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Research article

Effects of cytokinins, gibberellic acid 3, and gibberellic acid 4/7 on *in vitro* growth, morphological traits, and content of steviol glycosides in *Stevia rebaudiana*

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ABSTRACT

Steviol glycosides (SGs) and gibberellic acids share a part of their biosynthesis pathways. Despite the widespread studies on the effect of gibberellic acid 3 (GA_3), the effect of gibberellic acid 4 and 7 ($GA_{4/7}$) on *Stevia rebaudiana* has never been studied. This study aimed at a comparative evaluation of different hormone effects, i.e., 1 mg L^{-1} $GA_{4/7}$, 1 mg L^{-1} GA_3, or 0.5 mg L^{-1} kinetin and 0.5 mg L^{-1} 6-benzylaminopurine (BAP) (KB 0.5), on *in vitro* propagation, growth, morphological properties, and content of SGs in leaf samples of stevia. In comparison with the control group (hormone-free), the treatments of KB 0.5 or GA_3 produced the highest biomasses and largest leaf areas. The three hormonal treatments produced a similar number of leaves, the ratio of fresh to dry weight, and leaf length. $GA_{4/7}$-treated explants produced the highest ratio of leaf area to leaf length. The effect of $GA_{4/7}$ on shoot elongation was greater than that of the control or even GA_3. While the effect of GA_3 on rebaudioside-A (Reb-A) production was similar to that of the control (16.2 and 18.04 mg g^{-1}, respectively), $GA_{4/7}$ resulted in a lower amount of it (13.31 mg g^{-1}). Except for $GA_{4/7}$, which induced more stevioside accumulation, the treatments' effects were comparable to that of the control. The ratio of stevioside to Reb-A was the highest for $GA_{4/7}$ (2.62), followed by GA_3 (1.93), and then the two others. Sum of Reb-A and stevioside content was not changed by the use of any of the treatments.

1. Introduction

Stevia rebaudiana (Asteraceae), a short-day perennial bushy shrub originating from Paraguay, contains some sweet-tasting compounds about 300 times sweeter than sucrose (4 g L^{-1}). *S. rebaudiana* is the only species out of approximately 230 identified *Stevia* species that has sweetness compounds (Ceunen et al., 2013). Its sweetness comes from *ent*-kaurene-type diterpene steviol glycosides (SGs), e.g., stevioside (Ste) and rebaudioside-A (Reb-A) (Koheda et al., 1976; Kennelly, 2002; Martini et al., 2017), which range roughly between 4% and 20% of leaf dry weight (Yadav et al., 2011; Lemus-Mondaca et al., 2012).

SGs have the potential to outcompete artificial sweeteners because of their unique features, e.g., low-caloric, non-cariogenic, non-genotoxic, non-allergic, hypoglycemic, antioxidant, antimicrobial, antifungal activities, and high water solubility, as well as medicinal properties such as anti-inflammatory and antitumor effects (reviewed in Kennelly, 2002; Lemus-Mondaca et al., 2012). Although there are other alternative natural sweeteners (i.e., monk fruit), SGs owe their predominance over others to their higher yield and better taste. In addition, the other natural sweeteners have a delayed start and lingering sweetness (Kennelly, 2002; Lemus-Mondaca et al., 2012; Martini et al., 2017).

Among the SGs in stevia leaves, Reb-A is the most favorable sweetener. Even Ste, the most abundant SG in stevia leaves, has lagged behind Reb-A in the global market, since the latter has higher water solubility and a more pleasant taste with no bitter aftertaste (Kennelly, 2002). Therefore, the production of *S. rebaudiana* varieties with a higher Reb-A/Ste ratio is of great importance (Yadav et al., 2011; Yücesan et al., 2016a). It was stated that a negative correlation might exist between Reb-A and Ste (Yadav et al., 2011), while others observed that the ratio of Reb-A/Ste between different varieties (Ceunen et al., 2012) or after GA_3 treatment (Karimi et al., 2015; Hajihashemi and Geuns, 2017) was constant.

In vitro propagation of the crop is generally done by cutting internode and subculturing nodes. Each node normally produces two new branches with nodes (Yücesan et al., 2016b). Therefore, more nodes can

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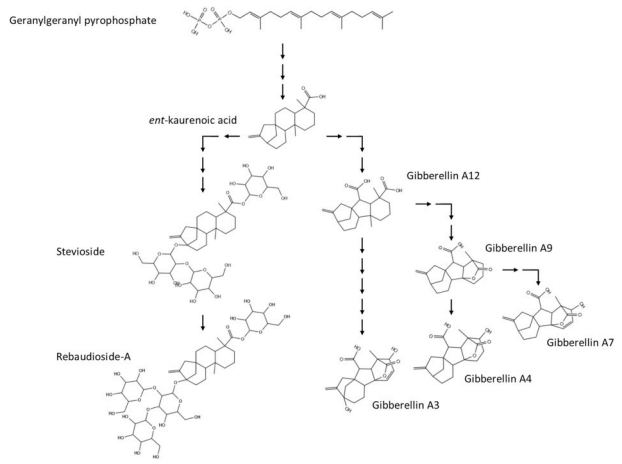

Fig. 1. Biosynthesis pathways of diterpene steviol glycosides (SGs) and gibberellic acids (GAs). Both SGs and GAs are synthesized from basic molecules, i.e., diterpene. The process begins with geranylgeranyl pyrophosphate. After three more modifications, *ent*-kaurenoic acid is synthesized. The process is divided into two sub-pathways: SG and GA. In the biosynthesis of SGs, after a few changes in *ent*-kaurenoic acid, firstly stevioside and then rebaudioside-A are produced. The same starting chemical, *ent*-kaurenoic acid, is processed through the other pathway (GA biosynthesis) to produce GA 3, 4, and 7 (Brandle and Telmer, 2007; Yamaguchi, 2008).

be the starting material for producing more shoots. In this regard, internodes of suitable length should be excised and subcultured onto a medium. Moreover, explants with longer internodes can have a higher survival rate, biomass, and leaf area (Dong et al., 2010). Cytokinins generally increase the number of nodes but shorten the shoot and consequently internodes, hence it is hard to handle short explants, which can virtually leave no internodes to excise. On the other hand, gibberellins (GAs) promote the growth of stem (Yoneda et al., 2018). In addition, since *ent*-kaurenoic acid is a common substrate (Richman et al., 1999) in the biosynthesis pathway of GAs and SGs that is further modified to produce the end products (e.g., GA_3, GA_4, GA_7, and Reb-A and Ste), there may be an effect on the production of SGs after the application of GAs (Fig. 1).

