

Daminozide Enhances Vigor and Steviol Glycoside Yield of Stevia (*Stevia rebaudiana* Bert.) Propagated in the Temporary Immersion Bioreactor

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Abstract

Stevia (*Stevia rebaudiana* Bertoni) contains sweet compound widely used as natural sweetener, steviol glycoside (SG). SG is a diterpenoid secondary metabolite synthesized from *ent-kaurenoic acid*, the same precursor of Gibberellin (GA). Therefore, in this study, a GA inhibitor, Daminozide (0, 10, 20 ppm) was used to block *ent-kaurenoic acid* conversion towards GA synthesis in attempt to increase SG content of stevia propagated in *Temporary Immersion Bioreactor* (TIB). Daminozide in 10 mg/L was observed to be the optimum concentration which increased biomass weight and SG content (stevioside and rebaudioside A) up to 40%. The treatment also increased transcripts accumulation of genes enrolled in SG biosynthesis, such as *SrKA13H*, *SrUGT85C2*, and *SrUGT76G1*, indicating SG pathway become more active due to the inhibition of GA pathway. Furthermore, the inhibition of GA was also indicated by the upregulated expression of GA biosynthesis gene (*GA3ox*) as the result of feedback regulation, and the downregulated expression of GA catabolism gene (*GA2ox2*) as the result of feed-forward regulation caused by inhibitor treatment.

Key Message

This study reports data regarding the influence of inhibition to the late-stage of GA biosynthesis by chemical treatment in modulating SG yield from stevia *in vitro* culture; SG and GA pathways are related.

Introduction

Diabetes is still a serious threat to global health. About 463 million people in the world living with diabetes, and it is estimated to be 51% increase by 2045, even 74% increase in south-east Asia (IDF 2019). This is fueled people nowadays to demand for sugar substitute which has lesser calorie and glycemic index. *Stevia* (*Stevia rebaudiana*), or also called honey leaves, provides natural image sweetener along with its zero calorie and zero glycemic index properties. Since receiving USA approval in 2008 and EU approval in 2011 (Yadav and Guleria 2012), stevia now has wider use in various food, beverage, and pharmaceutical products, as the global stevia market is expected a market growth of 8.2% during 2019 to 2025 (Industry Research 2019).

Due to high demand of stevia, efficient method for mass production of the plant materials is in urge. Hence, it should be done by *in vitro* propagation in the bioreactor system. *Stevia* is easily propagated by *in vitro* shoot cuttings, with a single cut of node growing 6-14 nodes in four weeks in media without growth regulators (Singh and Dwivedi 2014). Moreover, bioreactor is used for scale up purpose which provide more space for plant growth. However, hyperhydricity sometimes occurs in the liquid culture-based system, thus causing growth disorders (Debnath 2011). Therefore, bioreactor with temporary immersion system (*Temporary Immersion Bioreactor*, TIB) This system is featured with devices allowing automatic control for periodically contact of liquid media to the plant (Steingroewer et al. 2013), thus prevent hyperhydricity. Several types of TIB have been developed and used for micropropagation of industrial plants. One of them is RITA® Bioreactor System (<https://cirad.fr>, CIRAD, France). The RITA®

bioreactor consists of an autoclavable polypropylene container having two compartments separated by a table supported with grid and a central pipe. The upper container is for the plant while the lower container is for the liquid media. The container cap is supported with centric connected with air controlled using a timer, and secured with Millipore filters (Georgiev et al. 2014). The RITA® Bioreactor System have been successfully used for mass production of stevia (Ramírez-Mosqueda et al. 2016; Vives et al. 2017; Bayraktar 2019).

Furthermore, another concern in the production of stevia is to escalate its sweetness property, which come from the sweet compound, steviol glycoside (SG). SG is a steviol aglycon attached by sugar at its C13-hydroxyl site and C19-carboxylic acid site (Hellfritsch et al. 2012). SGs differ in their type and number of sugars attached to steviol. Stevioside (Stev) and rebaudioside A (Reb A) are the most abundant SG in stevia, account for 5,8 % and 1,8 % of leaves dy weight DW) (Gardana et al. 2010), thus 350 times sweeter than sugar (Mogra and Dashora 2017). These compounds are synthesized from *ent-kaurenoic acid* from methylerythritol 4-phosphate (MEP) pathway in chloroplast and endoplasmic reticulum. *Ent-kaurenoic acid* is then converted into steviol catalyzed by kaurenoic acid 13-hydroxylase (KAH) in cytoplasm (Yoneda et al. 2018). However, in cytoplasm, *ent-kaurenoic acid* is also metabolized into plant hormone Gibberellin (GA) catalyzed by ent-kaurenoic acid oxidase (KAO), followed by 2-oxoglutaric acid-dependent (2ODD) group enzymes such as GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox), and GA 2-oxidase (GA2ox) (Olszewski et al. 2002). Therefore, this correlation may lead to the idea that manipulating these pathways might affect synthesis of SG.

