit *Stevia rebaudiana* yang biasa digunakan sebagai pengganti gula. Elisitasi menggunakan metil jasmonat (MeJA) merupakan satu strategi yang boleh dilakukan untuk mengaruh kandungan steviol glikosida yang tinggi. MeJA boleh meningkatkan sintesis metabolit sekunder, namun begitu, ia juga boleh menjejaskan pertumbuhan *Stevia*. Objektif kajian ini adalah untuk menentukan kepekatan dan tempoh pendedahan elisitasi MeJA untuk memperoleh tahap penghasilan steviol glikosida paling tinggi di samping mencapai pertumbuhan terbaik bagi kultur pucuk *Stevia*. Kultur pucuk telah dielisitasi menggunakan MeJA pada kepekatan 10 µM, 50 µM dan 100 µM selama 24, 48 dan 72 jam. Kultur kemudiannya dipindahkan ke dalam medium tanpa MeJA selama 7 hari, seterusnya ciri pertumbuhan telah diukur. Analisis tahap steviosida dan rebaudiosida A dilakukan menggunakan HPLC daripada sampel yang diambil sejurus selepas rawatan MeJA dan 7 hari selepas elisitasi. MeJA didapati merencat pertumbuhan dari segi ketinggian, pembentukan pucuk adventitius dan saiz daun pada semua kepekatan. Walau bagaimanapun, bilangan daun, berat segar, bilangan tunas aksilari dan bilangan nod tidak menunjukkan perbezaan signifikan. Keadaan yang optimum bagi sintesis steviosida dicapai pada 50 µM MeJA selama 48 jam (0.73 mg/g), manakala keadaan yang optimum bagi sintesis rebaudiosida A pula dicapai dengan rawatan 100 µM MeJA selama 24 jam (0.63 mg/g). Analisis pertumbuhan tumbuhan dan penghasilan metabolit sekunder mencadangkan bahawa rawatan menggunakan 50 µM MeJA selama 48 jam didapati merupakan keadaan terbaik yang memberikan penghasilan metabolit sekunder yang tinggi di samping pertumbuhan tumbuhan yang juga tinggi. Penemuan ini boleh bertindak sebagai pendekatan asas dalam meningkatkan penghasilan steviol glikosida dalam industri.

Kata kunci: Kultur *in vitro*; pertumbuhan; rebaudiosida A; *Stevia;* steviosida

INTRODUCTION

Stevia sweetener has been utilised as a natural sweetener for years. Its sweetness comes from steviol glycosides, which are 250-300 times sweeter than sucrose (Peteliuk et al. 2021). These compounds possess a sweet taste, are non-toxic and non-mutagenic. Steviol glycosides are produced in the leaves of *Stevia rebaudiana*, a perennial plant cultivated in South America, particularly in Brazil and Paraguay, and sometimes referred to as 'Sweet Leaf', 'Sweet Herb', or 'Honey leaf'. *Stevia* is being grown and utilised in the culinary and pharmaceutical sectors in several nations, including Brazil, Central America, Uruguay, Thailand, China, Japan, Korea, and Australia (Jan et al. 2021).

The *Stevia* leaf extracts contain steviol glycosides such as stevioside, rebaudioside, steviolbioside, dulcoside, and steviol (Ahmad et al. 2020). Leaf extracts also contain austroinullin, thiamine, niacin, alkaloids, tannin, flavonoids, saponin, and quinone. Past research demonstrated the significant potential of *Stevia* leaves as a sweetener for expanding natural food market industries in the future (Peteliuk et al. 2021). Several studies also demonstrated that *Stevia* possesses antimicrobial and antioxidant properties, and may be beneficial in treating conditions including diabetes, obesity, tumours, and hypertension (Jan et al. 2021; Momtazi-Borojeni et al. 2017; Ruiz-Ruiz et al. 2015).

Several studies, including far-red LED treatment, polyploidy, and chitosan nanoparticles have been conducted on improving the production of stevioside and rebaudioside A, but the outcomes have not met expectations (Khalil, Zamir & Ahmad 2014; Pandey & Chikara 2014; Rameshsing, Hegde & Vasundhara 2015; Yoneda et al. 2017). One strategy that can be used to achieve high yields of steviol glycoside is elicitation. Elicitation induces stress in plants by introducing chemical compounds (elicitors) or modifying the environment, which triggers physiological changes such as the production of secondary metabolites as a defence mechanism. Elicitors can enhance secondary metabolite biosynthesis in various plant tissue cultures within a brief timeframe. Jasmonate is a potent elicitor commonly employed to induce the production of secondary metabolites (Sohn et al. 2022). Methyl jasmonate (MeJA) is a kind of jasmonate that regulates signal transduction to initiate plant growth and development. MeJA is one of the important phytohormones in plants involved in growth and development, secondary metabolic regulation, pollen germination, senescence, and defence in plants, both for biotic and abiotic stresses (Faizal & Sari 2019; Li et al. 2018). Plants also naturally synthesise MeJA under stress conditions in response to insect bites or environmental stress (Singh & Dwivedi 2018).