In *S. rebaudiana*, *ent*-kaurene synthase and *ent*-copalyl diphosphate synthase genes are highly expressed in mature leaves, where SGs are produced (Richman et al., 1999), whereas in *Arabidopsis* they are mostly expressed in young and growing tissues, where GAs are synthesized (Helliwell et al., 1998). In addition, it was suggested that GA could have negative control over the expression of *GA20ox* and *GA3ox*, two enzymes that produce active GAs from biologically inactive GAs (Hedden and Phillips, 2000). Among more than a hundred GAs identified from plants, a few of them are biologically active, i.e., GA_1, GA_3, GA_4, and GA_7 (Yamaguchi, 2008). However, there is limited information about the effects of GA_3 on stevia growth and the production of SGs. Moreover, to the best of our knowledge, no study has investigated the effects of $GA_{4/7}$ on *Stevia*. Based on the scholarly literature on *in vitro* regeneration of *S. rebaudiana* with the aim of enhancing propagation and SG yields, only a limited number of plant growth regulators from three phytohormone classes, i.e., BAP (Khalil et al., 2014; Tomaszewska-Sowa et al., 2015; Yücesan et al., 2016a, 2016b), 2,4-D, GA_3, (Khalil et al., 2014; Tomaszewska-Sowa et al., 2015), and kinetin (Yücesan et al., 2016a, 2016b), have been tried. Therefore, the present study was conducted to evaluate the effects of $GA_{4/7}$ for the first time in comparison with GA_3, kinetin, and BAP on *in vitro* propagation, morphological traits, and content of SGs (i.e. Ste and Reb-A) in *S. rebaudiana*.

2. Materials and methods

2.1. Plant materials

2.1.1. In vitro culture of seeds

Seeds of *S. rebaudiana* (0.5 mL in volume) were put into a microcentrifuge tube (2 mL) containing 1 mL of 70% (v/v) ethanol. After 3 min of vigorous vortexing, the sterilized seeds were spread over a few sterile filter papers under a laminar flow hood to be dried for 5 min. For germination, MS basal medium (Murashige and Skoog 1962) plus 30 g L^{-1} sucrose was used without any growth regulator. The pH of the medium was adjusted to 5.8 before adding 3 g L^{-1} Phytagel™ prior to autoclaving at 121 °C for 15 min. Afterward, the medium was poured into 90 × 15 mm disposable plastic Petri dishes (25 mL into each of them). For efficient germination, the Parafilm-sealed plates were kept in a growth chamber at constant temperature of 22 ± 2 °C (16/8 h photoperiod) under radiation of 35 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Master TL-D 840, Philips, Pila, Poland) at 70 ± 10% relative humidity.

2.1.2. Growing medium compositions and incubation conditions

For a comparative study on the *in vitro* growth, morphological characteristics, and SG contents of stevia, completely randomized designs with nine replicates for four discrete treatments were used. The treatments were as follows: control, MS medium supplemented with 30 g L^{-1} sucrose; KB 0.5, control supplemented with two plant growth regulators, i.e., 0.5 mg L^{-1} kinetin and 0.5 mg L^{-1} BAP; $GA_{4/7}$ treatment, 1 mg L^{-1} $GA_{4/7}$ added to control medium; and the last one consisted of control medium plus 1 mg L^{-1} GA_3. For solidifying, 3 g L^{-1} Phytagel™ was added to the media after adjusting pH to 5.8. After the media were autoclaved at 121 °C for 15 min, about 45 ± 5 mL of each medium was poured in autoclaved Magenta vessels under the laminar flow hood. At first, after excision of the radicle part of all the plantlets germinated in Petri dishes, they were transferred to a hormone-free primary medium (the same as the control) to produce 3–5 nodes. After excision of the internodes, the nodal segments were subcultured once more on the same medium and under the same conditions. Next, nodal segments (1.5 ± 0.5 cm in length) from 4-week-old stevia plantlets were excised and subcultured in vessels containing the four above-mentioned media. Afterward, the vessels were transferred to a growth chamber and kept in the same conditions as mentioned before for two weeks when the samples were used for data collection.

2.2. Observations

2.2.1. Fresh and dry weights

Immediately after being detached from the shoots, the leaves were weighed on a digital scale with 0.001 g readability. For dry weight measurement, the weighed fresh leaves were kept at 40 °C for 72 h in an oven. The dried matter was weighed on a digital scale with 0.01 mg readability (Mettler AT261 DeltaRange®, Switzerland).

2.2.2. Leaf growth parameters

After the detached fresh leaves of each explant were weighed, they were spread on a scanner straight away. The images were saved as Tag Image File Format (TIFF) files at a resolution of 300 × 300 dpi with a bit depth of 24. For the evaluation of leaf numbers, area, and length, the software Easy Leaf Area was used (Easlon and Bloom, 2014). A red scale of 4 cm^2 area in the same plane as the leaves was used for the program for comparison as a reference scale. To identify all leaf and scale areas, the setting sliders for leaf minimum Red RGB and scale red ratio were manually adjusted for each sample. After adjusting leaf minimum Green RGB to 220 and scale area value to four, the images were processed with the software. After images were highlighted using the software, they were saved in 96 × 96 dpi, a bit depth of 24, as uncompressed TIFF files. The data obtained from the software, i.e., each leaf area

individually, total leaf area, each leaf length individually, and total leaf length, were saved in a comma delimited (CSV) format of Microsoft Excel (2013).

