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Enhancement of stevioside production by using biotechnological approach in *in vitro* culture of *Stevia rebaudiana*

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Abstract: *Stevia rebaudiana* Bertoni, which is an important plant for the food and health sector, contains calorie-free natural sweet-tasting steviol glycosides (SGs). In the present study, the effects of different elicitors [methyl jasmonate (MeJA), salicylic acid (SA), or chitosan (CHI)] on the *in vitro* production of stevioside and rebaudioside A were carried out. For this purpose, 3-week-old *in vitro* plantlets were transferred into 250 mL flasks containing liquid woody plant medium (WPM) supplemented with MeJA, SA, or CHI at different concentrations (0, 50, 100, or 200 µM), and were exposed to these elicitors for 2 weeks. A HPLC method was developed to quantify the aforementioned SGs in the cultivated plantlets and all of the elicitor types and concentrations resulted in an increase in stevioside production ranged between 2.87 mg/g dry weight (DW) (Control) and 50.07 mg/g DW (100 µM MeJA). The highest number of shoot, node, leaf, leaf length, and biomass accumulation and shoot length were observed with application of 100 µM CHI and control, respectively. The present findings open new perspectives for increasing the stevioside production using a plant tissue culture system.

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1. INTRODUCTION

Stevia rebaudiana (Bertoni), native to the eastern Paraguayan rainforests, is a member of the family Asteraceae. Since being introduced to Europeans in 1899, steviol glycosides (SGs) have been of significant interest both commercially and scientifically, due to their intense sweetness [1]. SGs are tetracyclic diterpenes sharing the same kaurenoid precursor as

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gibberellic acid and are synthesized mainly in *Stevia* leaf tissue [1, 2, 3]. With an approximate sweetness of 300 times that of sucrose, a 4% concentration (w/v) [4], SGs are relied on as a non-caloric sweetener in many countries worldwide, including China, Japan, Korea, Australia, New Zealand, and many countries in the European Union [5, 6]. The 2 major SGs are stevioside and rebaudioside A, while other lower-concentration SGs include steviolbioside, rebaudioside B, C, D, E, F, and dulcoside A [5, 6]. These SGs, due to their intense sweetness, have become an attractive sugar alternative in the food industry [7] as well as in the health sector. Due to these benefits, increasing the production of SGs and understanding their mechanism are of great interest.

The production of secondary metabolites via plant tissue cultures, such as callus culture, cell culture, and organ culture, have some advantages over conventional agricultural production: 1) Only a small portion of tissue is necessary to establish the *in vitro* culture [8]. 2) Fluctuations in secondary metabolite concentrations due to geographical, seasonal, and environmental variations do not occur [9, 10]. 3) They offer a defined production system, which ensures a continuous supply of products, uniform quality, and yield [10, 11]. 4) It is possible to produce novel compounds that are not normally found in the parent plant. 5) These methods also provide opportunities for the production of plant products on a large scale and the improvement of secondary metabolite production [11]. It is well known that the biosynthesis of secondary metabolites in plants generally occurs in response to biotic and abiotic stresses [10, 12]. Therefore, one of the most common applications aimed at increasing the secondary metabolite production in plant tissue cultures is the use of elicitors [13, 14]. Elicitors are the signals that trigger the production of secondary metabolites [15]. Methyl jasmonate (MeJA), salicylic acid (SA) and chitosan (CHI) have been used as effective elicitors for secondary metabolite production *in vitro* for many years [12, 14, 15]. It was reported that both SA and MeJA are stress-signaling molecules that influence plant resistance to biotic and abiotic stress factors [14, 16]. Chitin, a structurally important component of many fungi, increases a plant's accumulation of soluble pathogenesis-related proteins [17]. Therefore, CHI has often been used as an elicitor in plant cell cultures to induce or stimulate the production of many secondary metabolites [18].