Research conducted by Faizal and Sari (2019) demonstrated that MeJA led to an increase in the concentration of saponin accumulation in adventitious root culture of *Talinum paniculatum*. MeJA treatment in *Aquilaria malaccensis* stimulated the production of several compounds in agarwood, including the sesquiterpenes; however, a long duration treatment (3 weeks) of MeJA at 50-150 µM caused necrosis in the *in vitro* shoot culture (Faizal et al. 2021). Application of MeJA to *in vitro* micropropagated *S. rebaudiana* culture was reported to enhance stevioside production, but prolonged exposure (4 weeks) at 50-200 µM had a detrimental effect on shoot growth (Bayraktar et al. 2016). Shorter exposure periods (2 weeks) of 50-200 µM MeJA in *Stevia* culture increased stevioside production, although it led to the formation of necrotic areas in leaves (Bayraktar et al. 2018). The shorter exposure period was predicted as the conditions that could induce the highest steviol glycoside level with the optimal shoot growth. The optimal shoot growth, particularly in leaves, will yield greater biomass, thus, increasing stevioside production for industry. Hence, the objective of this study was to establish the optimal concentration and duration of MeJA exposure to achieve the highest steviol glycoside production while attaining high growth of *S. rebaudiana* shoot cultures.

MATERIALS AND METHODS

SOURCE OF EXPLANTS

S. rebaudiana was acquired from the Indonesian Centre for Biotechnology and Bioindustry Research, located in Bogor, West Java. Apical shoots with three nodes from 4-week-old *S. rebaudiana* of approximately 30-50 cm tall were utilised as explants (Rahmawati et al. 2023). The explants were cultivated in the Tissue Culture Laboratory, Bandung Institute of Technology, Ganesha Campus.

INITIATION AND MULTIPLICATION

The initiation and multiplication steps followed the techniques from Rahmawati et al. (2023). The *Stevia* shoots were cleaned, submerged in 0.5% Antracol™ fungicide for 10 min, and disinfected using 70% alcohol, followed by treatment with 0.27% (v/v) NaClO supplemented with 2 drops of Tween 20 for 5 min. Afterwards, the explants were washed three times with sterile distilled water and blot dried.

The explants (0.5 g) were grown in semi-solid Murashige-Skoog (MS) with 30 g/L sucrose and 8 g/L agar (Murashige & Skoog 1962). Additionally, the medium was supplemented with 1.13 mg/L of benzylaminopurine (BAP) and 0.35 mg/L of indole-3-acetic acid (IAA) (Rahmawati et al. 2023). The pH of the medium was set at 5.6 to 5.8. The cultures were incubated at ambient temperature with a 12-h light and 12-h dark cycle with a light intensity of 1000 lux using 36-watt TLD lamps. The subculture was performed every 4 weeks using a multiplication medium. The multiplication medium consisted of 30 g/L sucrose, 8 g/L agar, and was enriched with 1 mg/L kinetin (Rahmawati et al. 2023). The newly regenerated shoots were isolated and transplanted into fresh media during the subculture procedure.

ACCLIMATISATION IN LIQUID MEDIUM

The explants of shoots employed for elicitation were 1.5-month-old explants. The liquid medium comprised a solution of half-strength MS medium, 30 g/L of sucrose, and 1 mg/L of kinetin. The acidity level of the medium was modified to a pH range of 5.6 to 5.8. The shoots were cut to obtain homogeneous height shoots. The cultures were acclimatised in a liquid medium for 5 days and agitated using a shaker at 40 rpm with 12 h photoperiods and a temperature range of 20 to 25 °C.

ELICITATION USING METHYL JASMONATE

The elicitation treatment was conducted in *in vitro* shoot, cultured using 10 mL of MS liquid medium in a culture tube with 1 mg/L kinetin and various concentrations of MeJA (10, 50, and 100 µM). MeJA was initially combined with alcohol in an equal volume to facilitate dissolution, after which water was added to achieve the necessary volume. Therefore, medium without MeJA and alcohol served as controls, and medium without MeJA but added with ethanol was designated as control + ethanol. The acidity level of the medium was modified to a range between 5.6 and 5.8 on the pH scale. The cultures were elicited for 24, 48, and 72 h (Lucho et al. 2018). The cultures were agitated using a shaker at 40 rpm with 12 h photoperiods and a temperature range of 20 to 25 °C.

Following the elicitation, the cultures were transferred to a MeJA-free liquid medium and allowed to grow for 7 days. The cultures were then harvested for analysis of the growth parameters and levels of stevioside and rebaudioside A. Furthermore, analysis of stevioside and rebaudioside A levels was conducted on the culture directly following MeJA elicitation.

ANALYSIS OF GROWTH

The growth parameters examined included leaf number, leaf size, height, node number, axillary bud number, adventitious bud number, and fresh weight. Measurements were conducted before elicitation and on the 7th day after elicitation. The value of the measurement on the 7th day was subtracted from the measurement before elicitation. Each treatment comprised 3 repetitions. Leaf size was measured using ImageJ software.

ANALYSIS OF STEVIOSIDE AND REBAUDIOSIDE A CONTENTS

The extraction methods followed the method described by Kolb et al. (2001). Oven-dried *Stevia* leaves (100 mg) were chopped and extracted using 10 mL of 70% ethanol. The mixture was then incubated in a 70 °C water bath for 30 min. The extract was filtered, dried, and dissolved in 1.5 mL of methanol before being analysed with HPLC.

The concentration of stevioside and rebaudioside A in the extract was determined using Shimadzu's High-Performance Liquid Chromatography (HPLC). A total of 20 µL of extract was injected into the HPLC. The mobile phase used was a mixture of methanol and water at a ratio of 8:2 (v/v). The UV detector operated at a wavelength of 210 nm, and the elution rate was set at 0.5 mL/min. The stationary phase employed was a C-18 column (250 × 4.6 mm; 5 µm) equipped with a protecting column. The injection volume was 1 µL using Hamilton Syringe Model 702 N SYR injectors.