2.2.3. Shoot growth parameters

After the leaves were removed, auxiliary shoot length was measured using a ruler from the branching point to the apex of the shoot. To investigate the effect of the plant growth regulators on shoot elongation, the length of the internodes was also recorded, to find out the specific effect of the applied hormones on the growth of basal to apical internodes. The internodes from the base to the apex were named the first, second, and third internodes.

2.3. Steviol glycoside extraction and high performance liquid chromatography (HPLC) analysis

The content of the samples was extracted by a modified method previously published (Yücesan et al., 2016b). The leaf samples were dried at 70 °C over 2 days. The dried leaves were triturated using a mortar and pestle. To extract the SGs, 20 mg of the corresponding powder was weighed (using a digital scale with 0.01 mg readability) and added to microcentrifuge tubes filled with 1 mL of 70% (v/v) methanol. The samples were ultrasonicated over 30 min at 50 °C. After sonication, the samples were centrifuged for 10 min at 12000 × *g*. The supernatants were filtered through a 0.2 μm PTFE filter mounted on a syringe.

The SG content of the samples was analyzed using a modified method adapted from Ceunen et al. (2012) and Yücesan et al. (2016a). In brief, 10 μL of methanolic extract was injected into an HPLC system (WPS-3000-SL SemiPrep-Autosampler; Thermo-Fisher Scientific). The mobile phase was a gradient of acetonitrile (ACN): water (t_0: 34% ACN, 4 min: 35% ACN, 10 min: stop), the pH of which was adjusted to 2.6 with phosphoric acid (H_3PO_4). The flow rate of 1 mL min^{-1} was adjusted with a binary pump (LPG 3400SD; Thermo-Fischer Scientific, Waltham, MA). Absorbance was detected (MWD-3100 UV–Vis Detector; Thermo-Fisher Scientific) at 210 nm, while 356 nm was the reference wavelength. The analytical column was an Inertsil® ODS-3, 150 × 4.6 mm with 5-μm particle size, obtained from GL Sciences Inc. (Tokyo, Japan). The column was kept warm at 40 °C in a column oven system (TCC-3000SD; Thermo-Fisher Scientific). For quantification, Ste and Reb-A (purity ≥ 98.5% and 96%, respectively) were dissolved in 70% methanol and used as external standards. The standards were prepared as a 1000 ppm solution, from which a series of two-fold dilutions was prepared (up to 15.625 ppm). The results were reported as milligrams of SGs per gram of dry leaves (mg g^{-1}).

2.4. Experimental design

The experiment was carried out in a completely randomized design (i.e., four hormonal treatments) with nine replicates. The results are expressed as milligram (fresh or dry weights), ratio (fresh to dry weight, specific leaf area or SLA, leaf length to its area), millimeter (leaf, auxiliary shoot, and internode lengths), centimeter square (leaves' area), and number of component for each plant (leaf).

2.5. Statistical analysis

For all data, the homogeneity of variances (assessed using Levene's test) and normality assumptions (assessed using Shapiro–Wilk (S-W) and Lilliefors corrected Kolmogorov–Smirnov (K-S) tests were explored. The data recorded for fresh weight, leaf area and length, auxiliary shoot length, specific leaf area (SLA), Ste, Reb-A, Ste/Reb-A, and Ste + Reb-A were subjected to one-way analysis of variance (ANOVA), and means were compared using Tukey's test at 5% level of significance ($p < .05$) to examine the differences between groups. For the data recorded for dry weight and the first internode length *Welch's* adjusted *F* ratio for one-way ANOVA and Games–Howell post hoc tests were run ($p < .01$). The analyses' results were used for estimating effect size (ES) of the independent variables (IV) through computing omega-squared and adjusted omega-squared values (ω^2 and $est.\omega^2$, respectively) (Cohen, 1988; Field, 2013). The data recorded for fresh to dry weight ratio (FW/DW), leaf number, and the second internode length were analyzed by the Bonferroni corrected Dunn's post hoc test to protect against inflation of the familywise Type I error rate after the Kruskal–Wallis (K-W) test, for which statistical analysis was two-sided, and $p < .05$ adjusted for multiple comparisons was considered significant. After performing the K-W test, to estimate the ES of the IVs, the value of epsilon-squared (ε^2) was calculated (King et al., 2011). To further investigate the effect of the treatments on Ste content, the value of a corrected measure of Cohen's *d* (Hedges' *g*) index was calculated using the means and standard deviations (Cohen, 1988; Hedges, 1981). To compare the results of the present experiment with others' records published previously, single-sample *t*-test analysis was run between them. A Windows™ based SPSS® program (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used for statistical analysis and graph drawing.

3. Results

3.1. Effect of the hormonal treatments on growth parameters

3.1.1. Leaves' fresh weight

S-W and K-S tests showed the assumption of normality was met and Levene's test showed the assumption of homogeneity of variance was met ($F(3, 32) = 1.918$, $p = .147$). FW of the leaves was highly affected by the hormonal treatments ($F(3, 32) = 11.25$, $p < .001$, $\omega^2 = 0.46$). ANOVA of the means and Tukey's post hoc test ($p < .01$) indicated that the treatment with kinetin and BAP both at 0.5 mg L^{-1} (KB 0.5) produced the highest FWs, while the control resulted in the lowest FWs (Fig. 2a). In the present experiment, the Kendall's tau-b (τ_b) correlation coefficient was computed to determine the relationship between FW and SG contents. Reb-A, Ste, and their ratio did not show any statistically significant relationships with FW (n = 12, $p = .131$, .337, and 0.891, respectively), but Reb-A + Ste values were positively correlated with FWs ($\tau_b = 0.485$, n = 12, $p = .028$). This is noteworthy because for the first time a relationship between FW and SGs has been statistically confirmed.

