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Stevioside and rebaudioside A production in treated hairy root culture of *Stevia rebaudiana* with elicitors

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ABSTRACT

Stevia rebaudiana is an important medicinal plant that is used by people with diabetes because of its steviol glycosides such as stevioside and rebaudioside A. Considering the limitation of seed production in this plant and the time-consuming nature of its propagation by stem cuttings, the use of a hairy root culture can be a suitable strategy for the production of these compounds. In this study, after the production of hairy roots by *Agrobacterium rhizogenes* strain ATCC15834 and their proliferation, different concentrations of methyl jasmonate (0, 50, 100, 150, and 200 µM) and salicylic acid (0, 30, 60, 90, and 120 mg/L) were applied and sampling was done at different times (24, 48, 72, and 96 h). The use of 100 µM methyl jasmonate for 48 h resulted in the accumulation of 47.31 mg/g DW, the production of 134.30 mg/L stevioside, the accumulation of 45.11 mg/g DW, and the production of 128.41 mg/L rebaudioside A and the use of 200 µM caused the accumulation of 45.56 mg/g DW and production of 120.47 mg/L rebaudioside A. While, among the different concentrations of salicylic acid, only the use of 90 mg/L for 72 h increased the accumulation of rebaudioside A (68.36 mg/g DW), and the other concentrations had a negative effect on the accumulation and production of stevioside and rebaudioside A. In conclusion, these findings showed that methyl jasmonate and salicylic acid can inhibit the growth of hairy roots and instead enhance the accumulation and production of stevioside and rebaudioside A.

***Key words*:** Candyleaf, Elicitor, High-performance liquid chromatography, Hairy root, Secondary metabolites.

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INTRODUCTION

S. rebaudiana is a natural sweetener and is used in food preparation (Thakur *et al.*, 2021). Studies show that the stevia plant has about 60 steviol glycosides, the most important of which are stevioside, rebaudiosides A-F, rubusoside, steviolbioside, and dulcoside A. Rebaudioside A is about 250-450 times sweeter than sucrose and has no bitter aftertaste (Abdelsattar *et al.*, 2023). As a result, people with diabetes and obesity can use them as sugar (Libik-Konieczny *et al.*, 2020). Due to the low viability of *S. rebaudiana* seeds, the impossibility of large-scale production, the need for more time for cutting and more manpower (Kazmi *et al.*, 2019; Simlat *et al.*, 2023)., it is not possible to meet the demand of industries. The current global production of *Stevia* is insufficient to meet the higher industrial demand, due to the lack of standardized protocols for the development of high-quality plants containing a higher amount of stevioside A and rebaudioside (Kazmi *et al.*, 2019). Therefore, it is necessary to prepare suitable protocols capable of producing high amounts of stevioside and rebaudioside A (Khan *et al.*, 2020). Therefore, researchers' focus on biotechnology techniques can be effective. Plant cell and tissue culture methods can be used in the sustainable production of secondary metabolites (Hussain *et al.*, 2012). *In vitro* culture techniques including callus induction, cell suspension culture, and hairy root culture have been already developed for many plants such as *Azadirachta indica* (Farjaminezhad and Garoosi, 2019; Farjaminezhad and Garoosi, 2021a, b), *Papaver bracteatum* (Zare *et al.*, 2014), *Melia azedarach* L. (Ahmadpoor *et al.*, 2022), *Stevia rebaudiana* (Mejía-Espejel *et al.*, 2018; Khan *et al.*, 2020), *Papaver armeniacum* L. (Sharifzadeh Naeini *et al.*, 2021), *Hyoscyamus niger* (Kareem *et al.*, 2019), *Salvia przewalskii* Maxim. (Li *et al.*, 2020), etc.

The use of hairy root culture is very efficient due to its rapid growth and higher biomass production, increased biosynthesis of secondary metabolites, and genetic stability in culture media without plant growth regulators (Srivastava and Srivastava, 2007). The *Agrobacterium rhizogenes* genes have the *rolA*, *rolB*, *rolC*, and *rolD* genes which are located in the Ri plasmid, that are responsible for the expression of hairy roots phenotype (Pistelli *et al.*, 2010).

The studies show that in the biosynthesis of secondary metabolites, the manipulation of *RolB* and *rolC* can be ideal. The *rolB* gene increases the production of secondary metabolites despite inhibiting cell growth; while, the *RolC* gene has less effect on the production of secondary metabolites and increases cell growth (Shkryl *et al.*, 2008). Therefore, hairy root culture is an important strategy for the production of secondary metabolites and primarily a source of secondary metabolites for studies on their biosynthesis via genetic manipulation of genes involved in this pathway (Libik-Konieczny *et al.*, 2020). Additionally, elicitation has been used in *in vitro* cultures to increase the biosynthesis of the secondary metabolites (Alvarado-Orea *et al.*, 2020).

Elicitors are different compounds from different sources that can cause physiological changes in the target organism. These compounds stimulate the production of secondary metabolites in the tissue culture of different plants in a short period of time (Rasouli *et al.*, 2021; Alcalde *et al.*, 2022). The most common elicitors used to produce secondary metabolites are salicylic acid (SA) and methyl jasmonate (MJ), which are involved in signal transduction mechanisms and can induce a response similar to the defense response of plants against stresses (Kandoudi *et al.*, 2022; Rakesh and Praveen, 2022; Jeyasri *et al.*, 2023; Rattan and Warghat, 2023). Production of MJ and SA causes a wide range of metabolic, physiological, and anatomical responses to external stimuli. MJ is an essential molecule in controlling many aspects of plant growth, development, and defensive response including secondary metabolism. Induction of hairy roots, as organ cultures, by *Agrobacterium rhizogenes* affords a persuasive and stable approach to elevate the biosynthesis of secondary metabolites (Chandra, 2012). Elicitation emerges as an efficient strategy for the improvement of secondary metabolite production in plant hairy root cultures which mimics the plant's natural response to environmental stress by stimulating JA biosynthesis leading to induction of the expression of genes for secondary metabolism and defense (Baenas *et al*., 2014). For example, MJ treatment increased thebaine, codeine, and morphine production in *P. armeniacum* L. hairy roots (Sharifzadeh Naeini *et al.*, 2021) and valtrate production in *Valeriana jaramansi* hairy root (Shuang and Hong, 2020), SA and MJ were used to induction the tropane alkaloid biosynthesis in the transgenic *Atropa baetica* (Jaber-Vazdekis *et al.*, 2008). The main purpose of this study was to examine the effects of MJ and SA on the growth, the accumulation of stevioside, and rebaudioside A in *S. rebaudiana* hairy roots.