The biosynthesis of SGs begins in the chloroplasts [1, 19] and there is a positive correlation between the development of the chloroplast membrane system and the biosynthesis of the SGs [19]. Therefore, it was thought that the synthesis of SGs takes place especially in specialized cells or structures, and plants with well-developed leaves are able to synthesize SGs in desired quantities and in the present study, *in vitro*-grown whole plantlets were used. In our previous research, we found that because of long exposure time (four weeks), SA and MeJA effected the shoot growth negatively; however, with the concentrations of 50 µM MeJA and 100 µM SA, we obtained approximately an 8-fold increase in the stevioside content when compared to the control. Although CHI applications resulted in better shoot growth, the stevioside content remained low from SA and MeJA applications [20]. Thus, we aimed to establish a suitable method by minimizing the exposure time and chancing the culture type. Therefore, we applied elicitors to 3-week-old *in vitro* plantlets transferred to liquid culture for two weeks. Herein, the results of this elicitation study are reported.

2. MATERIAL AND METHODS

2.1. Plant material

In vitro clonally propagated plantlets obtained from single seed descent seedlings of *S. rebaudiana* in the Bioengineering Department of Ege University were used as the plant material. To obtain a sufficient number of plants, nodal segments of 4-week-old *in vitro* grown plantlets, with one axillary bud and a length of approximately 1.0 cm to 1.5 cm, were cultured

into glass tubes (23/24 × 140 mm, Lab Associates b.v., Oudenbosch, Netherlands) with plastic caps, containing 10 mL of woody plant medium (WPM) [21] supplemented with 3% (w/v) sucrose, and solidified with 0.65% (w/v) plant agar (Duchefa Biochemie B.V., The Netherlands). For plant multiplication, they were transferred every 4 weeks to fresh solidified medium.

2.2. Initiation culture

For the elicitor applications, 3-week-old *in vitro* grown plantlets were used. Development of the shoots and roots from the nodal explants of *S. rebaudiana* cultured on the basal WPM occurred simultaneously. Root formation began after one week of culturing. For elicitor applications in agitated liquid culture, 3-week-old plantlets should be removed from the culture vessels without damaging their roots. To facilitate this operation, different substrates were tested. Nodal explants were transferred to the liquid culture in tubes with the filter paper bridge (LCFPB), loofa (Luffa cylindrica) sponge (LCLS), or perlite (LCPE) and in 250-mL flasks containing sterilized liquid WPM [liquid culture (LC)]. They were also cultured in glass tubes containing semi-solidified WPM in a reduced amount of agar (0.58% (w/v)) [solid culture in reduced agar (SCRA)] (Table 1; Figure 1). Per culture vessel, 10 explants for flask applications and one explant for other applications were used. The flasks were maintained on an orbital shaker at 100 rpm under daylight conditions. The experiments were performed in triplicate. Ten explants were used for each replication and 30 explants were tested in total per treatment. The data were recorded 3 weeks after starting the culture.

2.3. Preparation of the elicitors

MeJA, SA and CHI were prepared according to procedures described in our previous paper [20]. They were added individually in liquid medium as elicitors. For the stock solutions, MeJA and SA (Sigma-Aldrich) were dissolved in 96% ethanol. Next, the solutions were diluted with distilled water in concentrations as required. Finally, the pH of both solutions was adjusted to 5.8. In the case of CHI from crab shells (Sigma-Aldrich), it was dissolved in 2.0 mL of 1% (v/v) acetic acid (Sigma-Aldrich) and then diluted with distilled water. Finally, the pH of the solution was adjusted to 5.8.

2.4. Elicitor application

Nodal explants were cultured on WPM supplemented with 3% (w/v) sucrose and solidified with 0.58% (w/v) agar. After 3 weeks of culture, rooted shoots, approximately 6–8 cm long, with 14 leaves were carefully removed from the glass tubes and transferred into 250-mL flasks containing 25 mL of liquid WPM supplemented with different types (MeJA, SA, or CHI) and concentrations (0, 50, 100, or 200 µM) of elicitors and 3% (w/v) sucrose. The stock solutions of MeJA, SA, and CHI were sterilized by filtration through a 0.22-µm syringe Millipore filter (Minisart®, Sartorius, Germany), and then added to the autoclaved and cooled liquid WPM aseptically at the desired concentrations. The cultures were continuously agitated on a rotary shaker at 100 rpm. The plantlets were exposed to elicitors for 2 weeks. All of the applications were done in triplicate, and 10 explants were used for each replication. Thirty explants were tested in total per application. After 2 weeks of elicitor applications, either elicited or control plantlets were harvested, and the data regarding the stevioside and rebaudioside A contents were investigated using HPLC.