3.1.2. Leaves' dry weight

Normality assumptions were met according to S-W and K-S tests, whereas Levene's test showed the assumption of homogeneity of variance was not met ($F(3, 32) = 5.574$, $p = .003$). Therefore, *Welch's* adjusted *F* ratio analysis and Games–Howell post hoc test ($p < .01$) were used for comparison of means. The effects of the hormonal treatments on leaf dry weight were significantly different (*Welch's* $F(3, 16.5) = 16.956$, $p < .001$, $est.\omega^2 = 0.57$). The leaves treated with KB 0.5 and GA_3 produced the highest DWs ($p = .103$), while the lowest DWs were produced with the control and $GA_{4/7}$ treatments ($p = .334$) (Fig. 2b). Similar to the FW case, Reb-A, Ste, and their ratio did not show any significant relationships with DW (n = 12, $p = .217$, .493, and 0.891, respectively). Reb-A + Ste values with a weak probability of significance seemed positively correlated with fresh weights ($\tau_b = 0.364$, n = 12, $p = .100$).

3.1.3. The ratio of fresh weight to dry weight

The FW/DW results from three of the four treatments were normally distributed. Although based on Levene's test the assumption of homogeneity of variance was met ($F(3, 32) = 0.956$, $p = .425$), to guard against the bias of repeated testing effects, the treatments' effects were compared using one-way ANOVA on ranks. The K-W test showed that there was a statistically significant difference in the FW/DW ratio between the hormonal treatments, $\chi^2(3, N = 36) = 7.927$; $p = .048$, with

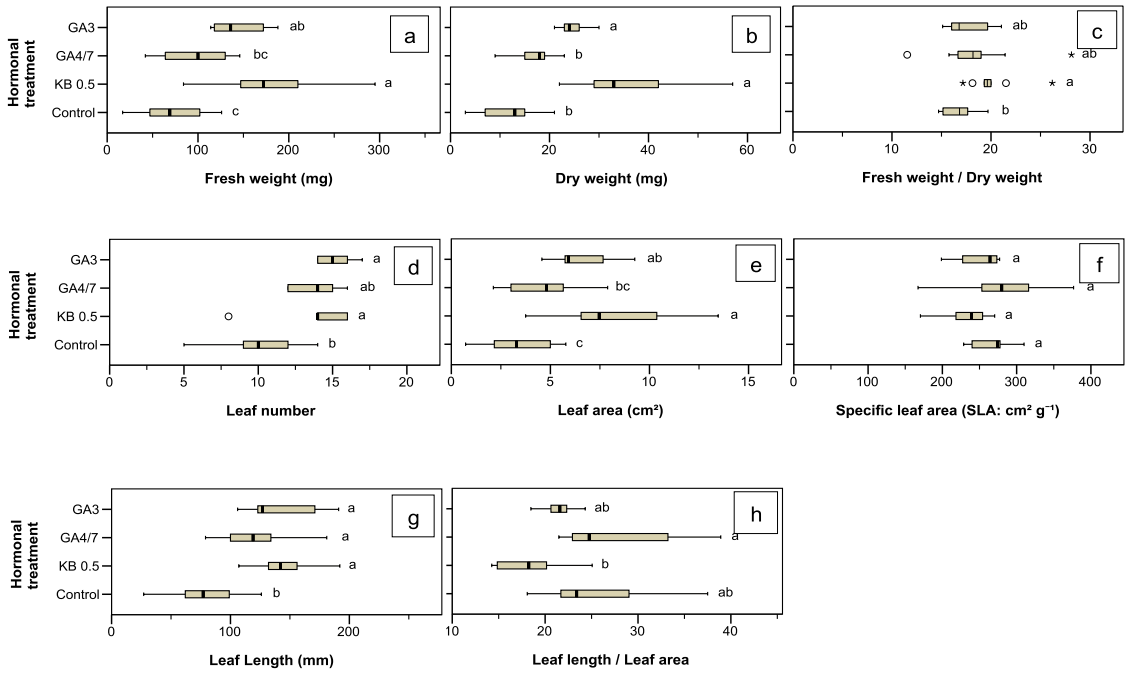

Fig. 2. Effects of hormonal treatments on *in vitro* stevia growth and morphology. The effects of treatments, i.e., control, 0.5 mg L^{-1} kinetin + 0.5 mg L^{-1} BAP, 1 mg L^{-1} $GA_{4/7}$ or GA_3, on: **a** leaves fresh weight, **b** dry weight, **c** fresh weight/dry weight, **d** leaf number, **e** leaf area, **f** specific leaf area, **g** leaf length, and **h** leaf length/leaf area were investigated. Level of significance for all the analyses was $p < 0.05$, except for dry weight ($p < 0.01$).

a mean rank FW/DW ratio of 12.72 for the control, 26.22 for KB 0.5, 18.72 for $GA_{4/7}$, and 18.33 for GA_3. A step-down post hoc with the Bonferroni corrected Dunn's test indicated that KB 0.5 had the highest ratio, while the control group had the lowest ratio (Fig. 2c). The ES of the treatments on the FW/DW ratio was $\varepsilon^2 = 0.227$.

3.1.4. Number of leaves

The data for the number of leaves that grew on each explant were tested for normality and homogeneity of variance. The S-W and K-S tests did not confirm normality for two of the four hormonal treatments, although Levene's test confirmed the homogeneity of variances ($F(3, 32) = 1.45$, $p = .247$). To avoid possible violation of ANOVA assumptions, a K-W test was conducted instead. The mean ranks for the control, KB 0.5, $GA_{4/7}$, and GA_3 were 7.83, 22.11, 18.89, and 25.17, respectively. A statistically significant difference between the hormonal treatments allowed us to examine the equality of the treatments using the Bonferroni corrected Dunn's post hoc test ($\chi^2(3, N = 36) = 14.846$; $p = .002$). GA_3 and KB 0.5 produced more leaves from each plantlet. However, the control medium resulted in the lowest number of leaves (Fig. 2d). The ES of the treatments on the number of leaves was $\varepsilon^2 = 0.453$.