Many researches attempted to increase SG biosynthesis by inhibiting certain stage of GA biosynthesis using retardants applied in *ex vitro* cultivation of stevia. Hajihashemi and Geuns (2017) used Paclobutrazol to block the conversion of ent-kaurene to ent-kaurenoic acid, resulted in decrement both GA and SG. In contrast, Yoneda et al. (2018) applied Daminozide to block the conversion of GA₁₂-aldehyde into bioactive GAs and their derivates, resulted in enhancement of SG level and its biosynthesis genes expressions. Daminozide has similar chemical structure with 2-oxoglutaric acid (2OG) thus can act competitively with 2OG in binding with 2ODD enzymes which catalyze GA late-stage biosynthesis (Rademacher 2000). Interestingly, the use of retardants is also corelated with the increment of plant yield (Karimi et al. 2014; Saptari et al. 2020). Accordingly, this study aimed to provide protocols for *in vitro* propagation of stevia with high biomass yielding and SG content by chemically manipulating SG and GA pathways using Daminozide treatment in TIB. *In vitro* propagation is a solution to cope with the problem of land, climate, and geographical limitations on the stevia cultivation which requires highlands areas for the best productivity.

Methods

Explant preparation

In vitro shoot of stevia was provided by Laboratory of Cell Culture and Micropropagation, Indonesian Research Institute for Biotechnology and Bioindustry (IRIBB). The shoots were cut into each nodal

segment and conditioned in the petridishes (16 cm of diameter) containing 10 mL of sterile liquid media. The media consists of Murashige and Skoog (MS) basal minerals, 30 g/L sucrose, and 0,5 g/L active charcoal, and was adjusted at pH of 5.7. Shoot conditioning was conducted for four days in the culture room equipped with LED lighting 20 $\mu\text{mol}/\text{m}^2\text{s}$ (12 h light/12 h dark photoperiod) and at temperature of 25°C.

TIB culture

TIB culture used the same composition of media as previous step devoid active charcoal. Daminozide treatment consisted of three concentrations, 0 ppm (control), 10 ppm, 20 ppm, added to the media. Media were then sterilized in autoclave with the temperature of 121°C and 1 kg/cm² of pressure, for 15 minutes, then were left overnight before used. Afterwards, 175 mL of sterile media were poured into each TIB vessel aseptically in Laminar Air Flow (LAF). Subsequently, every 40 of conditioned stevia shoots were then inoculated into the TIB. After that, TIB were installed to the system for automation of immersion period being used in the experiment. The experiment used immersion period of 30 minutes every 6 hours. The TIB system were incubated in the culture room equipped with LED lighting 20 $\mu\text{mol}/\text{m}^2\text{s}$ (12 h light/12 h dark photoperiod) and at temperature of 25°C.

Growth measurements

Growth parameter of stevia was observed after 40 days incubation in TIB, including shoot height, number and length of nodes, size of leaves, fresh and dry weight of shoot biomass. Shoot height was measured from base to the highest leaf tip. The leaves size was measured by the length and width of the leaves. Subsequently, weighing the biomass was carried out for the leaves only and total shoot biomass. Data was collected and subjected to the analysis of variance (ANOVA) following by Tukey's post hoc test at significance level of 0.05.

Stevioside and rebaudioside A accumulation

Accumulation of stevioside and rebaudioside A was analyzed using high-performance liquid chromatography (HPLC) based on method reported by Martono et al. (2016). Sample for analysis was prepared by drying the leaves of stevia in the oven at 105° C for five hours. The dry biomass was then mashed into powder and extracted using ultrasound, with 60% ethanol as the solvent. Afterwards, the extract was injected to HPLC system for analysis. The HPLC used Eurosphere C-18 (30° C of temperature) as stationary phase, and for the mobile phase it used the mixture of water-methanol (90:10 v/v, pH = 3,0), acetonitrile, and trifluoro acetic acid (TFA) (65 : 35 : 0,01 v/v/v), at flow rate 0.6 mL/min.

SG and GA metabolic genes transcription analysis

Analysis of SG and GA metabolic genes transcription was conducted using quantitative real time PCR (qPCR) to quantify the transcripts accumulation of genes related to SG and GA metabolism. Sample for the analysis was stevia leaves from 2nd – 3rd nodes of the shoot, after 40 days of culture incubation.