MATERIALS AND METHODS

Plant material and growth conditions

S. rebaudiana plants were obtained from the Faculty of Agriculture and Natural Resources of Tehran

University in Karaj and were grown in Imam Khomeini International University greenhouse at 25±2 °C. Young, fresh, and disease-free plantlets with a size of 10 cm were separated and transferred to the laboratory. The plantlets were washed with tap water for 30 min to remove dust particles, and then they were subjected to a disinfection cycle with 70% ethanol (v/v) for 1 min and sodium hypochlorite at 1.5% (v/v) for 15 min and, followed by five rinses with sterile distilled water for 6 min. The stems containing lateral buds were placed in MS basal medium (Murashige and Skoog, 1962) with 3% sucrose (w/v), and the cultures were maintained in a growth chamber with 16/8 light/darkness photoperiod of 25±2 °C. The light intensity was 5600 Lux.

Transformation of *S. rebaudiana*

The transformed plants were obtained by transformation with *Agrobacterium rhizogenes* strains A_4, A_7, ATCC11325, and ATCC15834. To prepare different strains of *Agrobacterium rhizogenes*, single colonies were prepared and cultivated in an LB medium containing 25 mg/L of rifampicin (Duchefa, Germany). For the infection, leaves of *in vitro* plantlets were cut into 1-2 cm^2 size with a sharp and sterile scalpel that was infected with *A. rhizogenes* strain colonies. After inoculation, the leaves were co-cultivated with *A. rhizogenes* on an MS medium containing 3% sucrose for 48 h at 25±2 °C in the dark. After washing of bacteria with sterile distilled water, the leaves were transferred onto an MS solid medium containing 300 mg/L of cefotaxime (Duchefa, Germany) and kept in a growth chamber at 25±2 °C at the dark. The leaves were subcultured every four days to the new MS medium with a gradual reduction of antibiotic concentration. After four subcultures, they were transferred onto the antibiotic-free MS medium. The grown hairy roots with a size of 2 cm were separated from the leaves, cultured in culture jars containing 10 ml of free-hormone MS liquid medium with 3% sucrose (w/v), and maintained on a rotary shaker at 120 rpm at 25±2 °C and sub-cultured every 10 days.

Confirmation of genetic transformation

For confirmation of the genetic transformation of the hairy roots, the extraction of plant genomic DNA was performed according to Japelaghi *et al.* (2011). The quality and quantity of extracted DNA from the hairy root were confirmed by 0.8% agarose gel electrophoresis and spectrophotometer, respectively. Plasmid extraction from *A. rhizogenes* strains was carried out according to Sambrook (2001). The DNA primers are shown in Table 1 (Fu *et al.*, 2005). The used PCR conditions were as the following: 94 °C for 5 min, followed by 35 cycles of 1 min at 60 °C, and 1 min at 72 °C; the amplification was finished with 10 min at 72 °C.

Table 1. The primers for *rol B* and *vir D* genes.

Gene	Sequence (5′ → 3′)
F *rol B*	TACTGCAGCAGGCTTCATGCA
R *rol B*	GCTTTCCCGACCAGAGACTG
F *vir D*	CCTGCCGTAAGTTTCACCTCACC
R *vir D*	CCTGCCGTAAGTTTCACCTCACC

Selection of the best hair root line

The six transgenic lines obtained from the *A. rhizogenes* strains are named lines A1, A2 (A_4), A3, A4 (ATCC15834), and A5, A6 (A_7). The 23 days after cultivation in MS liquid medium, the fresh weight of all lines was measured and the A4 line was selected for further study.

Preparation of growth curve

For this purpose, 100 mg of hairy root tissue was transferred to 100 ml Erlenmeyer flasks containing 20 mL MS liquid medium, and the tissue contents of 3 Erlenmeyer flasks were randomly taken every three days, and fresh and dry weights of hairy roots were measured. Finally, the growth curve was obtained during 30 days (Figure 1).

Preparation and application of MJ and SA

MJ and SA were applied to the hairy root elicitation. MJ (Sigma, USA) and SA (Sigma, USA) were dissolved in ethanol and filtered through a sterile filter, respectively (Li *et al.*, 2020). Eighteen days-old cultured hairy roots were treated with MJ and SA to the final concentrations of 50, 100, 150, and 200 µM for MJ and 30, 90, 60, and 120 mg/L for SA, and hairy root without elicitation was applied as a control. To acquire the best exposure time of hairy roots with elicitors, the hairy roots were acquired 24, 48, 72, and 96 h after adding the elicitors.

Extraction and HPLC determination of stevioside and rebaudioside A

To extract the stevioside and rebaudioside A, the methodology described by Kolb *et al.* (2001) was followed. Stevioside and rebaudioside A contents of the extracts were determined by a high-performance liquid chromatography (HPLC) system (Knauer, UV detector, Germany) equipped with a C18 column (Tosoh-TSKgel-ODS, 250 mm×4.6 mm, 5 µm, Japan) employing the procedure described by Serfaty *et al.* (2013). The detection was conducted at 210 nm.

Statistical analysis

All experiments were performed as a factorial

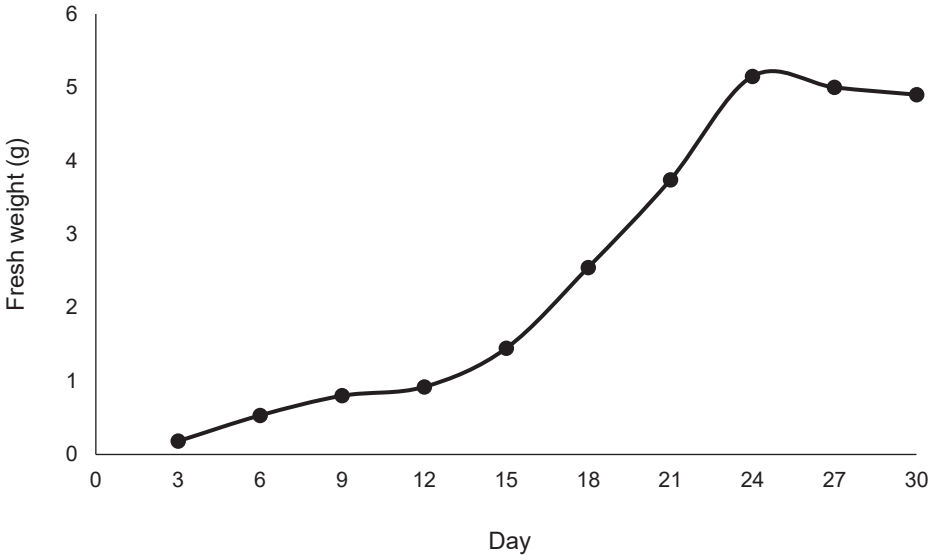

Figure 1. Growth curve of hairy root obtained from the *A. rhizogenes* strain ATCC15834.

experiment based on a completely randomized design with three replications. Data analyses were performed using IBM SPSS Statistics 24.0 (Armonk, NY, USA). Mean comparisons were carried out using Duncan's multiple range test at a probability level of 0.05.