2.5. Media and culture conditions

The pH of all of the media was adjusted to 5.8. The filter paper bridge, loofa sponge and perlite were placed in the glass tubes before autoclaving. Media were autoclaved at 121 °C with a pressure of 1.04 kg/cm^2 for 15 min. All of the cultures were maintained at 25 ± 1 °C and a 16 h photoperiod under a cool white fluorescent light (approximately 50 µmol/m^{2}s).

2.6. Stevioside and rebaudioside A analysis

The stevioside and rebaudioside A contents in the leaves of 5-week-old *S. rebaudiana* plantlets exposed to the elicitors for 2 weeks were analyzed using the HPLC method from Bayraktar et al. [20].

2.6.1. Chemicals

HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). The ultrapure water was supplied from an in-house ultrapure water system (Sartorius Arium 611, Sartorius Stedim Biotech, Göttingen, Germany).

2.6.2. HPLC sample preparation

Approximately 50 mg of leaf sample from each application was added to 5 mL of 70% (v/v) methanol and sonicated 3 times with this solvent for 20 min. The clear extracts were combined and diluted with methanol to 20 mL. Prior to the HPLC analysis, the samples were filtered with a 0.45 µm PTFE filter (Sartorius AG, Göttingen, Germany) to remove any non-dissolved particles.

2.6.3. LC-DAD Analysis

LC-DAD analyses were carried out with a Thermo Surveyor Plus HPLC instrument (Thermo Scientific, Bremen, Germany), equipped with a quaternary pump, an autosampler, a column oven and a diode array detector. For all separations, a Teknokroma RP C18 analytical column measuring 250 × 4.6 mm i.d., particle size being 5 µm (Teknochroma, Barcelona, Spain) was used. LC separations were conducted using following solvents: ultrapure water (A) and acetonitrile (B) and elution by gradient was according to the following scheme: 0-1 min 65A/35B, in 4 min to 63A/27B, in 2.5 min to 60A/40B, in 0.5 min to 5A/95B and kept at that composition for 3 min and changed to initial ratios (65A/35B) of method in 1 min. Prior to the next injection, the column was equilibrated for 3 min with the beginning conditions (65A/35B). The flow rate was 1 mL/min column temperature was 40 ºC. Detection was performed at 210 nm and UV spectra of all samples were scanned between 200-360 nm.

Quantifications of stevioside and rebaudioside A were performed using calibration curves generated by *Stevia* glycosides mixture which contains 50% rebaudioside A and 36% stevioside by mass. These percentages were determined according to relative peak areas of *Stevia* glycosides based on LC-DAD signals. Retention times of rebaudioside A was 5.75 and stevioside 6.15 minutes. Calibration curve for rebaudioside A were constituted with standard solutions between 1000.000 - 15.625 µg/mL and 720.00 – 11.250 µg/mL for stevioside. Regression coefficient of calibration curve for rebaudioside A was 0.998 and formula was peak area of rebaudioside A=8000.76 x rebaudioside A concentration (µg/mL). Similar to rebaudioside A, regression coefficient of stevioside was 0.998 and formula was peak area of stevioside=7850.76 x stevioside concentration (µg/mL).

2.7. Statistical analysis

The experiments were set up in a completely randomized design, and all of the applications were replicated in triplicate. Each replicate comprised of 10 explants. The statistical analysis of the data on various parameters was subjected to SPSS Version 16.0 (SPSS Inc., Chicago, USA). The significance of the differences among the means was carried out using the Student-Newman-Keuls (SNK) test at P = 0.05.

3. RESULTS AND DISCUSSION

3.1. Initiation culture

In the beginning, the influence of the different substrates on *in vitro* plantlet growth was determined (Table 1). The LC and LCLS showed 100% and 34.3% vitrification rate, respectively, and the results implied their unsuitability for the culture initiation (Figure 1a, b). The culture of the explants on LCPE resulted in the development of very thin shoots and small leaves, and the rooted shoots showed a 49% vitrification rate. Therefore, LCPE was not suitable for the initiation of the culture (Figure 1c). Although, in the LCFPB (Figure 1d), the development of the shoots was as good as that in the SCRA, the rooting rate was less than that in the SCRA (Figure 1e) (Table 1, 2). Consequently, the SCRA was found to be a more suitable system for culture initiation and the removal of rooted shoots with no damage.