Total RNA (50 mg fresh weight) was extracted from the samples using GeneAll® Ribospin™ Plant, according to the manufacturer's instruction. The RNA purity and concentration were determined using NanoDrop spectrophotometer, while the RNA quality and integrity were assessed with 1.0% agarose gel electrophoresis. Afterwards, the RNA samples with good quality, purity, and concentration were reverse transcribed into cDNA using AccuPower® RT PreMix – Bioneer, according to the manufacturer's instruction. qPCR was then performed using SensiFAST™ SYBR® Hi-ROX One-Step (Bioline) with the thermal cycling program ran at 95° C for 25 seconds of denaturation, 60° C for 15 seconds of annealing, and 72°C for 20 seconds of amplification, repeated for 45 cycles.

SG metabolic-related genes analyzed were *SrUGT76G1* (AY345974.1), *SrUGT74G1* (AY345982.1), *SrUGT85C2* (AY345978.1), *SrKA13H* (DQ398871.3), as for GA metabolic-related genes were *KAO*, *GA2ox1*, *GA2ox2*, *GA3ox*, and *ATH1*. Transcripts accumulation of those genes were compared to internal standard, *SrActin* (AF548026). Primers for amplification of *SrActin*, *SrUGT76G1*, *SrUGT74G1*, *SrUGT85C2*, *SrKA13H*, *KAO*, *GA2ox1*, *GA2ox2*, and *ATH1* were made from *in silico* study using Geneious Prime software, whereas primer for amplification of *GA3ox* was from Singh et al., (2017).

Results And Discussion

Stevia growth on TIB

Morphological growth of stevia cultured in TIB was affected by the Daminozide treatment. Plantlets treated with Daminozide were stunted and having shorter internodes (Fig. 1b) than control. Concentration of Daminozide showed positive correlation with the shortened plantlets height and internodes length (Fig. 1a & Fig. 2). The control has an average plantlets height of $14,6 \pm 2,1$ cm with $1,9 \pm 0,1$ of average internodes length, while the shortest plantlets was derived from 20 ppm Daminozide treatment with $6,5 \pm 1,0$ cm of average height and $1,0 \pm 0,1$ cm of average internodes length (Fig. 1a & Fig. 1b). Daminozide 20 ppm exhibited the strongest effect (55,5%) in shortening plantlets stem, so that plantlets in this treatment have fewer nodes ($6,5 \pm 1,1$) compared to other treatments (Fig. 1c).

These results indicate an interference of GA function in the stem development, whether because of low activities or low concentration of GA. In cells, GA was recognized by GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptor, promoting structural change of GID1 (Ueguchi-Tanaka et al. 2007). It allowed GID1 to interact with DELLA protein causing DELLA to be inactivated and degraded via proteasome SCF^{GID2/SLY1} (Sun 2011). DELLA function as repressors, one of which blocks PIF4 (PHYTOCHROME INTERACTING FACTOR 4), a transcription factor of genes involved in cell and hypocotyl elongation (de Lucas et al. 2008). Meanwhile, application of Daminozide may decrease GA level in cells, restraining DELLA from degradation. These GA-DELLA interaction in hypocotyl and stem elongation have been studied in several plants, such us *Arabidopsis thaliana*, *Oryza sativa*, and *Pisum sativum* (Ueguchi-Tanaka et al. 2007; de Lucas et al. 2008; Weston et al. 2008).

In contrast to the plantlet's height, Daminozide treatment has positive effect on the leave development of stevia cultured in TIB. Plantlets treated with Daminozide 10 and 20 ppm have larger leaves area than control (Fig. 1d and Fig. 2). The maximum leaves size ($3,02 \pm 0,28 \text{ cm}^2$) was obtained from the treatment of 20 ppm Daminozide. This result agrees with many studies reporting the use of GA inhibitor to increase plant vigor resulting in dwarf plants but with sturdier stem and bigger leaves (Tadesse et al. 2000; Kepenek and Karoglu 2011; Karimi et al. 2015; Sumaryono and Sintia 2016). Tadesse et al. (2000) reported Daminozide application into *S. tuberosum* in vitro culture multiplied leaves size of the plantlets.