The quantification was conducted by calibrating with standard solutions of stevioside and rebaudioside A. The stevioside compounds, with a purity of 95%, and rebaudioside A, with a purity of 99%, were obtained from Sigma Aldrich, USA. The standard curves for stevioside and rebaudioside A were constructed by plotting the concentration of the standard (mg) against the peak area (mili absorbance units per min).

STATISTICAL ANALYSIS

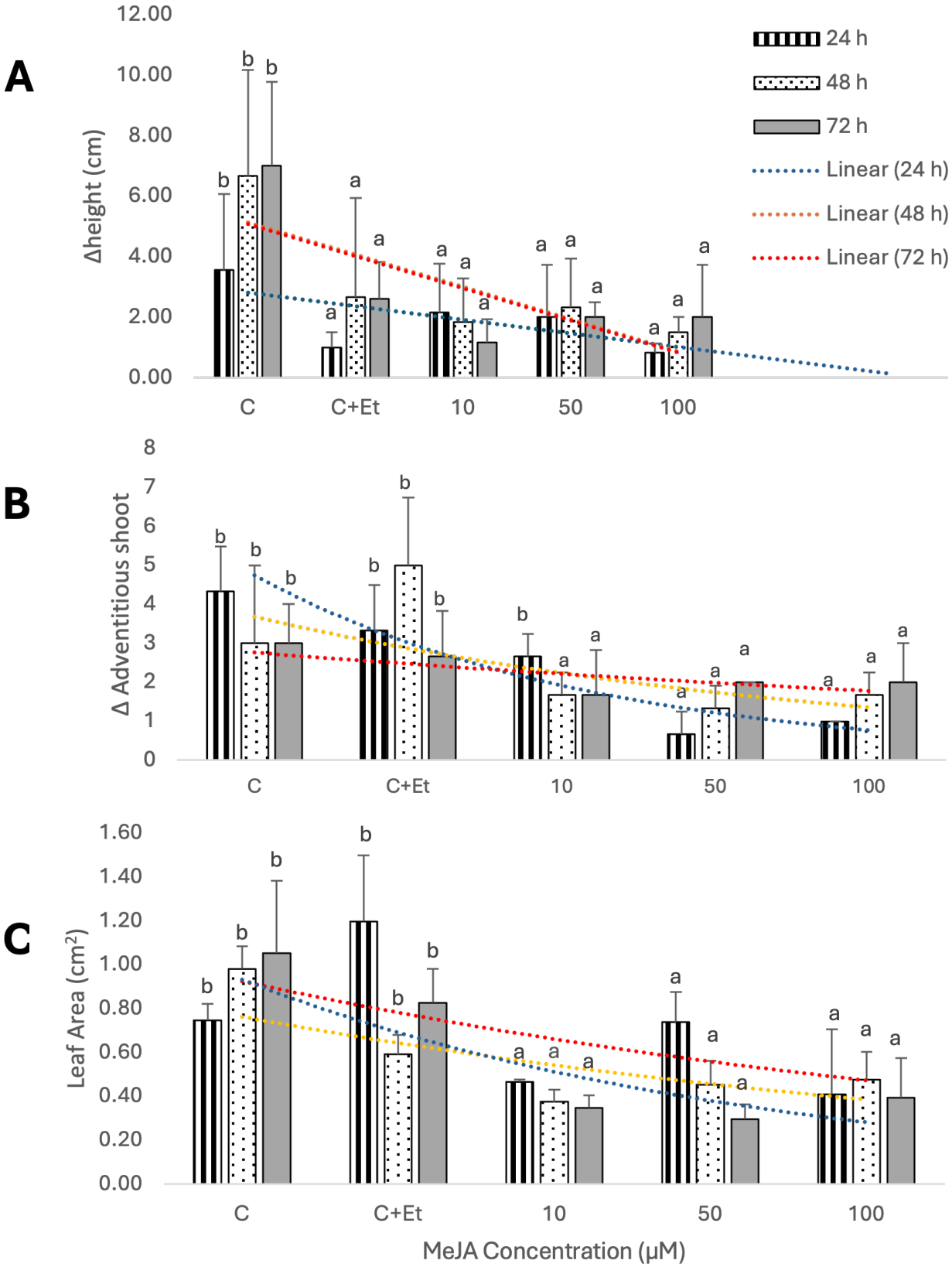
The data were obtained through the application of analysis of variance (ANOVA). The analysis proceeded to determine significant differences among treatments using the Duncan multirange range test (DMRT) with a confidence level of $\alpha < 0.05$. The statistical analysis was performed utilising IBM SPSS Statistics Version 26.

RESULTS AND DISCUSSION

MEJA ELICITATION ON *STEVIA* SHOOT CULTURE GROWTH

Exogenous MeJA can influence a great variety of morphological and physiological responses in *in vitro* culture. MeJA did not affect the growth of *Stevia's* shoot culture, except in height, leaf size, and number of adventitious buds (Figure 1). The presence of MeJA led to a notable reduction in plant height, regardless of the concentration and period of exposure (Figure 1(A)). All concentrations exhibited considerable inhibition of height compared to control, with the slowest height growth observed at 100 µM MeJA for 24 h. Other research showed that MeJA at 0.1-0.2 mg/L decreased shoot length of *Pistacia lentiscus* in shoot culture (Koc, Onay & Çiftçi 2014). The decrease in height had also been observed in soybean, tomato, and sunflower (Li 2018). The height of soybeans and tomatoes decreased at high concentrations (1 mM MeJA), while in sunflowers, the height decreased at low concentrations (0.1 mM) using hydroponic methods (Li 2018). Growth retardation might occur as a result of interference of the cell cycle in the meristem zone. The JA treatment induced cell cycle arrest at the G1 and G2 phases in By-2 tobacco cells (Świątek et al. 2004). Other research showed that MeJA inhibited M phase gene activation in Arabidopsis; thereby, cells were arrested at the G2 phase in the cell cycle (Pauwels et al. 2008).