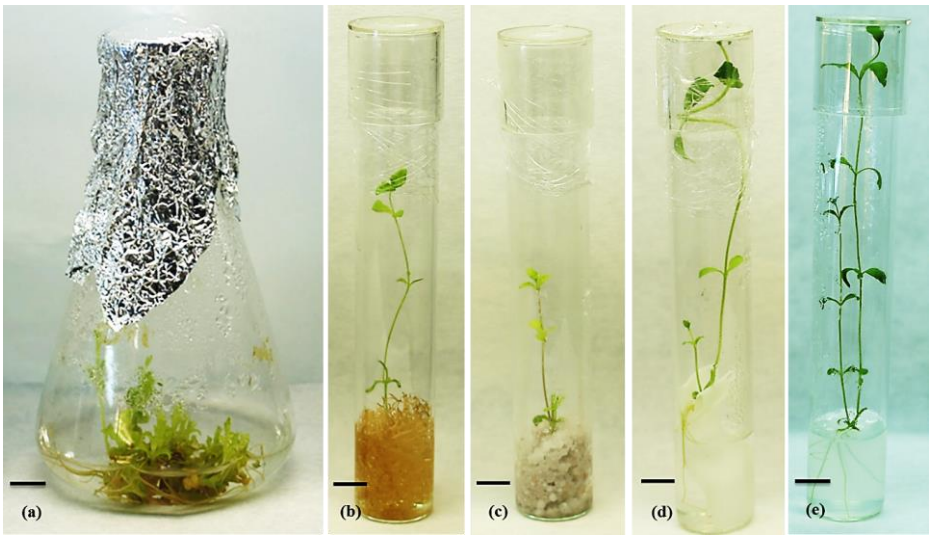

Figure 1. The growth of the plantlets after 3 weeks of the initiation culture: **(a)** Liquid culture in 250 mL flasks (LC); **(b)** Liquid culture in tubes with loofa sponge (LCLS); **(c)** Liquid culture in tubes with perlite (LCPE); **(d)** Liquid culture in tubes with filter paper bridge (LCFPB); **(e)** Solid culture in tubes with reduced agar (SCRA) (bars = 1.0 cm).

Table 1. Effect of different type of substrates on the *in vitro* shoot growth in *Stevia rebaudiana*

Substrate code	Number of shoots/explant ± SE	Shoot length (cm)/explant ± SE	Number of nodes/explant ± SE	Number of leaves/explant ± SE	Leaf length (cm)/explant ± SE	Stem diameter (mm)/explant ± SE	Vitrification (%) ± SE
LCSC	2.00 ± 0.08 b	9.89 ± 0.45 a	13.63 ± 0.88 a	29.13 ± 1.83 a	0.85 ± 0.03 a	0.62 ± 0.04 a	0.00 ± 0.0 d
LC	4.97 ± 0.54 a	1.99 ± 0.36 c	4.33 ± 0.63 c	10.67 ± 1.27 c	0.30 ± 0.02 c	0.67 ± 0.05 a	100.0 ± 0.00 a
LCFPB	1.60 ± 0.11 bc	8.43 ± 0.67 b	7.00 ± 0.39 b	16.00 ± 0.79 b	0.68 ± 0.06 b	0.56 ± 0.04 a	6.33 ± 0.17 c
LCPE	0.90 ± 0.13 c	3.10 ± 0.57 c	1.70 ± 0.25 d	4.80 ± 0.64 d	0.34 ± 0.05 c	0.25 ± 0.03 c	49.00 ± 4.13 b
LCLS	1.27 ± 0.08 bc	7.03 ± 0.50 b	2.87 ± 0.14 cd	7.73 ± 0.28 cd	0.61 ± 0.04 b	0.43 ± 0.02 b	34.33 ± 1.81 b

In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test

LCSC: Solid culture in tubes with reduced agar; **LC:** Liquid culture in 250 ml flasks; **LCFPB:** Liquid culture in tubes with filter paper bridge; **LCPE:** Liquid culture in tubes with perlite; **LCLS:** Liquid culture in tubes with loofa sponge

Table 2. Effect of different type of substrates on the root formation in *Stevia rebaudiana*