3.1.5. Leaf and specific leaf areas

The data for LA met the assumptions of both S-W and K-S tests of normality and Levene's test of homogeneity ($F(3, 32) = 2.308$, $p = .095$). ANOVA showed that the differences among the treatments' effects were statistically significant ($F(3, 32) = 9.068$, $p < .001$, $\omega^2 = 0.4$). Tukey's post hoc test indicated that while KB 0.5 and GA_3 induced leaves with the largest areas, the control and $GA_{4/7}$ treatments resulted in the smallest leaf areas ($p < .05$) (Fig. 2e). After being confirmed for normality and homogeneity ($F(3, 32) = 1.037$, $p = .389$), the means of SLA were subjected to ANOVA. The differences among the treatments' effects on SLA were not statistically significant ($F(3, 32) = 1.834$, $p = .161$) (Fig. 2f).

3.1.6. Leaf length

Lengths of all the leaves from each explant were summed, and the result was termed leaf length. The analysis of normality (S-W and K-S tests) and homogeneity (Levene's test) permitted us to compare the means of leaf lengths statistically ($F(3, 32) = 0.016$, $p = .997$). ANOVA and Tukey's post hoc test confirmed that the differences among the treatments were statistically significant ($F(3, 32) = 8.248$, $p < .001$, $\omega^2 = 0.38$). Leaves affected by the hormonal treatments were the longest, while the control induced the shortest ones ($p < .05$) (Fig. 2g).

3.1.7. Leaf length to leaf area ratio

The ratio of leaf length in a sum to leaf area in a whole was statistically evaluated. Although S-W and K-S tests confirmed the normality of ratios for most of the treatments, Levene's test based on mean did not confirm homogeneity ($F(3, 32) = 5.559$, $p = .003$). However, based on median, the assumption of homogeneity of variances was met ($F(3, 32) = 1.747$, $p = .177$). Therefore, a K-W test was conducted to rank the means. The test result was statistically significant ($\chi^2(3, N = 36) = 12.842$; $p = .005$). The mean ranks were 22.11, 9.56, 26.22, and 16.11 for the control, KB 0.5, $GA_{4/7}$, and GA_3, respectively. While $GA_{4/7}$ gave the highest ratio of leaf length to leaf area, KB 0.5 resulted in the lowest ratio (Fig. 2h). The ES of the treatments on the ratio was $\varepsilon^2 = 0.367$.

3.1.8. Auxiliary shoot length

The data for the length of the auxiliary shoots from different treatments were normally distributed and homogeneous ($F(3, 68) = 2.714$, $p = .052$). A significant ANOVA was followed by Tukey's post hoc test ($F(3, 68) = 55.152$, $\omega^2 = 0.69$). The control had the shortest (10.56 mm) auxiliary shoots, while $GA_{4/7}$ induced the longest ones (55.89 mm). KB 0.5 and GA_3 treatments had the same effect on the mean of auxiliary shoot length (Fig. 3a).

3.1.9. Internode lengths

The results of the treatments' effects on the first internode met the normality assumption, but the assumption of homogeneity of variance was not met ($F(3, 68) = 6.893$, $p < .001$). Therefore, *Welch's* adjusted F ratio was used to investigate significant differences between the groups. A statistically significant *Welch's* adjusted F test enabled us to

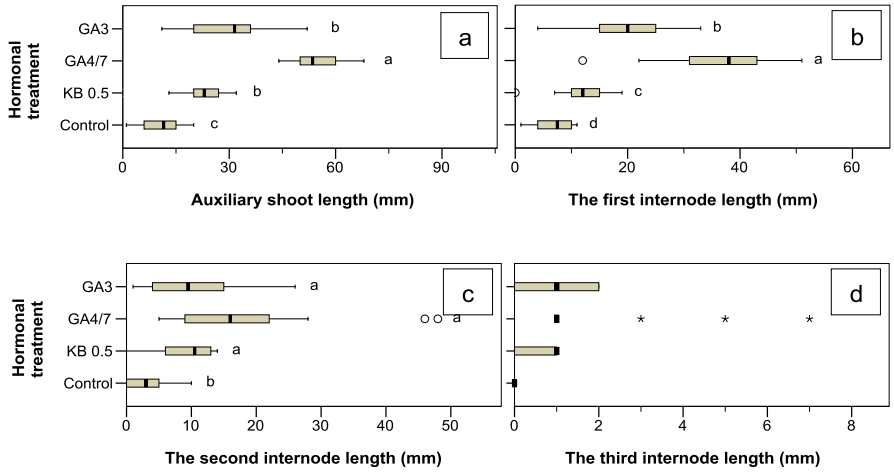

Fig. 3. Effects of hormonal treatments on *in vitro* stevia shoot growth. The effects of treatments, i.e., control, 0.5 mg L^{-1} kinetin + 0.5 mg L^{-1} BAP, 1 mg L^{-1} $GA_{4/7}$ or GA_3, on: **a** shoot length, **b** first internode length, **c** second internode length, and **d** third internode length were investigated. Level of significance for all the analyses was $p < 0.05$, except for first internode length ($p < 0.01$).

compare the means based on the Games–Howell post hoc procedure (*Welch's* $F(3, 35.032) = 55.524$, $p < .001$, $est.\omega^2 = 0.694$). While $GA_{4/7}$ produced the longest first internodes, the control gave the shortest ones (Fig. 3b).

The normality assumption was not met for the second internode length. After checking the homogeneity of variance based on median and with adjusted df ($F(3, 15.259) = 3.703$, $p = .035$), a K-W test was used instead. The ranks for the treatments were 16.75 for the control, 37.56 for KB 0.5, 52.64 for $GA_{4/7}$, and 39.06 for GA_3 treatment. After a significant difference was found among the mean ranks (χ^2 (3, $N = 72$) = 27.137; $p < .001$), they were further compared using Dunn's (Bonferroni adjusted) post hoc test ($p < .05$). The control resulted in the shortest second internodes, whereas the three others with longer internodes were statistically similar (Fig. 3c). The computed ES for the treatments on the variable was $\varepsilon^2 = 0.382$. This study is the first investigating changes in the growth of stevia internodes. Based on the results, the second internode length's contribution to auxiliary shoot elongation was less than that of the first.