High concentrations of MeJA inhibited adventitious shoot formation from shoot basal significantly in all MeJA



Means with the same letters are not significantly different at a 5% confidence level according to Duncan's test

FIGURE 1. MeJA suppressed growth in term of height (A), adventitious shoot (B), and (C) leaf area across all concentrations (C for Control and C+Et for Control and etanol)

treatments compared to the control, control with ethanol, and 10 µM MeJA for 24 h exposure (Figure 1(B)). Ethanol did not affect the growth of adventitious shoot formation. Research conducted by Moharramnejad et al. (2019) demonstrated that MeJA (50-200 µM) has a negative impact on the shoot and root development and growth of *Stevia* shoot culture in the Woody Plant Medium (WPM). In contrast to the findings of this investigation, Mahmood et al. (2012) demonstrated that the application of 40 µM of MeJA produced increments in adventitious shoot number for *Musa acuminata* cv. 'Berangan'. Both experiments showed that the plant response to the application of MeJA in terms of shoot development might be varied. It is probably caused by the concentration and composition of endogenous hormones present in plants (Kaminska 2021). Cytokinin plays a crucial part in the initiation, growth, and proliferation of shoots. At concentrations ranging from 0.1-1 µM, MeJA can elevate cytokine levels, hence promoting the growth and proliferation of shoots. However, at higher concentrations, MeJA can reduce the number of shoots (Kaminska 2021).

The leaf size in all treatments was significantly different from the control and control with ethanol (Figure 1(C)). It showed that MeJA alone affected the leaf size. The smallest leaf size was found at 50 µM MeJA for 72 h. Bayraktar et al. (2018) showed that the application of MeJA for 2 weeks decreased fresh weight, dry weight, and leaf length of *Stevia*. In tomato, soybean, and potato, exogenous application of MeJA did not affect the leaf size (Li et al. 2018; Rohmawati & Dewi 2019). The application of 50 µM MeJA in *A. thaliana* led to the inhibition of leaf growth due to a decrease in both the number and size of cells (Noir et al. 2013). The inhibition of leaf growth by MeJA application was suggested as a result of the suppression of GA-related pathways. GA regulates the leaf size by the modulation of cell division and increasing water absorption (Ritonga et al. 2023). Exogenous application of MeJA caused the breakdown of JASMONATE ZIM-domain (JAZ) and the release of DELLA repressors, which in turn decreased GA activity and inhibited leaf growth (Song et al. 2014). Several studies indicated that GA deficiencies led to a reduction in leaf size due to a reduction in cell length (Chen et al. 2020; Mariotti et al. 2022).

The number of leaves, fresh weight, axillary bud, and number of nodes showed no significant difference (Figure 2(A)-2(D)). The number of newly formed leaves remained consistent across different concentrations for both 48 h and 72 h treatments, with no significant difference observed between the control and treatment groups (Figure 2(A)). Treatment of 100 µM MeJA for 24 h produced the lowest number of new leaves. There was no statistically significant difference in fresh weight between control and treatment (Figure 2(B)). Similar results showed that MeJA did not affect the fresh and dry weights of *Ziziphora persica* organ culture (Zare-Hassani et al. 2019). However, MeJA (5-16 µM) caused a reduction in leaf length, root length, and total fresh weight in *Curcuma longa* micro rhizomes *in vitro* (Cousins & Adelberg 2008).

The number of new axillary buds was not significantly different, as depicted in Figure 2(C). Research in pear by Cheng et al. (2023) showed that the application of exogenous JA-MeJA to gamma-ray mutants did not significantly affect the number of axillary buds. However, when JA-MeJA was applied to the non-mutants, the number of axillary buds was decreased. Gene expression in the mutant was associated with the increase of PcCOI1 as a transcription factor that will suppress PcJAZ activation, the repressor of jasmonates. This suggested that the signalling pathway of jasmonate has a role in suppressing the formation of axillary buds. MeJA also did not affect the number of nodes (Figure 2(D)), which is contradictory to Bayraktar et al. (2018), where the treatment of 50 µM MeJA for 3 weeks decreased the number of nodes in *Stevia*.

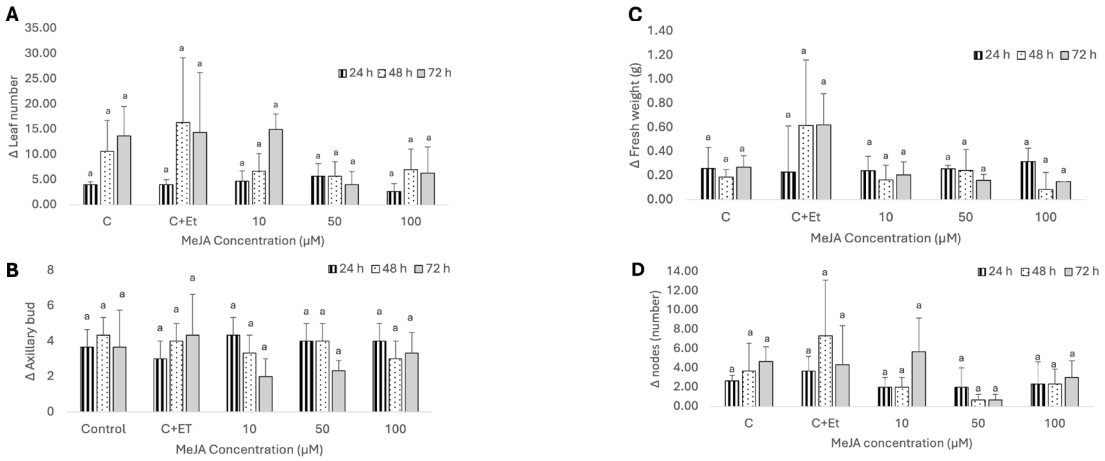


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FIGURE 2. The number of leaves (A), fresh weight (B), axillary buds (C), and number of nodes (D) showed no significant difference (C for Control and C + Et for Control and etanol)