Substrate code	Percent of explants forming roots ± SE	Number of roots/ rooted explant ± SE	Root length (cm)/ rooted shoot ± SE
LCSC	100.00 ± 0.00 a	4.30 ± 0.23 a	3.54 ± 0.14 b
LC	70.00 ± 5.77 b	2.97 ± 0.26 b	5.73 ± 0.25 a
LCFPB	80.00 ± 5.77 b	4.27 ± 0.21 a	3.41 ± 0.14 b
LCPE	56.67 ± 3.33 c	2.87 ± 0.25 b	0.98 ± 0.08 c
LCLS	96.67 ± 3.33 a	3.10 ± 0.21 b	2.99 ± 0.22 b

In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test

LCSC: Solid culture (reduced agar) in tubes; **LC:** Liquid culture in 250 ml flasks; **LCFPB:** Liquid culture in tubes with filter paper bridge; **LCPE:** Liquid culture in tubes with perlite; **LCLS:** Liquid culture in tubes with loofa sponge

3.2. *In vitro* shoot growth and biomass accumulation

The effect of some elicitors (SA, MeJA and SA) on the *in vitro* shoot growth, biomass and SGs production of *S. rebaudiana* was investigated. For this purpose, 3-week old *in vitro* plantlets of *S. rebaudiana* were exposed to elicitors for 2 weeks in the liquid culture.

Information on the growth parameters of shoot cultures exposed to elicitation for 2 weeks was presented in Figure 2. Plantlets exposed to CHI at different concentrations showed the best response regarding shoot number among the applications (Figure 3a, b). The highest number of shoots per explant was obtained in the application with 100 µM CHI, which led to a shoot number of 3.60, followed by application with 200 µM CHI, with 3.10 shoot number (Figure 2a). Especially in plantlets with shoot tip necrosis, the effect of apical dominance on lateral buds was removed and shoots were induced from lateral buds. This resulted in more than two shoots formation (Figure 3c).

No significant differences were obtained in the shoot length per explant between applications (Figure 2b). Two-week elicitor treatment did not suppress shoot elongation. The maximum shoot length (8.67 cm/explant) was observed in the control medium (liquid WPM) followed by all of the elicitor applications except with 200 µM MeJA, and they were statistically placed in the same group. The shortest shoot length was obtained as 6.97 cm from 200 µM MeJA application.

Plantlets exposed to CHI at the concentration of 100 µM had the highest number of nodes (6.93 nodes per explant), number of leaves (23.67 leaves per explant), and leaf length (0.68 cm) (Figure 2c, d, e). Elicitor applications induced more leaf necrosis than non-treated plantlets (control). The necrosis rate was predominantly observed in the SA and MeJA treatments at the concentrations of 100 and 200 µM and CHI treatment at the concentration of 200 µM. Percentage of leaves showing necrosis ranged between 8.35% (control) and 24.07% (200 µM MeJA) (Figure 2f). As regards biomass production, CHI induced the highest plantlet fresh (153.7 mg per explant) (FW) and dry (35.7 mg per explant) weight (DW) at the 100 µM concentration (Figure 2g, h). According to our previous study, 4-week elicitation with SA and MeJA, especially at the 100 and 200 µM concentrations, resulted in high level necrosis formation in MeJA applications and the development of very thin and short shoots with short internodes and small leaves in SA applications. CHI applications gave generally best response regarding plant growth and biomass production. In the present study, the negative effect of MeJA and SA on plant growth was minimized by reducing exposure times of these elicitors. CHI also showed the same responses with our previous study.