The data for third internode length violated all assumptions (normality, homogeneity, and equality of variances). The explants on the control did not produce the third internode. However, the hormonal treatments mostly induced the third internode. Among the treatments, all the explants subjected to the $GA_{4/7}$ treatment grew the third internodes and at the same time the longest ones (Fig. 3d).

3.2. Steviol glycoside content analysis

The results of HPLC analysis of leaf extracts were subjected to statistical analysis. The analyses were performed on Ste and Reb-A contents, the ratio of Ste to Reb-A (Ste/Reb-A), and the sum of the SGs (Ste + Reb-A). K-S and S-W tests confirmed that all the data were normally distributed. In addition, Levene's test confirmed homogeneity of variance based on means for all the data (Ste: $F(3, 8) = 0.058$, $p = .980$; Reb-A: $F(3, 8) = 0.169$, $p = .914$; Ste/Reb-A: $F(3, 8) = 1.57$, $p = .271$; Ste + Reb-A: $F(3, 8) = 0.024$, $p = .994$). The means of the data were, therefore, compared using ANOVA. The results of the analysis for Reb-A and Ste/Reb-A were statistically significant (Reb-A: $F(3, 8) = 23.559$, $p < .001$; Ste/Reb-A: $F(3, 8) = 124.115$, $p < .001$); however, for Ste and Ste + Reb-A ANOVA was not statistically significant (Ste: $F(3, 8) = 2.785$, $p = .11$; Ste + Reb-A: $F(3, 8) = 1.407$, $p = .31$) (see Fig. 4). Post hoc analysis of Reb-A contents showed that while the control and KB 0.5 produced the highest amount of Reb-A, $GA_{4/7}$ resulted in the least amount of it (Fig. 4a).

Although ANOVA showed that the Ste content of the extracts was not significantly different ($p = .099$) (Fig. 4b), the ratio of Ste to Reb-A was significantly different between the treatments. This ratio was the largest for $GA_{4/7}$-treated explants while it was smallest for the control and KB 0.5-treated explants (Fig. 4d). However, Ste + Reb-A contents in leaves of treated explants were not significantly different ($p = .336$) (Fig. 4e). Since post hoc analysis suggested that the probability of significant differences among Ste contents of the treated explants was promising ($p = .099$), it was analyzed further using independent-samples *t*-test. In spite of the fact that the pair-wise comparison was not statistically significant ($p > .05$), the ES of $GA_{4/7}$ was statistically promising (Hedges' $g = 1.788$, SE = 0.97, 93.5% CI [0.006, 3.57], $t(4) = 2.744$, $p = .052$). Hedges' *g* test suggested that $GA_{4/7}$ had a significantly large effect on the Ste content of stevia (Fig. 4c).

4. Discussion

Supplementing *in vitro* medium with chitosan tripled FWs after four weeks compared with the control (Bayraktar et al., 2016); however, KB 0.5 increased FW 2.5-fold after 2 weeks. It was observed that elicitors had no effect on dry weight (Bayraktar et al., 2016). Spraying GA_3 at 250–1000 mg L^{-1} onto *S. rebaudiana* leaves did not change dry weight significantly (Karimi et al., 2015), while in the present experiment GA_3 at 1 mg L^{-1} significantly increased dry weight under *in vitro* conditions. In contrast, 1 mg L^{-1} $GA_{4/7}$ *in vitro* supplementation gave a result comparable to that of the control.

In a previous study, kinetin or BAP produced the highest number of leaves at 0.5 mg L^{-1}, while a higher or a lower amount of the chemicals produced the lower number (Singh and Dwivedi, 2014). After one month, the explants in 2 mg L^{-1} BAP produced a very similar number of leaves (M = 10.5, SE = 1.19) in comparison with the number of leaves produced in the control (M = 10.11 mm, SD = 3.14) in the present experiment (0.399, 95% CI [−2.8 to 2.02], $t(8) = -0.372$, $p = .720$). The similar number of leaves despite growing over half of the duration mentioned in Singh and Dwivedi's (2014) report might be ascribed to varietal differences or different ambient conditions.

Yoneda et al. (2018) reported that *ex vitro*-grown stevia plants soaked weekly with 4 mg L^{-1} GA_3 after 4 weeks produced smaller leaf areas than the control group. Their observation is not consistent with ours, possibly due to a shorter growing period. Similarly, the effect of half out of about 15–20 different treatments on stevia plants after 4 weeks was not statistically significant (Yoneda et al., 2017, 2018). Bayraktar et al. (2016) applied some elicitors and most of them shortened the length of the leaves.

Yücesan et al. (2016b) subjected stevia nodal explants to hormone-free, kinetin 1 mg L^{-1}, and BAP 1 mg L^{-1} treatments. Only their hormone-free treatment was similar to ours, for which a single-sample *t*-test comparison suggested that the samples from the present experiment records (M = 10.56 mm, SD = 5.7) and Yücesan et al.'s (2016b) records (M = 26 mm, SE = 1) for shoot length in hormone-free medium could not be from the same population. The statistically significant