There were reports on the antagonistic interaction between GA and cytokinins (CK), the key hormones responsible for leaf morphogenesis (Jasinski et al. 2005; Fleishon et al. 2011; Shwartz et al. 2016). GA inhibits CK signaling (Fleishon et al. 2011). Therefore, GA inhibition might allow optimal function of CK on the leaf morphogenesis, as reported on the in vitro culture of tomato, in which elevated its GA level through exogenous GA application emerged tall plantlets with small and less complexity leaves. Meanwhile, reducing GA level or activity resulted in dwarf plantlets with bigger and more complex leaves (Fleishon et al. 2011). Similar result was also observed in this study where GA inhibition through Daminozide application increased leaves size of stevia plantlets. The enhancement of leaves production, both its number or size positively affect plant productivity, especially the plant harvested for its leaves such as stevia. Moreover, leaves are the main organ to produce SG.

Leaves size was responsible for leaves biomass weight. Daminozide also increased fresh biomass weight of the leaves (Fig. 3a), consequent to the increase of leaves size. However, Daminozide did not affect leaves dry biomass weight (Fig. 3). Further measurement showed Daminozide treatment also increased ratio between leaves biomass and stem & root biomass (Fig. 3). The biomass in control was consisted more of stem and root (4:6), while with Daminozide treatment plantlets consecutively yielded more leaves biomass (5:5 and 6:4) (Fig. 3), in correlation with the stem growth inhibition by Daminozide (Fig. 1a & 1b) which also promoted leaves growth (Fig. 1d & Fig. 2). The result is in accordance with Karimi et al. (2019) in which Daminozide maintained leaf yield and harvest index of stevia treated with drought stress.

The enhancement of plant weight occurs either by the increment of cell matters due to photosynthates allocation (Poorter et al. 2012) or by the increment of cell water content (Aharon et al. 2003). Karimi et al. (2015) reported Daminozide application into stevia cultivated in the green house shifted photosynthates allocation to leaves, demonstrated by the increase of leaf dry weight. Therefore, in this study, Daminozide only increased fresh biomass weight while the dry biomass weight was unaffected, indicating in the *in vitro* condition Daminozide might increase cell water absorption and storage capacity, especially the leaf cells. The plant capacity to absorb and store water could maintain plant vigor (Aharon et al. 2003), which often attempted by the application of GA inhibitor such as paclobutrazol (Sumaryono and Sintia 2016; Flores et al. 2018). Daminozide exhibited similar effect with paclobutrazol in maintaining plant vigor. Nevertheless, having different stage of pathway in blocking GA biosynthesis, application of Daminozide in stevia was more beneficial since it also potentially increases SG yield (Yoneda et al. 2018; Saptari et al. 2020).

Sg Yield By The Treatment Of Daminozide

Stevia grown in TIB treated with 10 ppm Daminozide had the highest stevioside ($8,1 \pm 0,4$ mg/g of dry weight) and rebaudioside A content ($14,6 \pm 1,2$ mg/g of dry weight) among treatments, while those in 20 ppm Daminozide treatments had no significant difference with the control. Daminozide 10 ppm significantly increased 42,6% of stevioside and 40,67% of rebaudioside A than control (Fig. 4). The yield of stevioside and rebaudioside A was calculated by multiplying stevioside and rebaudioside A contents with the leaf dry biomass weight. The measurement showed that maximum stevioside ($4,09 \pm 1,05$ mg) and rebaudioside A ($7,45 \pm 1,39$ mg) yields were obtained from stevia TIB cultures treated with 10 ppm Daminozide, about two times higher than control (Table 1). Meanwhile, those treated with Daminozide 20 ppm only exceeded 50,4% of rebaudioside A yield than control.

Table 1

Total stevioside and rebaudioside A yield from stevia after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 5$.

Daminozide concentration (mg.L ⁻¹)	Total stevioside yield (mg)	Total rebaudioside A yield (mg)
0	2.12 ± 0.20 b	3.79 ± 0.57 c
10	4.09 ± 1.05 a	7.45 ± 1.39 a
20	2.67 ± 0.71 b	5.71 ± 0.90 b

The result was slightly different from those reported by Saptari et al. (2020) that applied Daminozide in thin layer liquid culture, in which 10 ppm and 20 ppm Daminozide treatments exhibited similar performance in modulated stevioside and rebaudioside A yields. It indicates specific optimal condition of Daminozide treatment is required for each culture type. Therefore, results from these *in vitro* studies complemented the *ex-vitro* study reported by Yoneda et al. (2018). The optimum concentration for *ex-vitro* application of Daminozide in stevia to modulate SG yield was 200 ppm (Yoneda et al. 2018), thus for *in vitro* application it required about 10 times lower concentration.