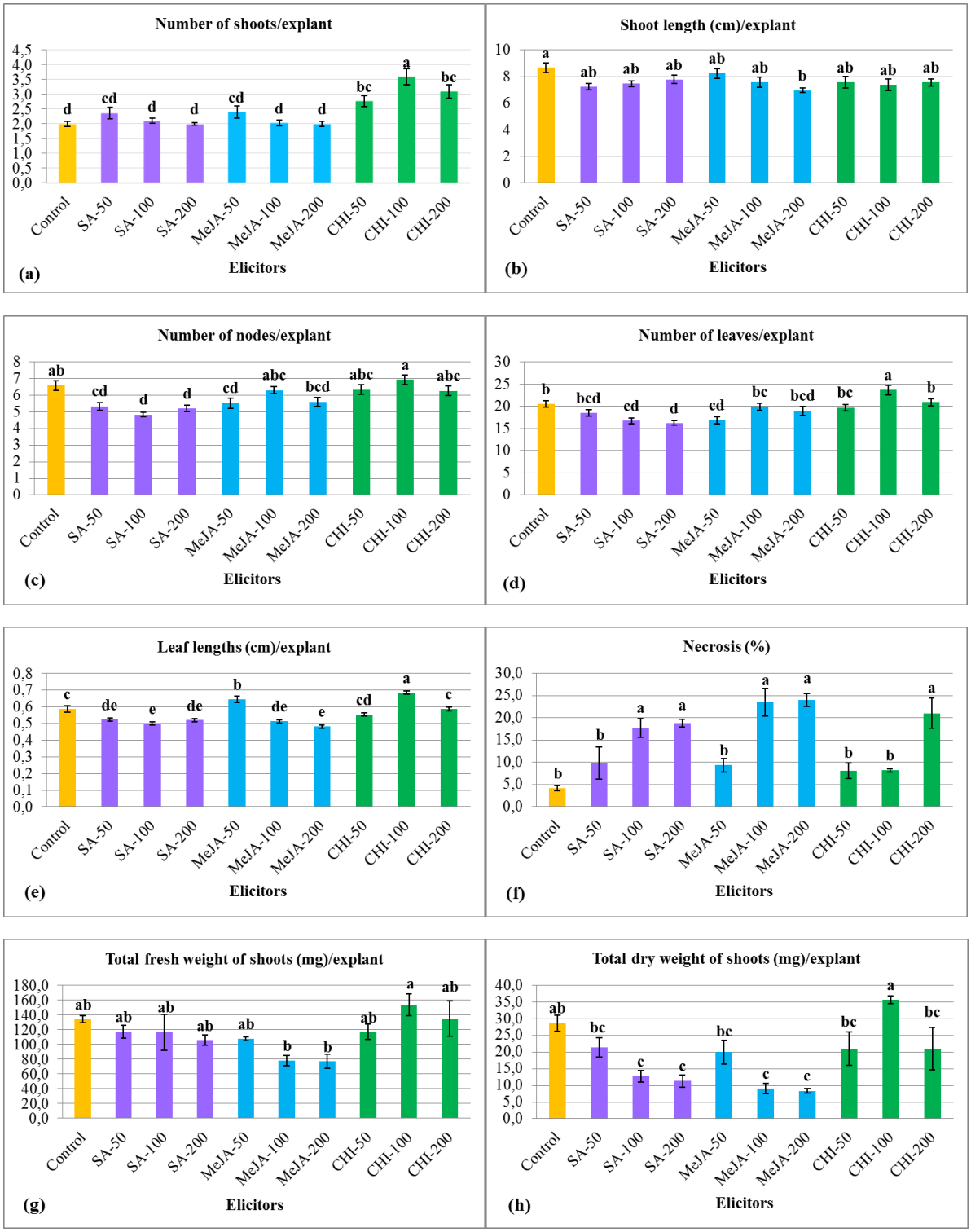

Figure 2. Effect of different type and concentrations of elicitors on *in vitro* shoot growth (a-f) and biomass production of *Stevia rebaudiana* after 2 weeks of culture. In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test.

Figure 3. The growth of the plantlets after 2-week CHI elicitation **(a, b)** and plantlet showed shoot tip necrosis **(c)**.

3.3. Steviol glycosides production

There are several methods to increase the production of secondary metabolites in *in vitro* plant culture systems, such as elicitation, two-phase culture, hairy root culture, and immobilization, but elicitation is the most widely used method to enhance the secondary metabolite production. The elicitors mimic the effects of stresses, and so stimulate the plant defense system, which results in increased biosynthesis of secondary metabolites in plant tissues [22]. To date, a lot of plant tissue culture studies have proven that elicitors increased secondary metabolite production of medicinal plants [14]. In the present study, similar stimulatory effects were also observed for the *in vitro* plantlets of *S. rebaudiana*, resulting in increased yields of stevioside production.