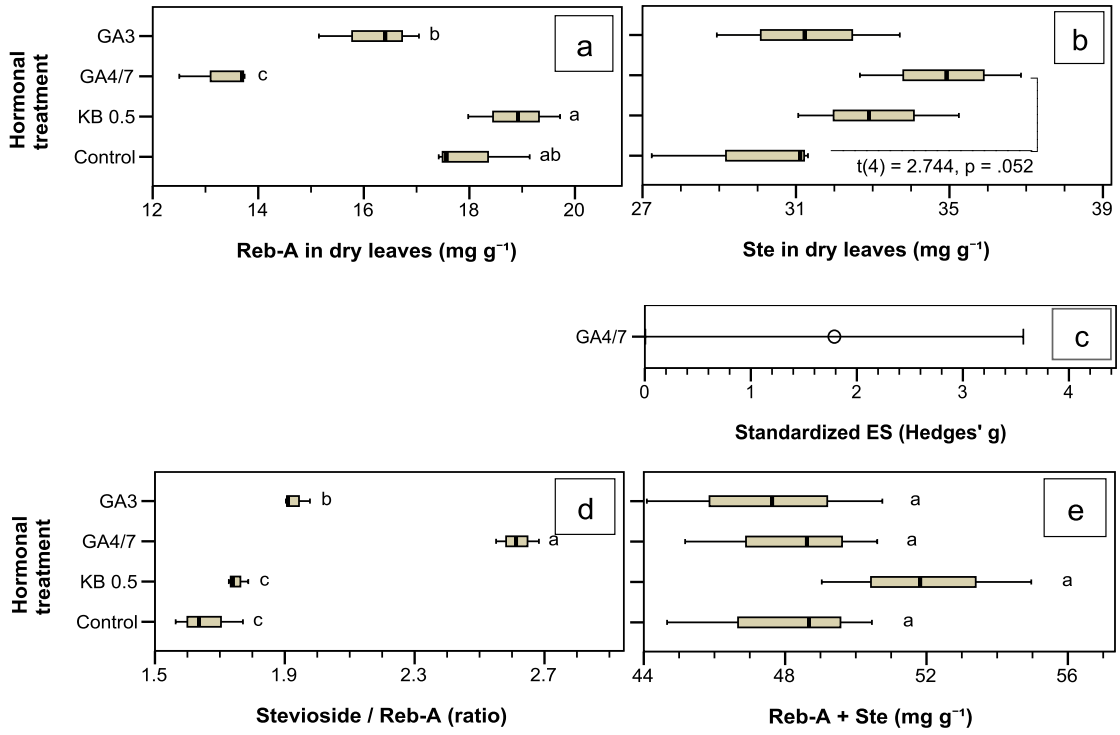

Fig. 4. Effects of hormonal treatments on *in vitro* stevia's steviol glycoside content. The effects of treatments, i.e., control, 0.5 mg L^{-1} kinetin + 0.5 mg L^{-1} BAP, 1 mg L^{-1} $GA_{4/7}$ or GA_3, on: **a** rebaudioside-A in dry leaves, **b** stevioside in dry leaves, **c** $GA_{4/7}$ effect size on stevioside in dry leaves (93.5% CI [0.006, 3.57], $p = .065$), **d** rebaudioside-A/stevioside, and **e** rebaudioside-A + stevioside were investigated. Level of significance for all the analyses was $p < 0.05$.

difference (15.44 mm, 95% CI [12.61 to 18.28], $t(17) = -11.494$, $p < .001$) may have been due to the difference in treatment duration (2 vs. 3 weeks). Karimi et al. (2015) sprayed GA_3 at 250–1000 mg L^{-1} onto stevia plants, and found that GA_3 at a concentration more than 250 mg L^{-1} could significantly increase the height of plants (LSD, $p \leq .05$). Yoneda et al. (2018) soaked stevia plants once a week with GA_3 solutions at 4 or 10 mg L^{-1} and measured shoot length after 4 or 6 weeks. They observed that the control and 4 mg L^{-1} GA_3 treatments had similar effects; in comparison GA_3 solution at 10 mg L^{-1} significantly increased shoot lengths, whereas after 6 weeks shoot elongation was not dosage dependent (effects of GA_3 solutions at 4 or 10 mg L^{-1} were similar, $p < 0.05$). After 6 weeks, GA soaked plants grew 1.8 and 2 times taller than the control. However, GA_3- and $GA_{4/7}$-treated explants in the present experiment increased shoot length 3 and 5.5 times, respectively, after 2 weeks ($p < 0.001$). The higher differences between our experiment and the aforementioned one might be because of the optimum condition of *in vitro* media or higher activity of $GA_{4/7}$ than GA_3, if it was not due to intervarietal differences.

Hedden and Phillips (2000) concluded that GA itself could have a negative feedback effect on *GA20ox* and *GA3ox* gene expression. Both genes play a major role in biosynthesizing biologically active GAs (Yamaguchi, 2008). On the other hand, *GA2ox* converts the active GAs, e.g., GA_4 but not GA_3, to inactive compounds. It was observed that *GA2ox* activity is higher in the shoot apex (Sakamoto et al., 2001). Therefore, we presume that after using the GAs, particularly $GA_{4/7}$, internode elongation decreased from the basal to the apical internode, perhaps due to *GA2ox* mediated inactivation of the GAs.

Reb-A was firstly isolated from *S. rebaudiana* by Koheda et al. (1976) as much as 1.4% of dry matter. This amount was significantly lower than the Reb-A yield recorded from the control in our experiment (0.404%, 95% CI [0.167 to 0.642], $t(2) = 7.326$, $p < .05$). A comparison of the highest Reb-A induction (KB 0.5: M = 1.887%, SD = 0.87) with the lowest amount of Reb-A recorded for *ex vitro* grown stevia plants in Yücesan et al.'s (2016b) experiment (M = 4.7%, SD = 0.3) showed that the *in vitro* plants could not produce as much as the *ex vitro* plants (2.813%, 95% CI [2.596 to 3.029], $t(2) = -55.916$, $p < .001$). Bayraktar et al. (2016) could not detect Reb-A from about 20 *in vitro* and an *ex vitro* treatments. They detected 0.55 mg g^{-1} dry leaf, which was significantly lower than ours (18.322, 95% CI [16.157 to 20.486], $t(2) = 36.422$, $p = .001$). Karimi et al. (2015) reported that spraying GA_3 at concentrations greater than 250 ppm onto *S. rebaudiana* plants significantly increased Reb-A, while in our experiment GA_3's effect was not greater than that of the control, and $GA_{4/7}$'s effect was actually the worst ($p < 0.05$). Stevia plants soaked with 10 mg L^{-1} GA_3, although not statistically analyzed, produced 3.7-fold more Reb-A than the control (Yoneda et al., 2018). However, we found that treating *in vitro* explants with 1 mg L^{-1} GA_3 over 2 weeks did not change Reb-A content significantly (0.9 times lower than the control, $p = .123$). In addition, $GA_{4/7}$ significantly decreased Reb-A content (1.35-fold higher than the control, $p = .001$).