Elicitation of MeJA induced yellowing and necrotic in leaves (Figure 3). In the 50 µM MeJA treatment, the leaves turned yellow after being exposed for 72 h (Figure 3(B)). In the 100 µM MeJA treatment, the leaves turned brown after being exposed for 72 h (Figure 3(C)). Bayraktar et al. (2018) also observed necrotic leaves in *Stevia* following the exposure to 200 µM MeJA for 2 weeks. The MeJA induced senescence of the leaves (Sohn et al. 2022) and also necrosis in the cell culture of *A. malaccensis* (Faizal et al. 2021).

The mechanism of MeJA inhibiting growth, particularly in terms of height, adventitious shoots, and leaf size, is not yet clear. The influence of MeJA on growth and development is a complex matter because it is related to physiological conditions and also the influence of other hormones. Several studies indicated that there are differences in plant response and sensitivity to MeJA, depending on the type of plant and the concentration of MeJA (Kaminska 2021). MeJA can disrupt the cell cycle process, resulting in the suppression of height (Świątek et al. 2002). MeJA was also linked to suppression of gibberellin activity, which resulted in the inhibition of leaf growth (Kazan & Manners 2012). Furthermore, MeJA can trigger senescence in plants, resulting in a reduction of growth, yellowing, and necrosis in the leaf (Kim, Chang & Tucker 2015).

THE IMPACT OF MEJA ELICITATION ON STEVIOSIDE AND REBAUDIOSIDE A PRODUCTION

The stevioside and rebaudioside A contents were measured both directly after elicitation and 7 days post-elicitation. The stevioside content increased in samples directly harvested after elicitation, particularly for 50 and 100 µM MeJA (Figure 4(A)). The highest increment was observed in the 100 µM treatment for 24 h (2.2 times compared to the control) and the 50 µM treatment for 48 h (1.9 times compared to the control). Rebaudioside A content also increased, particularly with 50 µM and 100 µM MeJA, as shown in Figure 5(A). However, rebaudioside A levels in 10 µM MeJA did not show significant differences from the control. Another study by Bayraktar et al. (2018) demonstrated that a longer treatment of MeJA elicitation (2 weeks) increased the stevioside production about 17.4-fold higher than control. A similar effect in *Stevia* using the hydroponic system also showed an increment of stevioside after 100 µM MeJA elicitation (Lucho et al. 2018).

Samples collected 7 days post-elicitation using 50 and 100 µM MeJA showed significant increase in stevioside compared to the control (Figure 4(B)). The high increment in stevioside levels was observed at 100 µM MeJA for 24 h (3.8 times higher than control) and 72 h (4.5 times higher than control). However, the rebaudioside A levels were not significantly different compared to the controls. It was thus suggested that rebaudioside A was produced spontaneously after elicitation (Figure 5B).

MeJA has already been widely utilised as an elicitor to enhance the production of numerous secondary metabolites. MeJA could enhance the stevioside content, both from samples that were harvested immediately after elicitation and 7 days later. According to Lucho et al. (2018), the application of MeJA to *Stevia* in a hydroponic system increased the expression of genes involved in the stevioside synthesis, including kaurene synthase, kaurene oxidase, and UGT76G1. The UGT76G1 enzyme catalyses the conversion of stevibioside into stevioside. It was also shown that MeJA did not have an impact on the UGT74G1 enzyme, which is responsible for converting stevioside into rebaudioside A (Lucho et al. 2018).

Exogenous application of MeJA had been shown to enhance the levels of phenolic compounds (Ahn et al. 2014), alkaloids (Sohn et al. 2022), terpenoids (Faizal & Sari 2019), and other compounds in several plant species. MeJA elicitation can enhance the activation of transcription factors that regulate gene expression by specifically binding to the target gene promoter, resulting in the accumulation of metabolites induced by JA. These transcription factors consist of various families, including AP2/ERF, bHLH, MYB, and WRKY (Sohn et al. 2022). MeJA elicitation has been shown to cause the upregulation of flavonoid biosynthesis genes in *Carthamus tincorius* (Chen et al. 2020) and rubber biosynthesis genes in *Hevea brasiliensis* (Liu et al. 2018).

COMPARISON OF GROWTH AND PRODUCTION OF SECONDARY METABOLITES IN MeJA TREATMENT

In general, almost all growth parameters did not exhibit a significant difference compared to the controls. However, treated samples showed a tendency to be lower than those of the controls. High concentrations of MeJA tended to inhibit the growth of *Stevia,* particularly in height, adventitious bud number, and leaf size. Growth is a factor related to high fresh weight, which in turn maximises the yield of secondary metabolites. Stevioside and rebaudioside A are produced in the leaves; thereby, leaf growth is necessary to produce high yields of these metabolites.

Table 1 and Figure 6 showed plant growth and secondary metabolite content after MeJA elicitation. Leaf growth occurs through the process of cell division and expansion, increasing leaf number and size. The treatments that resulted in high leaf growth were 10 µM MeJA for 72 h and 50 µM MeJA for 24 h (Table 1). Meanwhile, the treatments that yielded high fresh weight were 100 µM MeJA for 24 h and 50 µM MeJA for 48 h. The plant growth results showed that 50 µM MeJA for 48 h produced high leaf growth as well as high fresh weight.