RESULTS

Induction of hairy roots

The first hairy roots were observed 15 days after infection by *A. rhizogenes* strains A_4, A_7, and ATCC15834. After 2 months, the hairy root was not observed on the leaves inoculated with *A. rhizogenes* strain ATCC11325. The frequency of hairy root induction among these strains was not different, but in terms of morphology and growth, the hairy roots obtained from the ATCC15834 strain were better. The 2 lines of hairy roots from each bacterial strain were transferred to MS liquid medium and maintained on a rotary shaker at 120 rpm at 25±2 °C in the dark and sub-cultured every 10 days. Based on the amount of growth of hairy roots in the MS liquid medium, the A4 line obtained from the *A. rhizogenes* strain ATCC15834 was selected (Figure 2).

Investigating the transformation of hairy root lines

The confirmation of transformation of hairy root lines was performed using PCR and the results of PCR on 0.8% agarose gel showed that the obtained lines were transgenic (Figure 3). Among the 6 lines, the A4 line obtained from the *A. rhizogenes* strain ATCC15834 had the highest biomass compared to other lines and selected as the superior line for more study.

Effect of MJ on growth and steviol glycosides synthesis in hairy roots

Significant differences ($p \leq 0.05$) were found among the MJ concentrations, sampling time, and their interactions in terms of the fresh and dry weight of hairy roots, and accumulation and production of stevioside and rebaudioside A (Table 2). To evaluate the effect of MJ on growth and steviol glycoside production, five concentrations of MJ were added to the culture medium. Figure 4 shows the effect of MJ on the growth of the hairy roots during 96 h of exposure. Hairy roots without elicitors weighed 44.73 gFW/L and 2.36 gDW/L and decreased fresh weight after elicitation with MJ, but dry weight increased with using 100 µM MJ (2.52 gDW/L) (Figure 4A). The elicitation using MJ showed inhibition of the hairy root growth compared to the control treatment. Also, the maximum biomass was 36.61 gFW/L and 2.55 gDW/L after 48 h in control (Figure 4B). Hairy roots in control conditions weighed 17.77 gFW/L and 0.89 gDW/L on the first day and reached a biomass accumulation of 55.28 gFW/L and 3.40 gDW/L after 96 h of cultivation, while for hairy roots treated MJ, the maximum biomass was 39.60 gFW/L and 2.84 gDW/L after 24 h and 48 h of elicitation with 200 and 100 µM MJ, respectively (Figure 4C).

To analyze the elicitation effect on steviol glycoside production and accumulation, the same concentrations of MJ were tested. Figure 5 shows the accumulation and production of stevioside depending on exposure time after elicitation with MJ. It was observed that the addition of 100 µM MJ increased the accumulation and production of stevioside (18.97 mg/g DW and 51.12 mg/L), compared to the control without elicitation

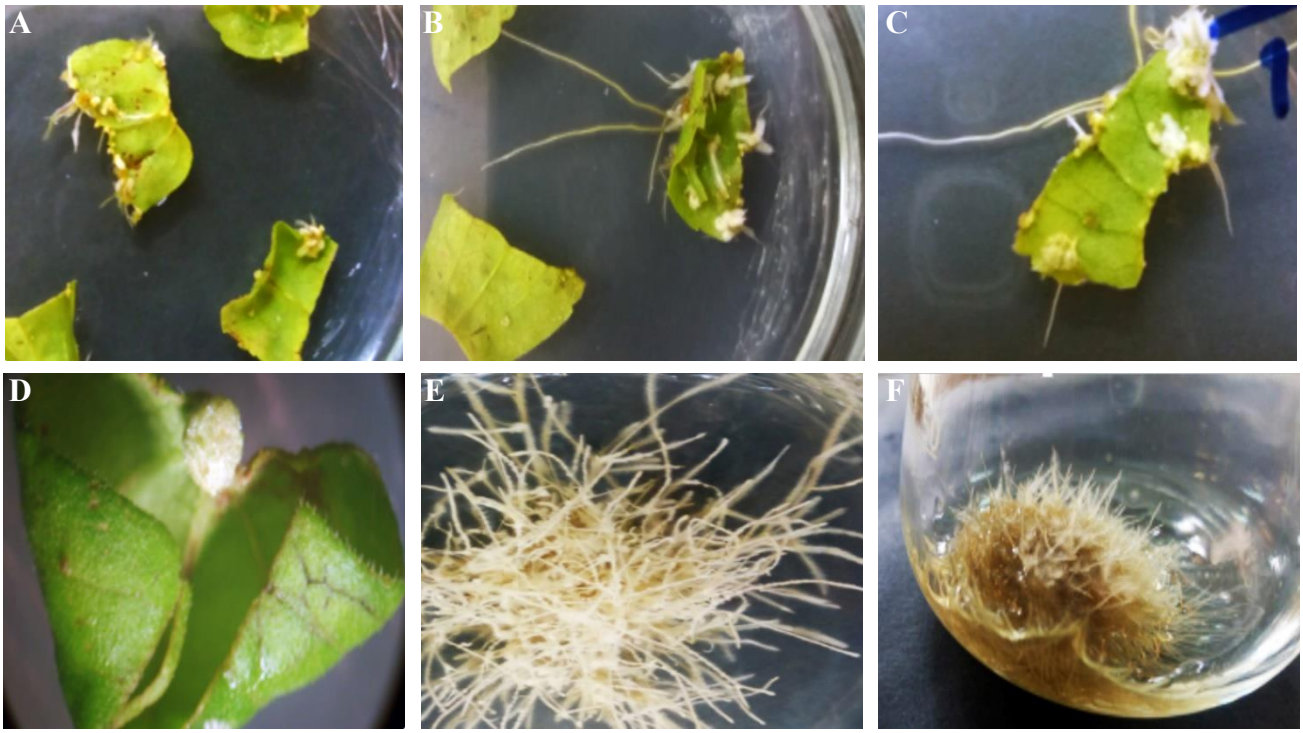

Figure 2. Induction and propagation of hairy roots in a basal MS medium. **A:** Hairy roots obtained from inoculation with *A. rhizogenes* A_4 strain, **B:** Hairy roots obtained from inoculation with *A. rhizogenes* ATCC15834 strain, **C:** Hairy roots obtained from inoculation with *A. rhizogenes* A_7 strain, **D:** Cell mass created by inoculation with *A. rhizogenes* ATCC11325 strain after two months, **E and F:** Propagation of hairy roots obtained from inoculation with *A. rhizogenes* ATCC15834 strain in the liquid medium.