Declarations

Acknowledgement

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Author contributions

RTS conducted the experiments, carried out the experimental analysis, and prepared the manuscript. RRE conceptualized the research design and analysis of *in vitro* propagation and metabolite analysis, and evaluated the manuscript. RAP conceptualized the research design and analysis of molecular study, evaluated and edited the manuscript. All authors read and approve the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

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Figures

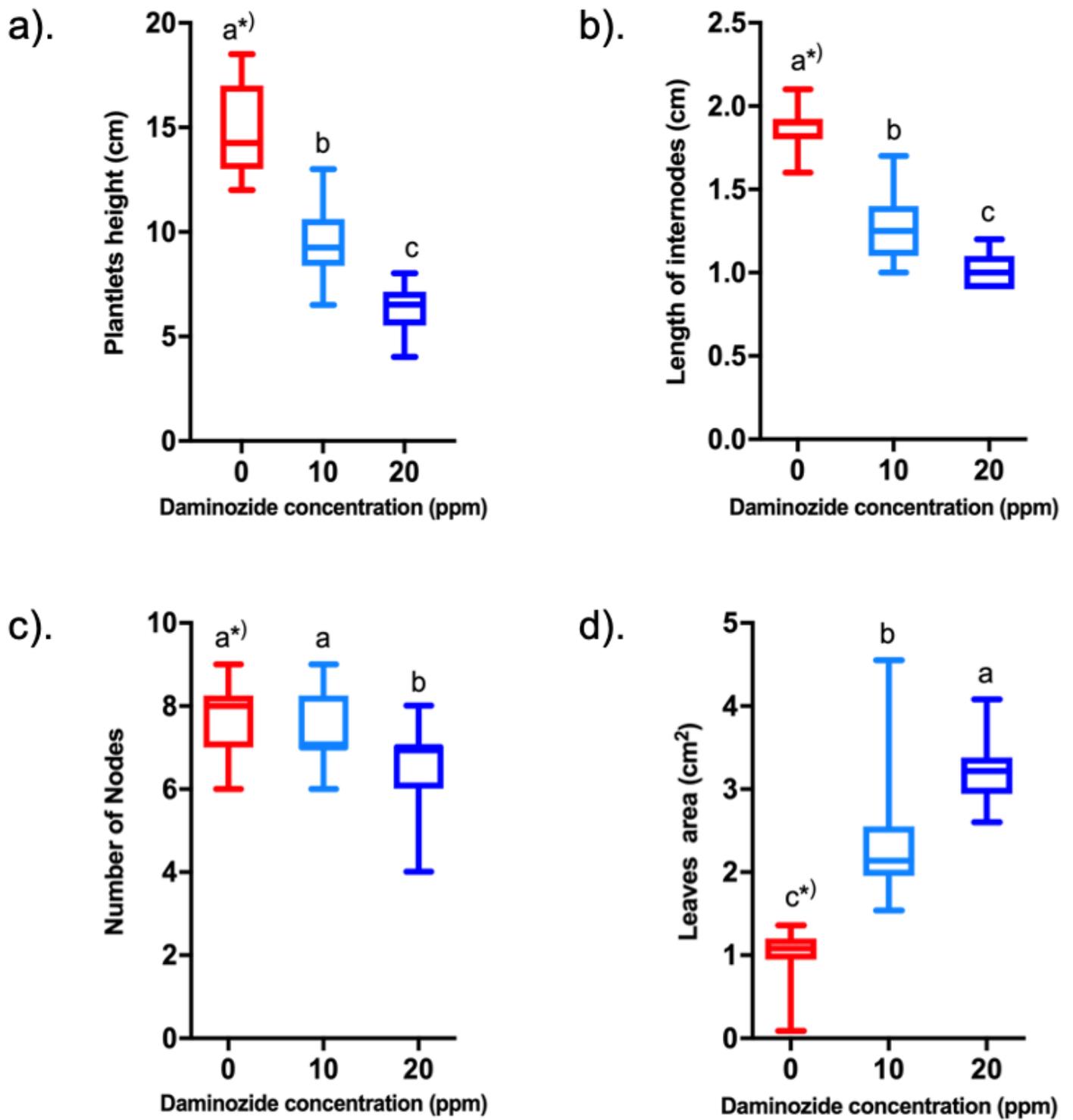


Figure 1

Growth measurements of stevia after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 40$.