All of the MeJA doses provided higher metabolite content than the control plants, and the 100 µM dose was more efficient than the other concentrations with regards to the metabolite yield. The highest quantity of stevioside (50.07 mg/g DW) was 17.4-fold higher than the controls (2.87 mg/g DW) (Figure 4). SGs in *S. rebaudiana* share a common pathway with gibberellic acid and both are synthesized via the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway that occurs in plastids and mainly in leaves [1, 3, 23]. It has been proven extensively that Jasmonates, a phytohormone, induce plant secondary metabolites with incredible structural variety [23]. It has been reported that MeJA alters gene expression in the MEP pathway as well as the concentrations of products derived from these genes [23]. The pot plants of *S. rebaudiana* were inoculated with *Rhizophagus fasciculatus* (Thaxt.) C. Walker & A. Schüßler, an arbuscular mycorrhizal fungus, to improve the yield of SGs. It was observed that arbuscular mycorrhizal fungi colonized *Stevia* plants showed a significant increase (more than three-fold) in the concentration of JA when compared with the controls [23]. *Stevia* plants originated from *in vitro* culture and growing in a hydroponic system were exposed to MeJA, spermidine (SPD), SA, and paclobutrazol (PBZ) at the concentration of 100 µM for 24, 48, 72, and 86 h to examine the effect of these elicitors on SGs' contents and transcriptional levels of fifteen genes responsible for biosynthesis of SGs. It was found that MeJA and SPD showed positive effect on the transcription of SGs biosynthetic genes at 48 h of exposure [3]. In the present study, MeJA applications to *in vitro* plantlets of *S. rebaudiana* at different concentrations showed a similar effect and increased the stevioside production when compared to the control plants (Figure 4).

When the data were evaluated in the SA-treated plants, stevioside production was stimulated by SA treatments. At the 3 different concentrations (50, 100, and 200 µM), 100 µM dose of SA was more effective than the 50 and 200 µM concentrations with regards to the stevioside yield and the stevioside content reached up to 26.66 mg/g DW (9.3-fold higher) with the 100 µM dose (Figure 4). Belonging to the glycosyltransferase (GT) family, UDP-glycosyltransferases (UGTs) are a very diverse group of plant enzymes able to transfer a sugar residue from an activated donor to an acceptor molecule. UGTs play a role in the production of SGs in *S. rebaudiana* [24]. It has been reported in many studies that some elements, such as SA, JA, or some foreign compounds, can regulate GTs [25]. This may be because the stevioside biosynthesis increased in the SA-treated *in vitro* grown plantlets.

In the case of the CHI, compared to the controls, the 50 µM and 200 µM CHI applications affected the production same and all the SA applications raised apparently stevioside production. The stevioside content increased from 2.87 mg/g DW to 25.13, 31.24 and 25.50 mg/g DW at the 50, 100 and 200 µM concentrations, respectively (Figure 4). CHI is a linear polysaccharide that originates from the deacetylation of chitin, which is the main structural component of the cell wall of plant pathogen fungi. It is known that CHI mimics the effects of several pathogenic fungi and so activates the biosynthesis of defense-related secondary metabolites in plants [18]. Similar to other secondary plant metabolites, SGs may also behave in a defensive manner as feeding deterrents or antimicrobial agents against specific herbivores, pests, or pathogens [2]. Therefore, application with CHI increased the level of stevioside production when compared with the controls. In our previous work, the long exposure time (four weeks) of CHI resulted in better shoot growth than the MeJA and SA-treated explants and the stevioside content was lower than that of these applications [20]. In the present study, we improved the stevioside contents by reduced the exposure time of CHI.