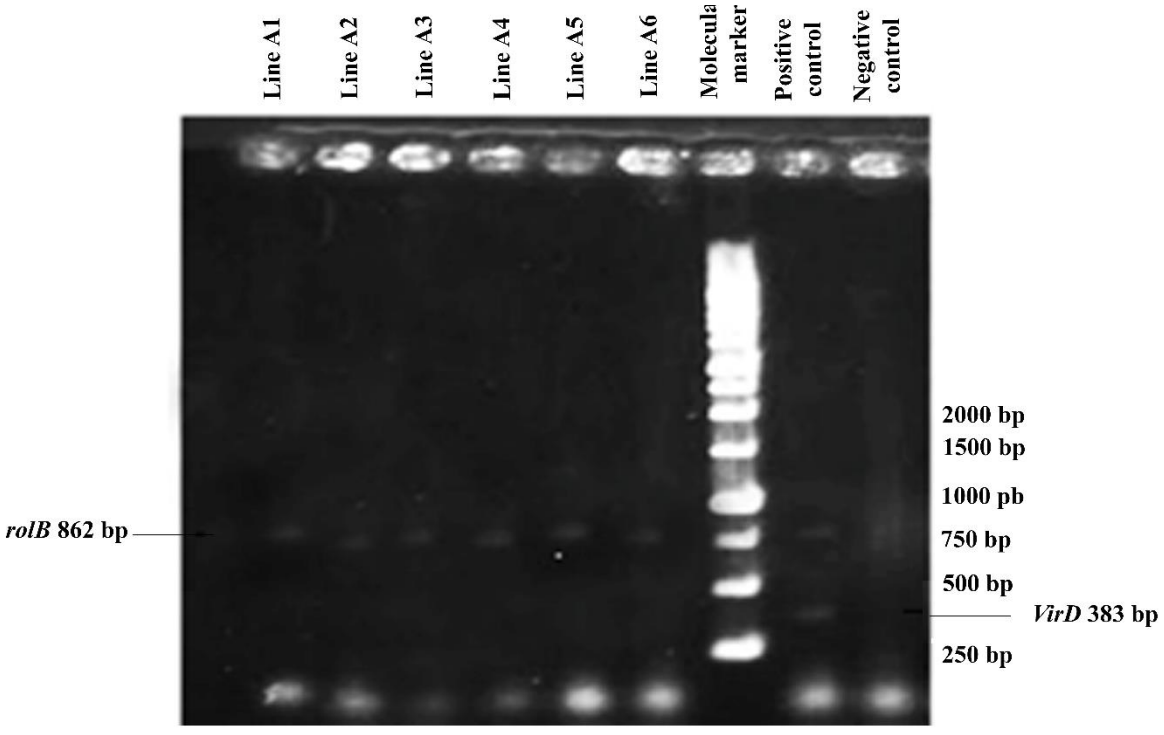

Figure 3. Electrophoresis of PCR product of *rolB* and *virD* genes. The molecular marker is 1 kb ladder.

Table 2. Analysis of variance for the effect of MJ and sampling time on growth and steviol glycosides synthesis in hairy roots of *S. rebaudiana*.

Source of variation	df	Mean of square					
		Fresh weight	Dry weight	Stevioside accumulation	Stevioside production	Rebaudioside A accumulation	Rebaudioside A production
MJ	4	967.469**	1.386**	66.499**	1111.056**	504.122**	2621.506**
Sampling time (ST)	3	273.813**	2.065**	1387.133**	10335.25**	1733.403**	12454.121**
MJ×ST	12	388.383**	1.204**	237.510**	2084.106**	384.362**	2589.541**
Error	40	27.799	0.156	2.019	56.879	2.448	108.938
Coefficient of variation (%)		17.35	18.74	8.83	21.9	6.81	20.85

**: Significant at 1% probability.

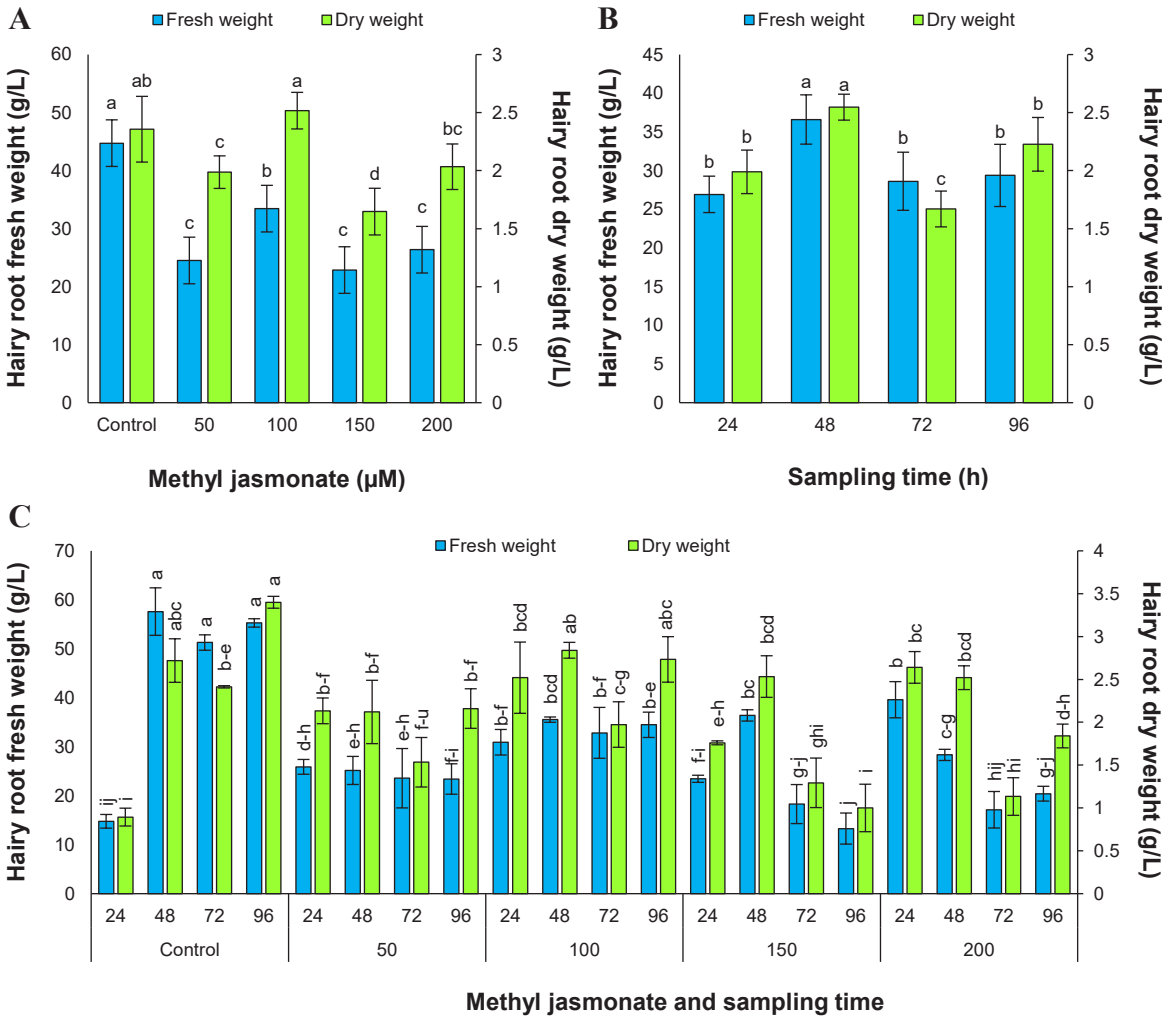

Figure 4. Effect of **A:** Methyl jasmonate, **B:** Sampling time, and **C:** their interaction on hairy root fresh and dry weight. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

(17.82 mg/g DW and 36.01 mg/L). After 48 h, the stevioside accumulation and production increased up to 1.24- and 1.63-fold of the control and reached the maximum in the experiment (26.59 mg/g DW and 69.81 mg/L). In these results, the hairy root cultures treated with 100 µM MJ for 48 h increased their stevioside accumulation and production and reached 47.31 mg/g DW and 134.30 mg/L, respectively.