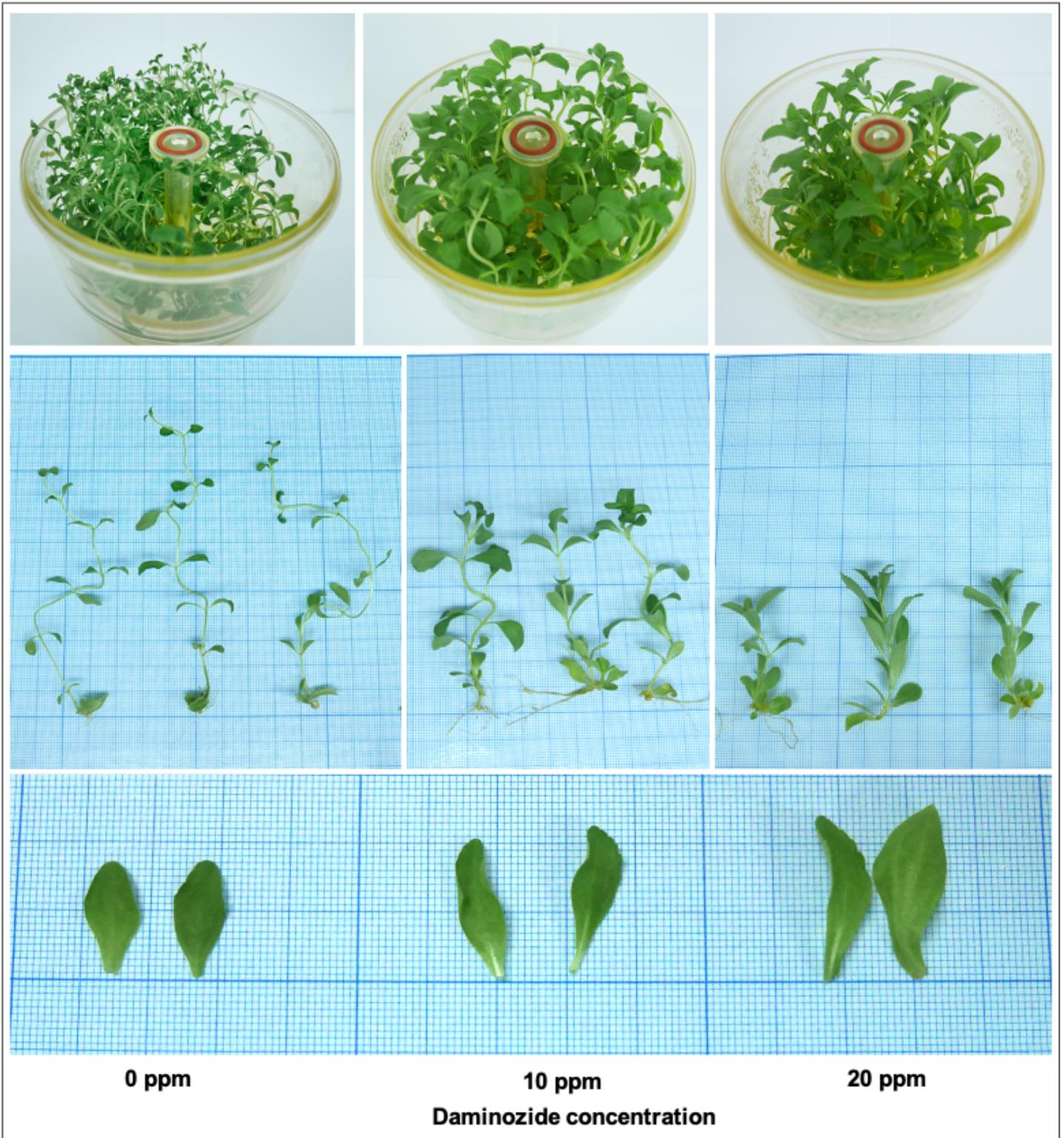


Figure 2

Morphological growth of stevia after six weeks of incubation in TIB with Daminozide treatments.