Samples harvested directly after treatment produced the highest stevioside when treated with 50 µM MeJA for 48 h, while that for rebaudioside A was at 100 µM MeJA for 24 h (Figure 6). In samples harvested 7 days post elicitation, the highest stevioside content was produced in

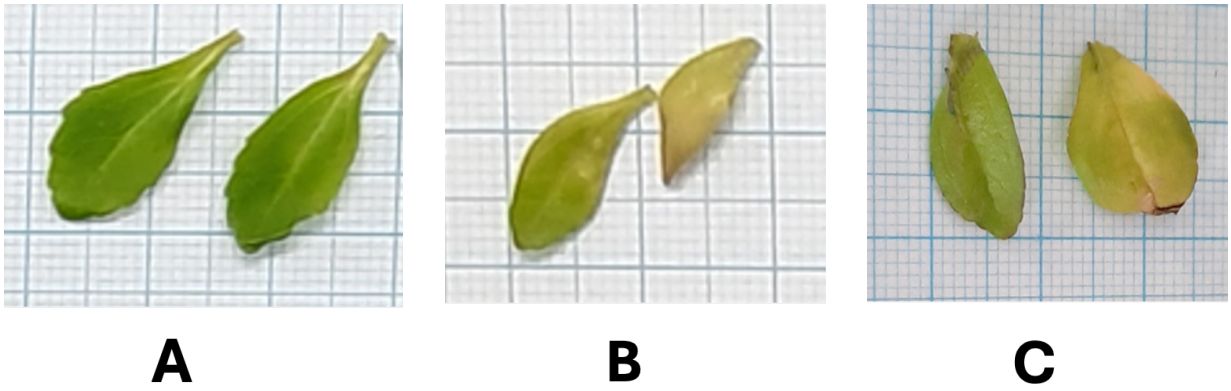
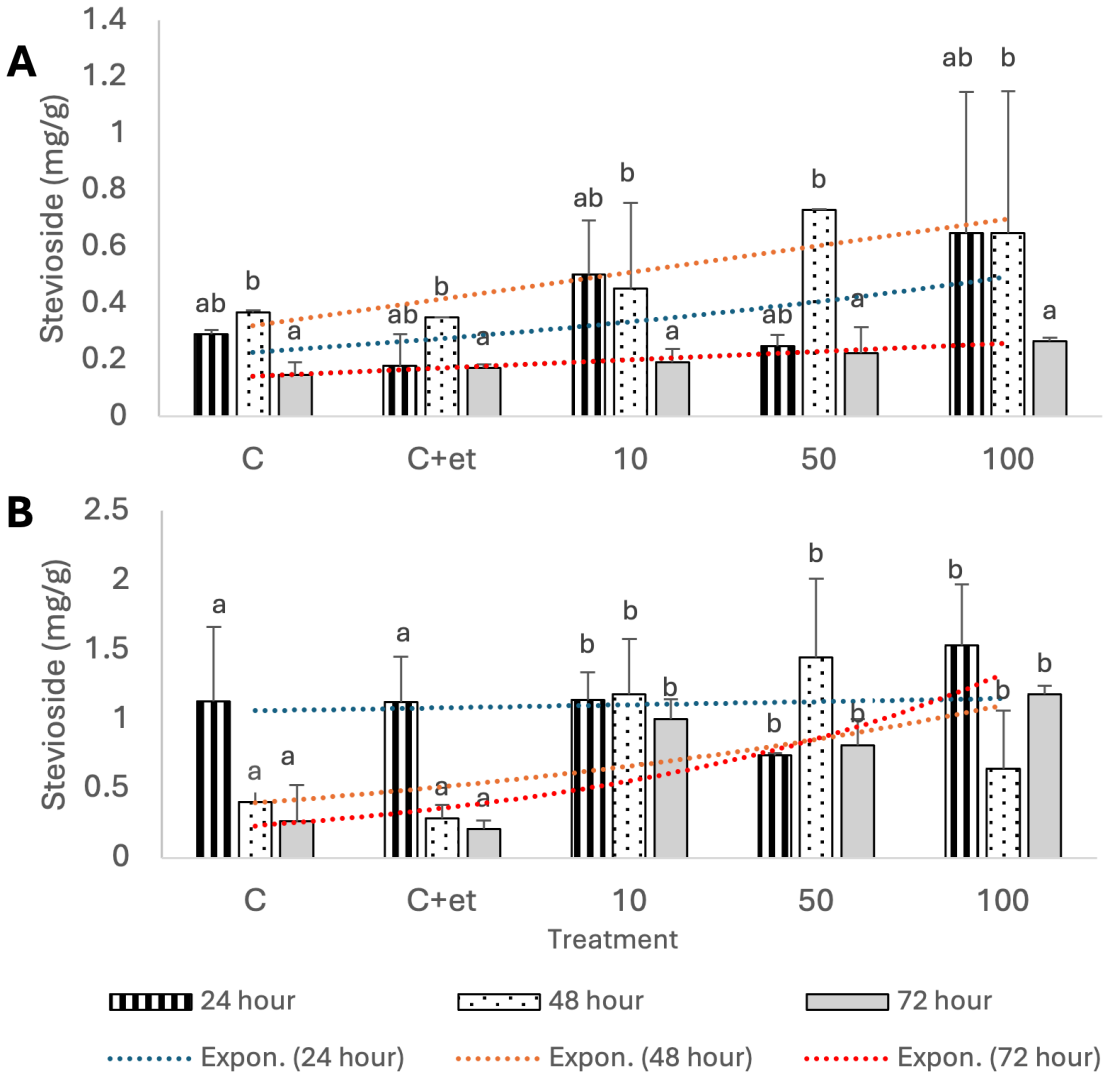
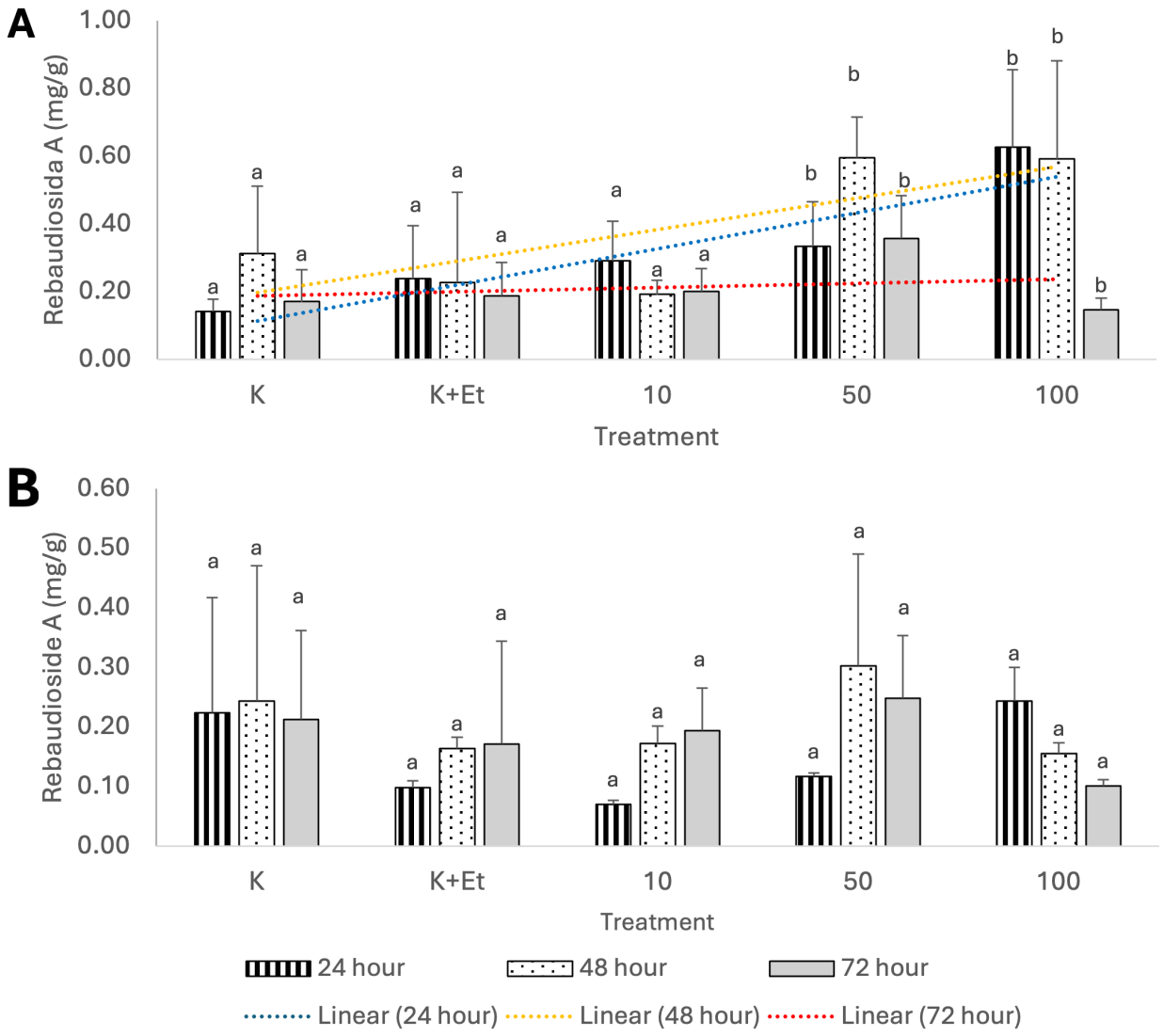