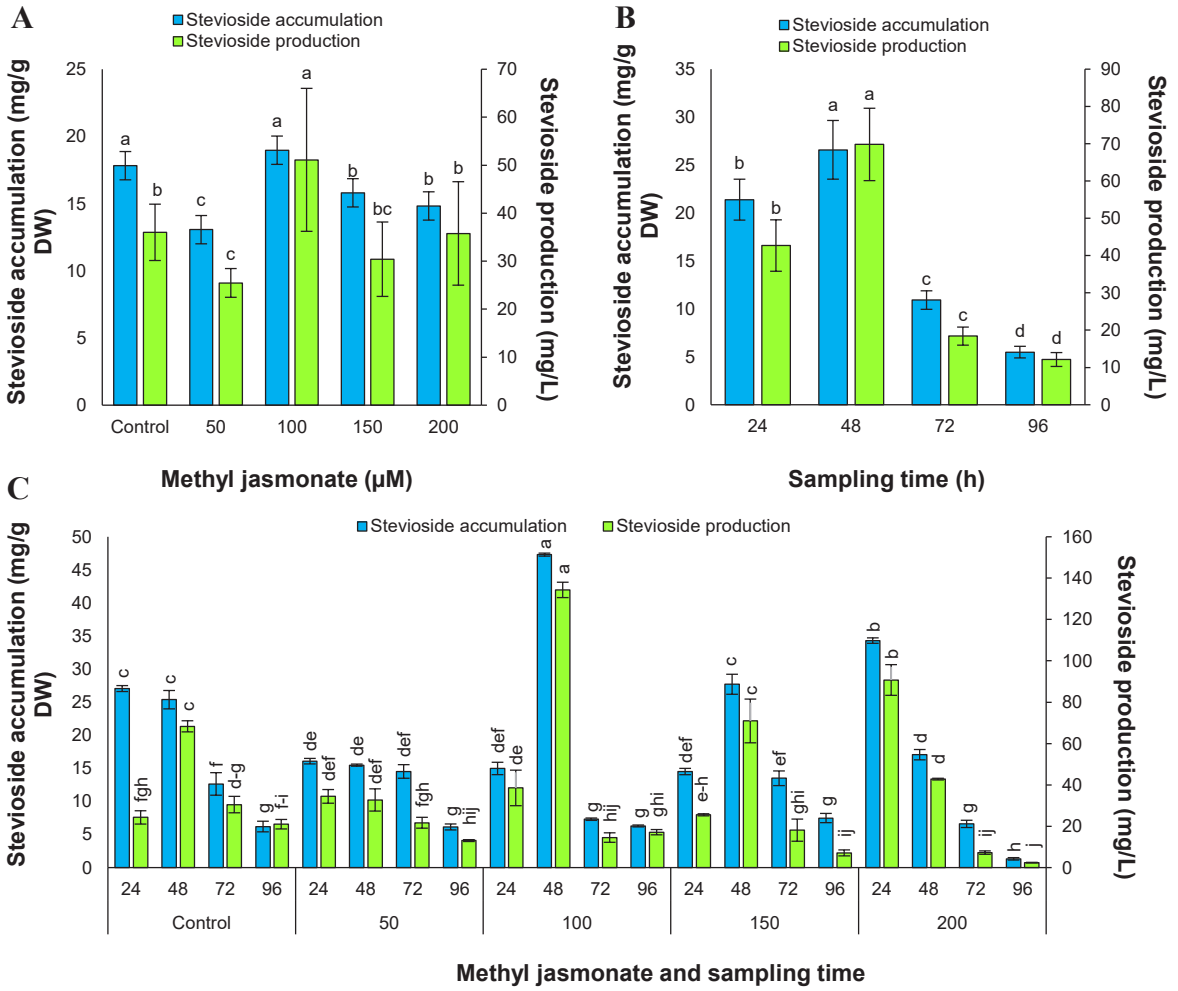

Figure 5. Effect of **A:** Methyl jasmonate, **B:** Sampling time, and **C:** their interaction on stevioside accumulation and production. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

The rebaudioside A accumulation and production was also modified by the presence of the MJ (Figure 6). The maximum accumulation and production of rebaudioside A was in the treatments with 50 and 100 µM MJ (28.71 mg/g DW and 67.20 mg/L, respectively). This resulted in 2.41- and 2.35-fold increases with 50 and 100 µM, respectively, compared to roots without elicitation. Among different exposure times, the highest rebaudioside A accumulation and production was 31.22 mg/g DW and 81.15 mg/L obtained 48 h after elicitation. The highest rebaudioside A accumulation (45.56 mg/g DW) was reached 24 h after the addition of 200 µM MJ and increased 5.16-fold compared to the control (8.82 mg/g DW). The highest rebaudioside A production was reached 48 h after the addition of 100 µM MJ and increased 1.51-fold compared to the control (84.97 mg/L).

Effect of SA on growth and steviol glycosides synthesis in hairy roots

Significant differences were found among the SA concentrations, sampling time, and their interactions in terms of the fresh and dry weight of hairy roots, and accumulation and production of stevioside and rebaudioside A (Table 3). Significant differences were shown in the hairy root fresh and dry weight by exposed to 30, 60, 90, and 120 mg/L SA compared to the control. The hairy root fresh weight and dry weight of these four treatments were 2.62 - 4.35 and 1.86 - 2.68 times lower than the control (44.73 g/L; Table 2; Figure 7A). Significant differences were not found among the sampling time in terms of the hairy root fresh and dry weight. The results of the analysis of variance presented in Table 2 indicated that different effects of SA and sampling times on the hairy root fresh and dry weight were significant. Means comparison of the results showed that the highest fresh hairy root fresh weight (57.60 g/L) and dry weight (3.40 g/L) was obtained in control after 48 and 96 h, while the lowest rate (5.33 g/L and 0.69 g/L) was related to the 60 mg/L SA after 96 h.