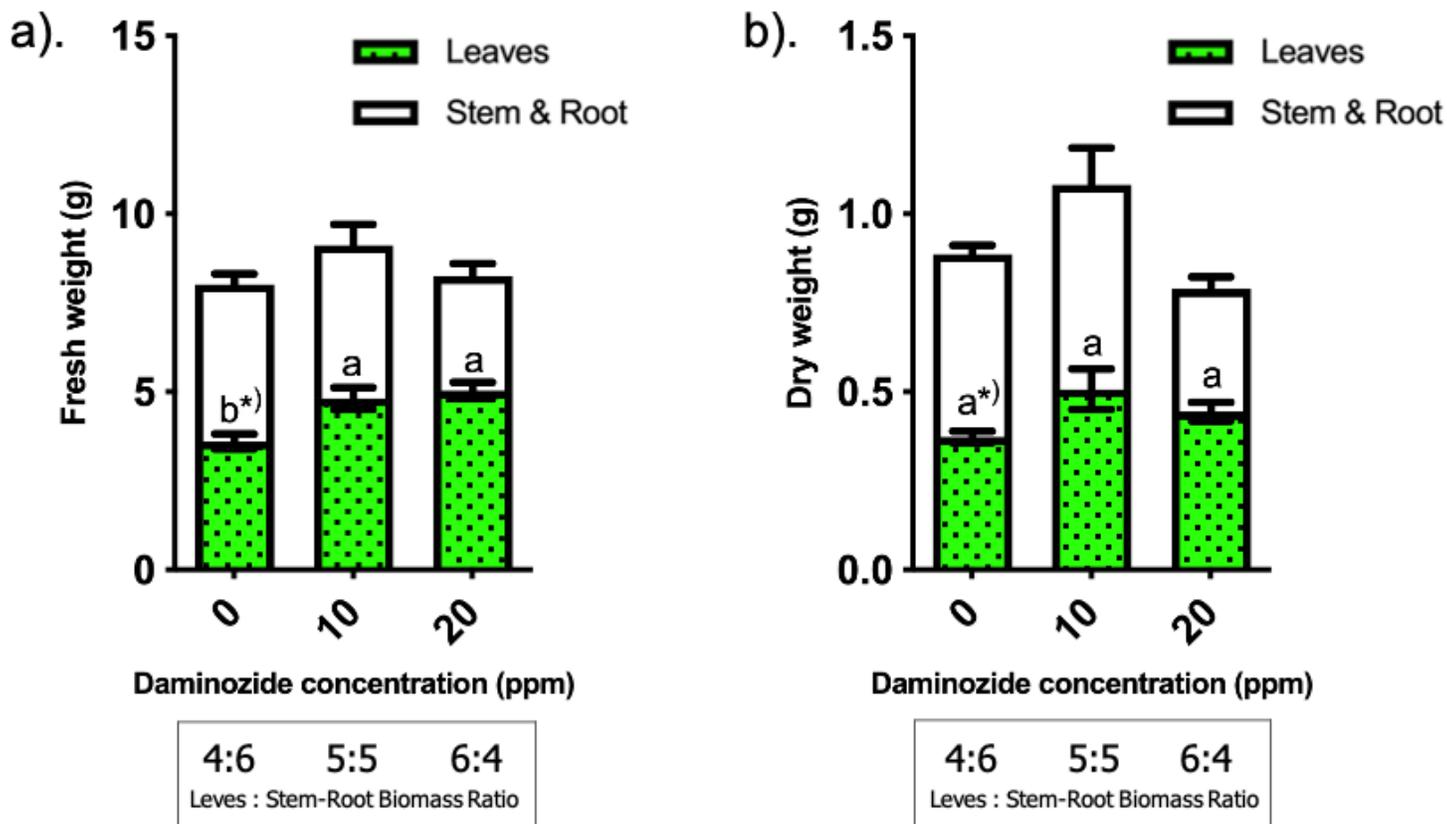


Figure 3

Stevia biomass after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means of leaves biomasses are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 5$.