Although a number of published papers have discussed the effects of exogenously applied GA_3 on the SG content of stevia, the effect of $GA_{4/7}$ has never been investigated. The result of the present experiment suggests that the lower amount of Reb-A produced on $GA_{4/7}$ supplemented *in vitro* medium might be explained by the higher amount of Ste produced under the same conditions. Comparing the ratio of Ste to Reb-A from the $GA_{4/7}$ treatment (M = 2.615, SD = 0.066) with *ex vitro* grown *S. rebaudiana* as reported by Yücesan et al. (2016b) indicated that the ratio in the present experiment was significantly higher (1.147, 95% CI [0.983 to 1.311], $t(2) = 30.089$, $p = .001$). Since genes in the SG pathway are highly expressed in mature leaves, the lower ratio *in vitro* could be due to spatial and temporal expression differences (Richman et al., 1999). However, Karimi et al. (2015) and Hajihashemi and Geuns (2017) observed that GA_3 did not change the ratio significantly. In comparison, in the present experiment the highest ratio recorded for $GA_{4/7}$ treated explants was still significantly lower than the ratio reported by Ceunen et al. (2012) (0.715, 95% CI [0.551 to 0.879], $t(2) = -18.837$, $p = .003$). Considering Richman et al.'s (1999) report, the lower ratio we observed might have been due to the shorter duration of growth (2 vs. 4 weeks) and growth condition (*in* vs. *ex vitro*). *In*

vitro explants excised from *S. rabaudiana* were stimulated using various elicitors. Although the results were not statistically analyzed, in comparison with the control Ste content increased 9-fold (Bayraktar et al., 2016). However, Singh and Dwivedi's (2014) result is far from expected, because they observed that *in vitro* plants produced more Ste than *ex vitro* plants (7.017 ± 0.058 vs. 9.236 ± 0.046). In the present experiment, only *in vitro* grown explants were researched; hence no comparison was made with *ex vitro* ones. Vives et al. (2017) compared different *in vitro* propagation media, i.e., liquid or semisolid media and a temporary immersion bioreactor (BIT®). BIT® significantly produced more total SGs in 1 g of dry matter than the other media ($M = 43.4$, $SE = 3.11$). However, the amount of Reb-A + Ste in the present experiment was significantly higher than that of BIT® (5.473, 95% CI [3.456 to 7.49], $t(11) = 5.972$, $p < .001$), which also might have been due to varietal differences.

Consistent with our result, others also observed that GA_3 treatment did not change Ste content significantly (Karimi et al., 2015). Supplementing GA_3 and its biosynthesis inhibitor (paclobutrazol) into *in vitro* media to research their effects on expression levels of six genes in SG biosynthesis indicated that after GA_3 treatment kaurene oxidase was upregulated, whereas expressions of *UGT76G1* and *UGT85C1* were almost stable. However, GA_3 (and SGs) biosynthesis inhibitor downregulated the expression of all three genes (Hajihashemi et al., 2013). Yoneda et al. (2018), in contrast, reported that 4 or 10 g L^{-1} GA_3 did not change kaurene oxidase or *UGT76G1* expression levels, but upregulated *UGT85C2*. In addition, they recorded 1.4 times more Ste production in comparison with the control (not statistically analyzed), while in the present experiment the effect of GA_3 and the control on Ste production was very similar ($p = .865$). Interestingly, in comparison with the control, $GA_{4/7}$ resulted in a significantly lower amount of Reb-A ($p = .001$), while the GA_3 effect was not significantly different ($p = .123$). A few groups studied the effect of a GA biosynthesis inhibitor (daminozide, inhibits the pathway after divergence from the SG pathway). Daminozide did not change the total amount of SGs significantly, which suggested that GA biosynthesis is not a limiting factor for SG biosynthesis (Karimi et al., 2015). GA_3 in the present experiment was used at 1 mg L^{-1} level, while the others used levels as high as 10 mg L^{-1} (Hajihashemi et al., 2013; Hajihashemi and Geuns, 2017; Yoneda et al., 2018), 21.5 mg L^{-1} (Kumar et al., 2012), and 1000 mg L^{-1} (Karimi et al., 2015). Therefore, the reason behind the contradictory reports might have been the use of a diverse amount of GA_3, which may have resulted in negative feedback in GA and SG biosynthesis feedback.

5. Conclusion

The results of the present experiment showed that the effects of GA_3 and $GA_{4/7}$ on stevia growth were statistically similar, except for auxiliary shoot growth, which with $GA_{4/7}$ grew more than with GA_3 treatment. However, the effects of the GAs on the content of SGs were different. $GA_{4/7}$ treatment decreased Reb-A while increased the amount of Ste yields in comparison with the control and GA_3-treated explants. As a result, the ratio of Ste to Reb-A significantly increased in $GA_{4/7}$-treated plants compared with the other treatments. However, the total amount of Ste and Reb-A did not change in any treatments. The effects of the applied cytokinins (kinetin and BAP) on almost all growth parameters were statistically similar to those of GA_3 treatment. However, the effects of cytokinins on Ste and Reb-A yields, their ratio, and the total amount of them were not statistically different from those of the control. Therefore, it seems that the application of GAs in stevia plants has a negative effect on Reb-A accumulation but such an effect on Ste is not highly significant. In addition, the negative effect of $GA_{4/7}$ on Reb-A and Ste yields was significantly greater than that of GA_3. The results suggest that the GAs may change the ratio of Reb-A to Ste but not the total content of SGs in *S. rebaudiana*.

Contributions

A.P. and F.A. have contributed equally to this work. A.P. and F.A. conceived and designed the research, performed the experiments, analyzed the data, and wrote the article; B.Y. provided the seeds and helped with HPLC analysis; B.Y. and S.G. improved and commented on the article. S.G. supported the research. All authors read the article and approved the final version.

Declarations of interest

The authors declare no conflict of interest.

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