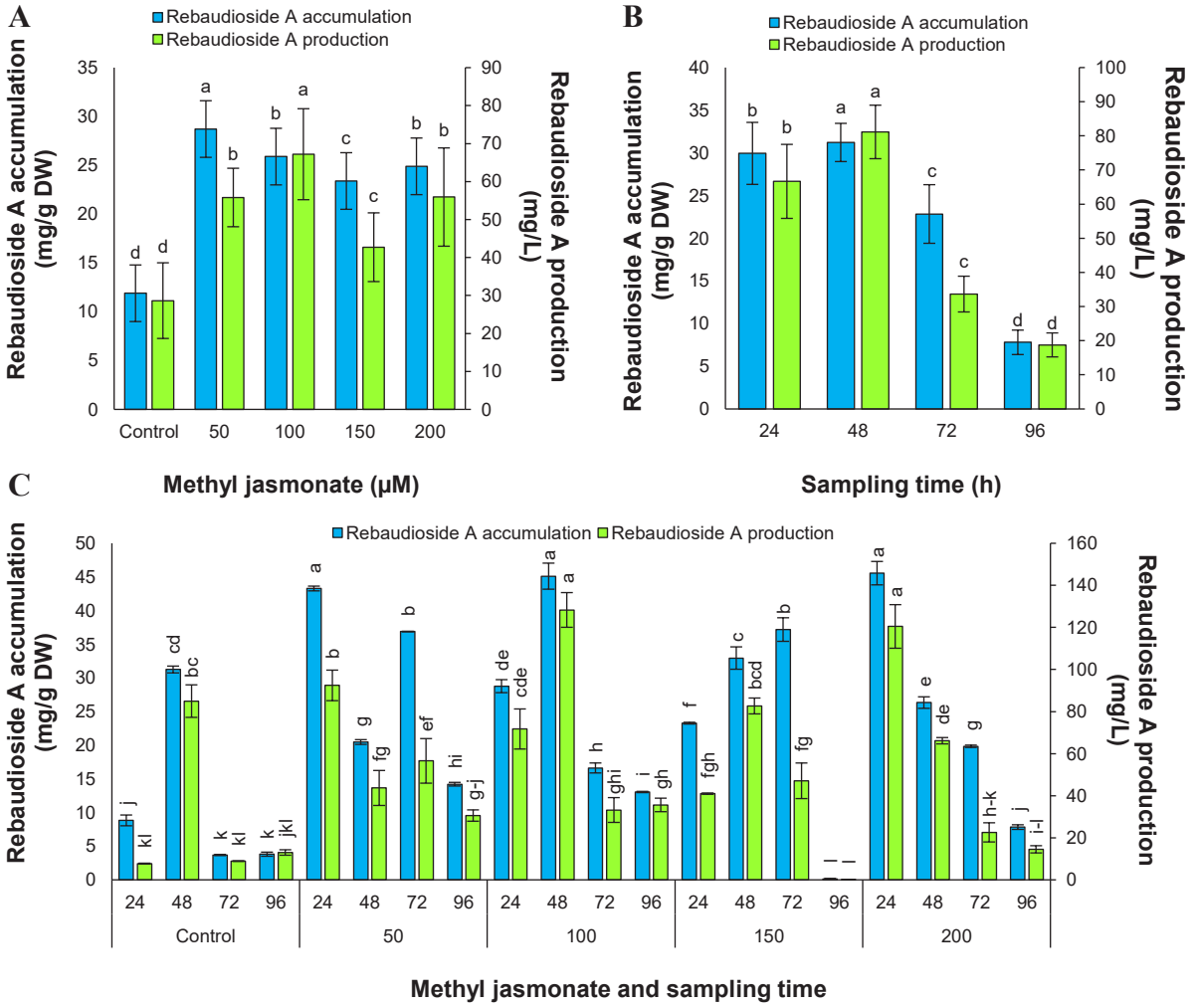

Figure 6. Effect of **A:** Methyl jasmonate, **B:** Sampling time, and **C:** their interaction on rebaudioside A accumulation and production. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

The stevioside accumulation and production in the hairy were significantly different among the treatments. Hairy roots treated with 120 mg/L SA accumulated (17.65 mg/g DW) and produced (22.27 mg/L) approximately 3.34 and 4.50 times more stevioside compared to those cultured with 60 mg/L of SA, but it was lower than the control (17.82 mg/g DW and 36.01 g/L). In addition, after 24 h and 48 h of treatment with SA the stevioside accumulation and production reach the highest. In general, the stevioside accumulation and production that was detected in the hairy roots were higher at 24 h and 48 h after using SA (27.06 mg/g DW and 68.33 mg/L; respectively) (Figure 8).

Rebaudioside A was detected in all concentrations of SA. In medium with 90 mg/L and 30 mg/L SA, the accumulation and production of rebaudioside A (40.86 mg/g DW and 41.68 mg/L, respectively) was 3.44 and 1.45 times greater than that control, respectively (Figure 9). The maximum amount of rebaudioside A were obtained 72 and 48 h after elicitation with SA (45.81 mg/g DW and 50.26 mg/L, respectively). The results showed that the highest rebaudioside A accumulation and production was obtained in 90 mg/L SA after 72 h and control condition after 48 h, respectively (68.36 mg/g DW and 84.97 g/L).

DISCUSSION

Secondary metabolites are synthesized by plants in biotic and abiotic stress conditions and elicitors. The use of medicinal plants has increased in recent years. Therefore, growing demand has put many medicinal plant species at risk of extinction (Yousefian *et al.*, 2020). Plant tissue culture techniques such as induction of hairy root by *A. rhizogenes* and elicitation can accumulate and stimulate secondary metabolites for the production of important pharmaceutical compounds

Table 3. Analysis of variance for the effect of SA and sampling time on growth and steviol glycosides synthesis in hairy roots of *S. rebaudiana*.

Source of variation	df	Mean of square					
		Fresh weight	Dry weight	Stevioside accumulation	Stevioside production	Rebaudioside A accumulation	Rebaudioside A production
SA	4	2457.82**	4.045**	418.28**	1858.08**	1418.31**	421.44**
Sampling time (ST)	3	32.47^{ns}	0.163^{ns}	539.89**	1009.79**	4798.89**	5712.85**
SA×ST	12	365.38**	1.031**	47.23**	331.10**	479.10**	1364.99**
Error	40	11.719	0.065	6.761	31.179	19.705	92.978
Coefficient of variation (%)		17.36	18.75	22.41	22.96	14.77	27.08