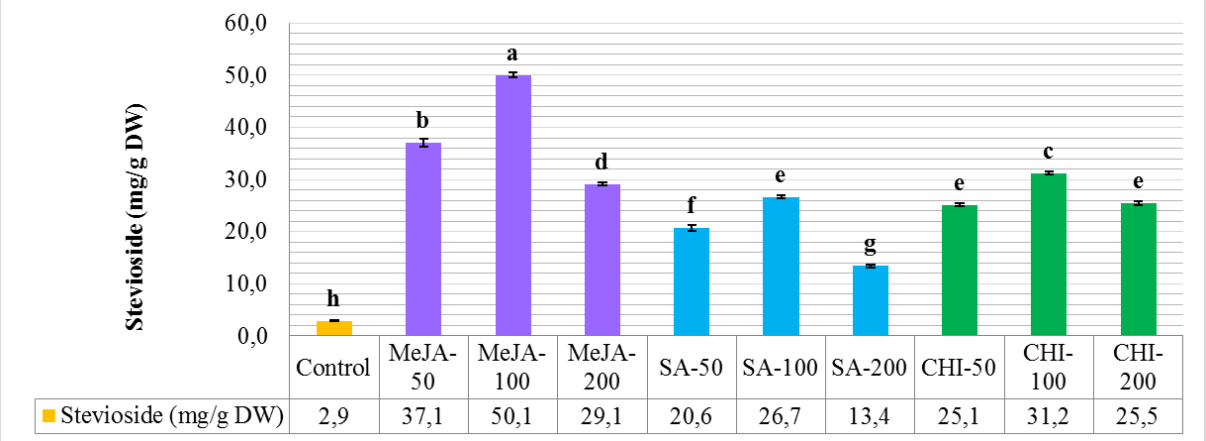

Figure 4. Effect of different type and concentrations of elicitors on stevioside production of *Stevia rebaudiana* after 2 weeks of elicitation culture. In each column, mean ± SE followed by the same letter was not significantly different (p=0.05) according to Student-Newman-Keuls test.

In the previous studies aimed at the increasing the SGs yield with elicitors in *in vitro*, Mejía-Espejel et al. [26] applied the SA (10 and 100 mM) and MeJA (10 and 100 mM) to the calli obtained from leaf segments of *S. rebaudiana* and increased the stevioside content 9.8 times compared to that of the leaves of plants grown under greenhouse conditions. They obtained the highest rebaudioside A contents (34.6 times higher than in leaves) by applying 10 mM of SA. Álvarez-Robles et al. [27] elicited *in vitro* shoot cultures of *S. rebaudiana* with methanol at different concentrations. They determined the stevioside and rebaudioside A production as 42 mg/g DW and 10.50 mg/g DW, respectively. Golkar et al. [28] enhanced the

stevioside and rebaudioside A concentrations up to 32.34 mg/g DW and 3.40 mg/g DW in callus culture exposed to 45 mg/L silver nanoparticles (Ag NPs) and 0.25 mg/L SA, respectively. Javed et al. [29] cultured the nodal explants of *S. rebaudiana* on the media containing different concentrations of Copper oxide (CuO) nanoparticles (NPs) and determined the highest stevioside and rebaudioside A production as 2.06% and 4.17% in the shoots exposed to 10 mg/L CuO NPs, respectively. In the present study, much higher contents of stevioside could be achieved and the stevioside content reached its maximum value with the 100 µM MeJA-treated *in vitro* plantlets (50.07 mg/g DW). In our previous work, with 50, 100 and 200 µM concentrations of MeJA and SA, we obtained tiny shoots with necrotic leaves from 4 weeks MeJA and SA applications in agar culture. The stevioside contents of the 100 and 200 µM MeJA- and 200 µM SA-treated plantlets were not determined due to the week growth and the necrotic formation in the leaves. The stevioside content increased 8.05-fold (12.53 mg/g DW) and 8.89-fold (13.84 mg/g DW) with the 50 µM applications of both MeJA and SA, respectively [20]. In the present study, the exposure time was reduced up to two weeks and the stevioside content was improved without inhibiting plant growth.

Among the three elicitors, the stevioside content reached its maximum value (50.07 mg/g DW), which was 17.4-fold higher than that of the control plants, in the 100 µM MeJA-treated *in vitro* plantlets. When the concentrations of all off the elicitors were compared, the 100 µM dose was found to be more effective than the other concentrations in stevioside production. After 2 weeks of elicitation, no rebaudioside A production was observed in all the applications and control.

4. CONCLUSION

In the present study, all of the elicitors (MeJA, SA, and CHI) tested resulted in an increase in the stevioside synthesis when compared to the untreated *in vitro* plantlets. *In vitro* plantlets synthesized the highest stevioside content (50.07 mg/g DW) when they were exposed to 100 µM MeJA. This result is 17.4-fold higher than in the control plants. Elicitor application can be made to plants grown in bioreactors and field or hydroponic system for increasing SGs contents.

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