FIGURE 3. Leaves appearance: (A) Control; (B) 50 µM MeJA for 72 h; and (C) 100 µM MeJA for 72 h



Means using the same letters mean no significantly difference at a 5% confidence level by Duncan test

FIGURE 4. The effect of MeJA on stevioside content: (A) Directly after elicitation; and (B) 7 days post-elicitation



Means using the same letters mean no significantly difference at a 5% confidence level by using the Duncan test

FIGURE 5. The effect of MeJA on rebaudioside A content: (A) Directly after elicitation; and (B) 7 days post-elicitation

100 µM MeJA for 24 h, while the highest rebaudioside A content was treatment with 50 µM MeJA for 48 h. Even though both the 50 µM MeJA for 48 h and 100 µM MeJA for 24 h treatments were able to increase stevioside and rebaudioside A content, the 50 µM MeJA for 48 h treatment was suggested as an optimal condition for achieving a high growth and production of steviol glycosides. This suggestion was based on the findings that the leaves as the source of the secondary metabolites showed more pronounced growth after 48 h treatment with 50 mM MeJA compared to 24 h of treatment with 100 mM MeJA (Table 1).

In contrast to this study, Bayraktar et al. (2018) demonstrated that 100 µM MeJA administered for 2 weeks was more efficacious compared to other concentrations in the formation of stevioside, although no rebaudioside A production was noted in the treatment. In this study, the period of elicitation is essential for plant growth, which enhances stevioside content. A further study demonstrated that the equilibrium between growth and bacoside production in the shoot culture of *Bacopa monnieri* was attained with a 7-day MeJA treatment; prolonged elicitation resulted in reduced biomass and bacoside production (Sharma et al. 2015). MeJA is a crucial regulator of plant growth and development during biotic or abiotic stress. MeJA modulates signal transmission by interacting with other genes in plants, thus facilitating suitable metabolic responses in both normal and stressful situations. It has been identified as an excellent chemical elicitor for the biosynthesis of naturally occurring secondary metabolites (Sohn et al. 2022).

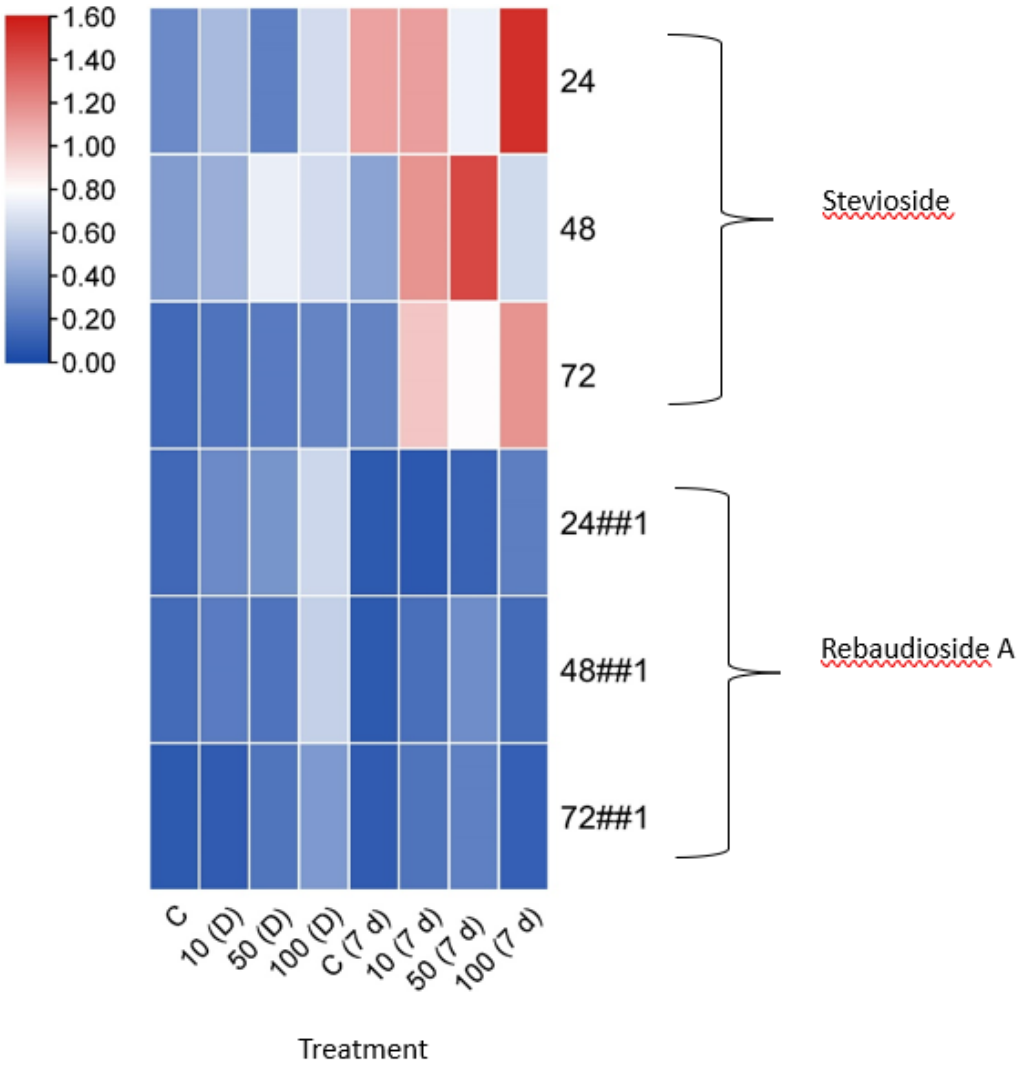


FIGURE 6. Heatmap of stevioside and rebaudioside A production after MeJA elicitation. D = direct sampling after treatment; 7 d = 7 days after treatment (DAT). Duration of elicitation is indicated by '24' for 24 h, '48' for 48 h, and '72' for 72 h

TABLE 1. Matrix of plant growth

Parameters	Duration (h)	Treatment			
		Control	10 µM MeJA	50 µM MeJA	100 µM MeJA
Leaf number*leaf size	24	3.502	2.1794	4.1958	1.0947
	48	10.45	2.5346	2.5515	1.7616
	72	14.35	5.25	1.2	2.4687
Plant fresh weight (g)	24	0.26	0.24	0.26	0.32
	48	0.19	0.16	0.24	0.08
	72	0.27	0.21	0.16	0.15

CONCLUSIONS

MeJA had a negative impact on *Stevia* growth in terms of height, leaf size, and the formation of adventitious buds. Furthermore, the increase in inducible secondary metabolites at 24 h and 48 h, followed by a decrease after 72 h, suggested a time-dependent response that might reflect the transient nature of the induction process. Treatment at a concentration of 50 µM effectively elevated high levels of stevioside and rebaudioside A without hindering growth. This finding can serve as a fundamental approach for enhancing the production of steviol glycosides in the industrial sector, particularly by utilising MeJA as a stimulant for secondary metabolite production.

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