**: Significant at 1% probability, ns: non-significant.

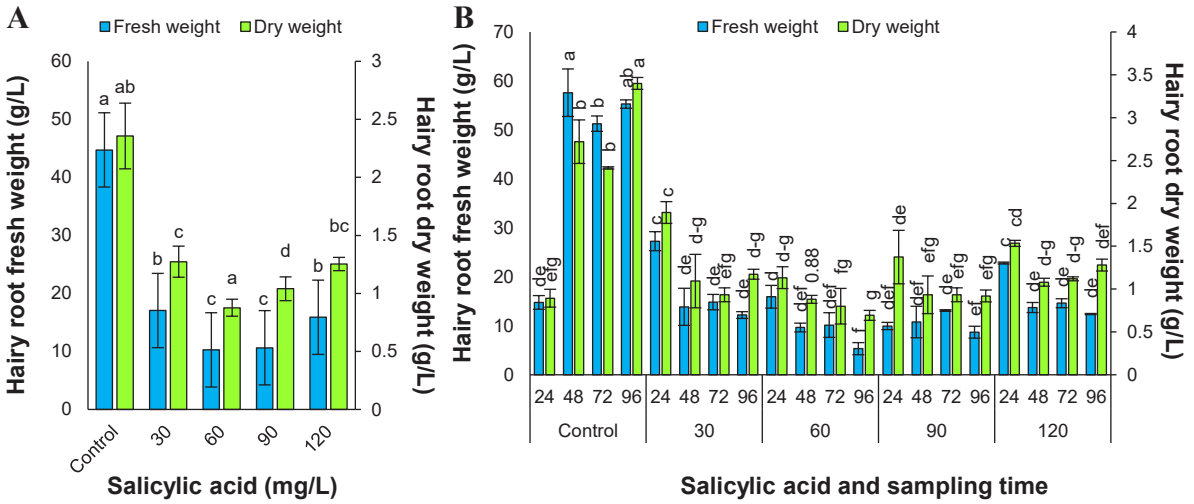

Figure 7. Effect of **A:** Salicylic acid and **B:** their interaction on hairy root fresh and dry weight. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

(Gonçalves and Romano, 2018). The obtained hairy roots by infection of explant with *A. rhizogenes* have high growth and genetic stability (Li *et al.*, 2020). In the current study, hairy root cultures of *S. rebaudiana* were successfully obtained by using *A. rhizogenes* strains A_4, A_7, and ATCC15834, and the presence of *rolB* genes in hairy root lines was detected by PCR analysis. In this study, to investigate the hairy root growth and steviol glycosides synthesis in response to MJ and SA treatment in hairy root cultures of *S. rebaudiana*, the fresh and dry weight of hairy roots, as well as the accumulation and production of stevioside and rebaudioside A were investigated at different sampling times. The results showed that the treatment with MJ and SA has a negative effect on hairy root growth. MJ (100 µM after 48 h) enhanced the stevioside and rebaudioside A accumulation and production, but SA inhibited stevioside accumulation and production and rebaudioside A production. From these findings, it can be inferred that higher levels of MA and SA might not have been effective in regulating growth of hairy roots of *Stevia*. The other reason could have been a long-time exposure of *stevia* to high levels of MJ and SA. MJ and SA are signaling molecules, and play an important role in plant resistance against biotic and abiotic stress (Pankaew *et al.*, 2023; Silva-Santos *et al.*, 2023). Salicylic acid and methyl jasmonate (Xu *et al*., 2015), are potential signal biomolecules involved effectively in signal transduction mechanisms and can regulate a number of responses related to plant defense against a variety of biotic stresses. The role of MeJ and SA in attaining an increased amount of secondary metabolites has been shown in tissue cultures of a variety of plants (Xu *et al*., 2015), but their influence was found to be species specific (Sivanandhan *et al*., 2013). It is known that MJ is converted to jamonic acid (JA) and then it

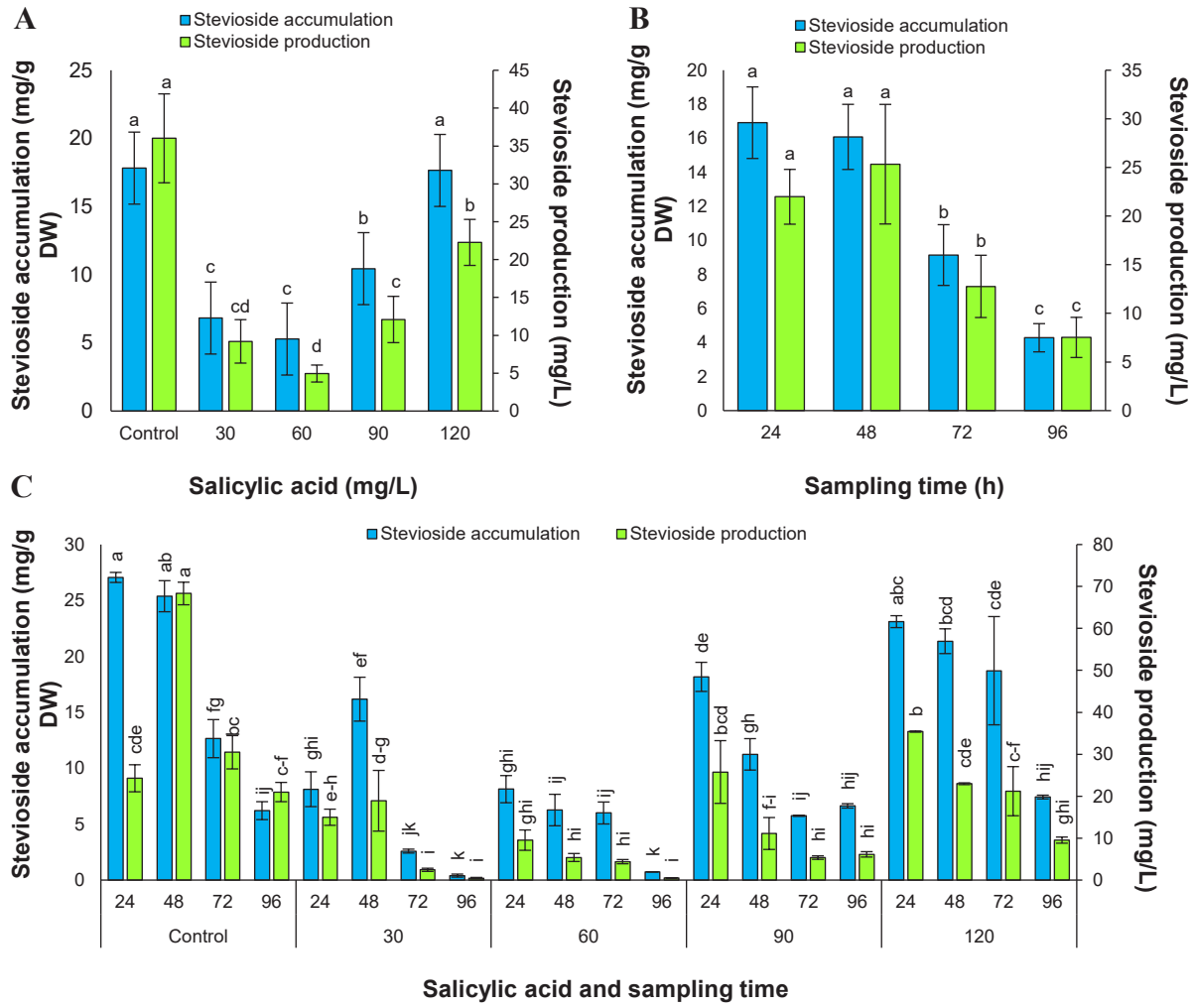

Figure 8. Effect of **A:** Salicylic acid, **B:** Sampling time, and **C:** their interaction on stevioside accumulation and production. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

is conjugated to JA-Ile (7–iso-jasmonyl-L-isoleucine) (Wasternack and Hause, 2013). COI1 (CORATINE INSENSITIVE 1), an F-box protein directly binds to JA-Ile and serves as a receptor for jasmonates (Yan *et al*., 2009). COI1 protein interacts with other proteins to form SCF−COI1 complex (Skp1, Cullin/F-box−COI1), an E3 ubiquitin ligase complex; it recruits JAZ proteins which are negative regulators of JA-induced genes. Once JAZ proteins are degraded, the MYC2 transcription factor is released and can bind to JA-responsive genes which are coded to secondary metabolites (Wasternack and Hause, 2013). It is reported that MJ and SA enhance lignin production and plant cell development in plants (Zhao *et al*., 2005). Also, it has been found that the application of MJ and SA increased the stevioside of *Stevia*. Therefore, the application of MJ as an elicitor is an effective strategy for increasing secondary metabolite production (Moharramnejad *et al.*, 2019). Bayraktar *et al.* (2016) showed that the addition of 50 µM MJ increases 8.05-fold stevioside content compared with the control plants of *S. rebaudiana*. Also, they reported that using 100 µM SA increased the stevioside content to 13.84 mg/g DW. MJ and SA have been used to improve the total phenols content in *Bletilla striata* (Yang *et al.*, 2016). MJ also enhanced the total flavonoid and phenolic contents of the callus of *Phyllanthus pulcher* (Danaee *et al.*, 2015). SA increased the phenolic acid accumulation in *Salvia miltiorrhiza* cell culture (Dong *et al.*, 2010). The researchers have shown that elicitation with SA inhibits the growth of the hairy root cultures of *Silybum marianum* (Khalili *et al.*, 2009). In that study, the application of SA inhibits the growth of hairy roots. In addition, SA enhanced the production of withanolide A, withanone, and withaferin A in the hairy root cultures of *Withania somnifera* (L.) Dunal (Sivanandhan *et al.*, 2013).

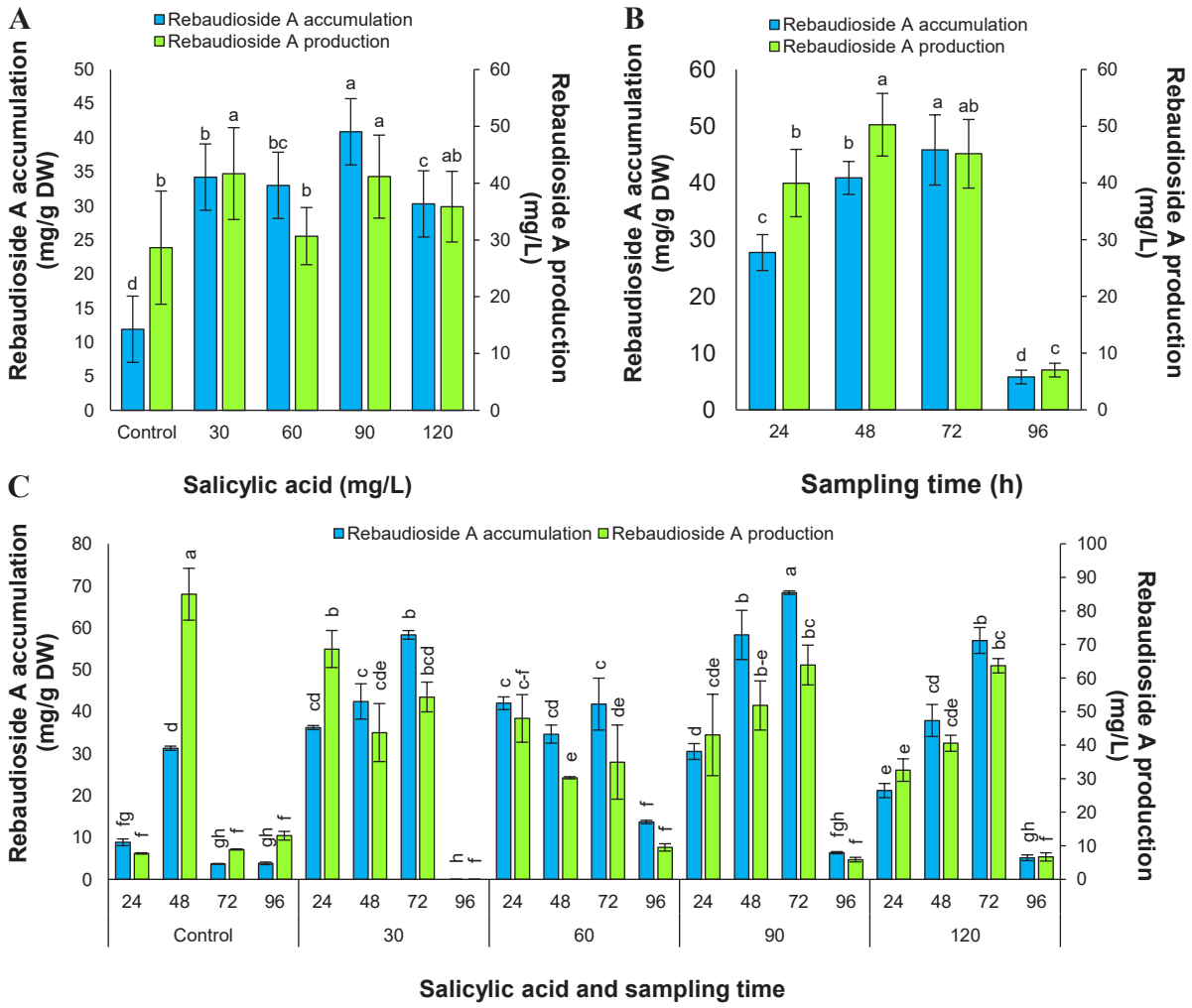

Figure 9. Effect of **A:** Salicylic acid, **B:** Sampling time, and **C:** their interaction on rebaudioside A accumulation and production. The bars with at least the same letters don't have significant differences. The bars are based on Standard Error (SE).

CONCLUSIONS

The current research established the effects of MJ and SA on hairy root growth, stevioside, and rebaudioside A accumulation and production. Our results demonstrated that employing MJ in *S. rebaudiana* hairy roots inhibited the growth and enhanced accumulation and production of stevioside and rebaudioside A. While the exposure of the hairy root cultures to different concentrations of SA inhibits their growth and stevioside accumulation and production and rebaudioside A production, higher concentrations of SA considerably stimulated the rebaudioside A accumulation.

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