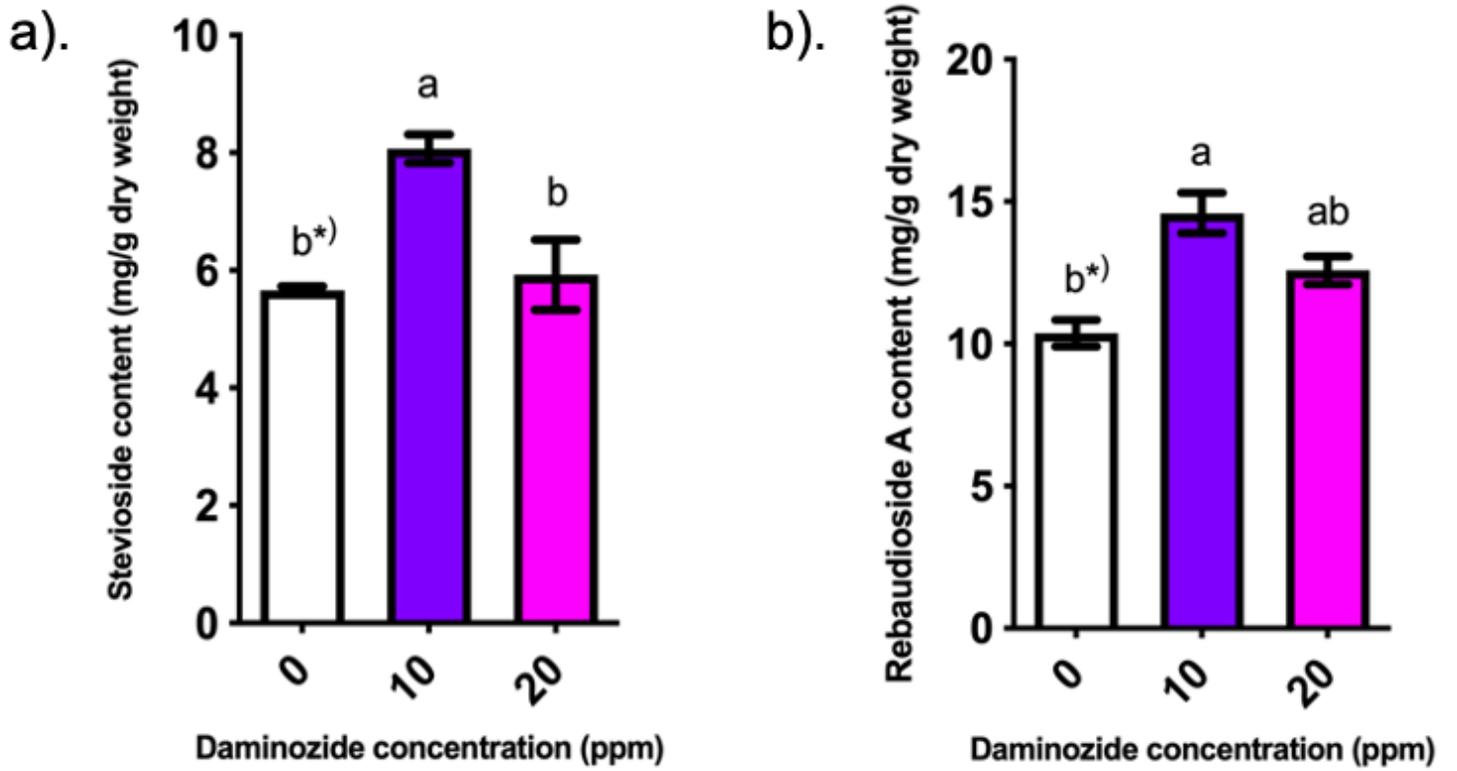


Figure 4

Stevioside (a) and rebaudioside A (b) content from stevia after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 5$.

GGDP



Ent-kaurenoic acid



Steviol



Steviolmonoside



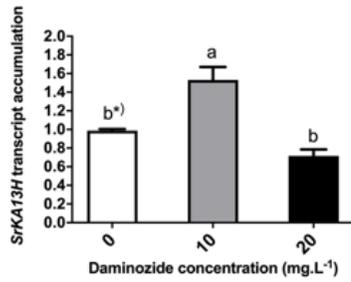
Steviolbioside



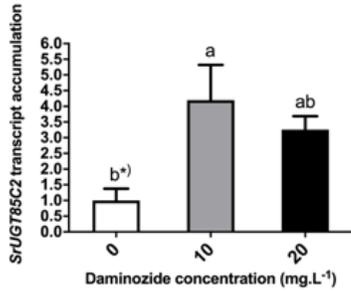
Stevioside



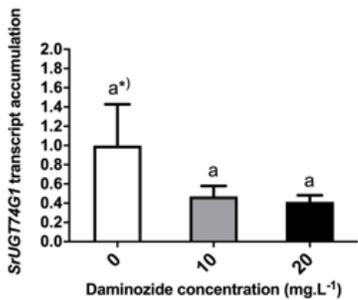
Rebaudioside A



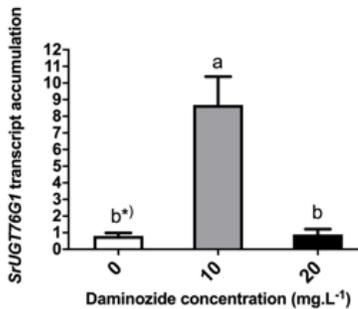
SrKA13AH



SrUGT85C2



SrUGT74G1



SrUGT76G1

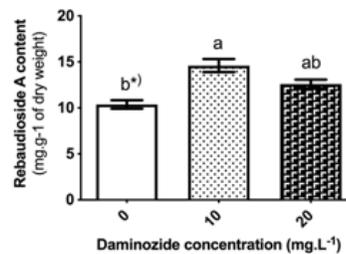
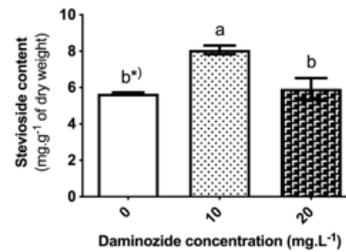


Figure 5

Steviol glycoside-related genes transcript accumulations from stevia after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 3$.

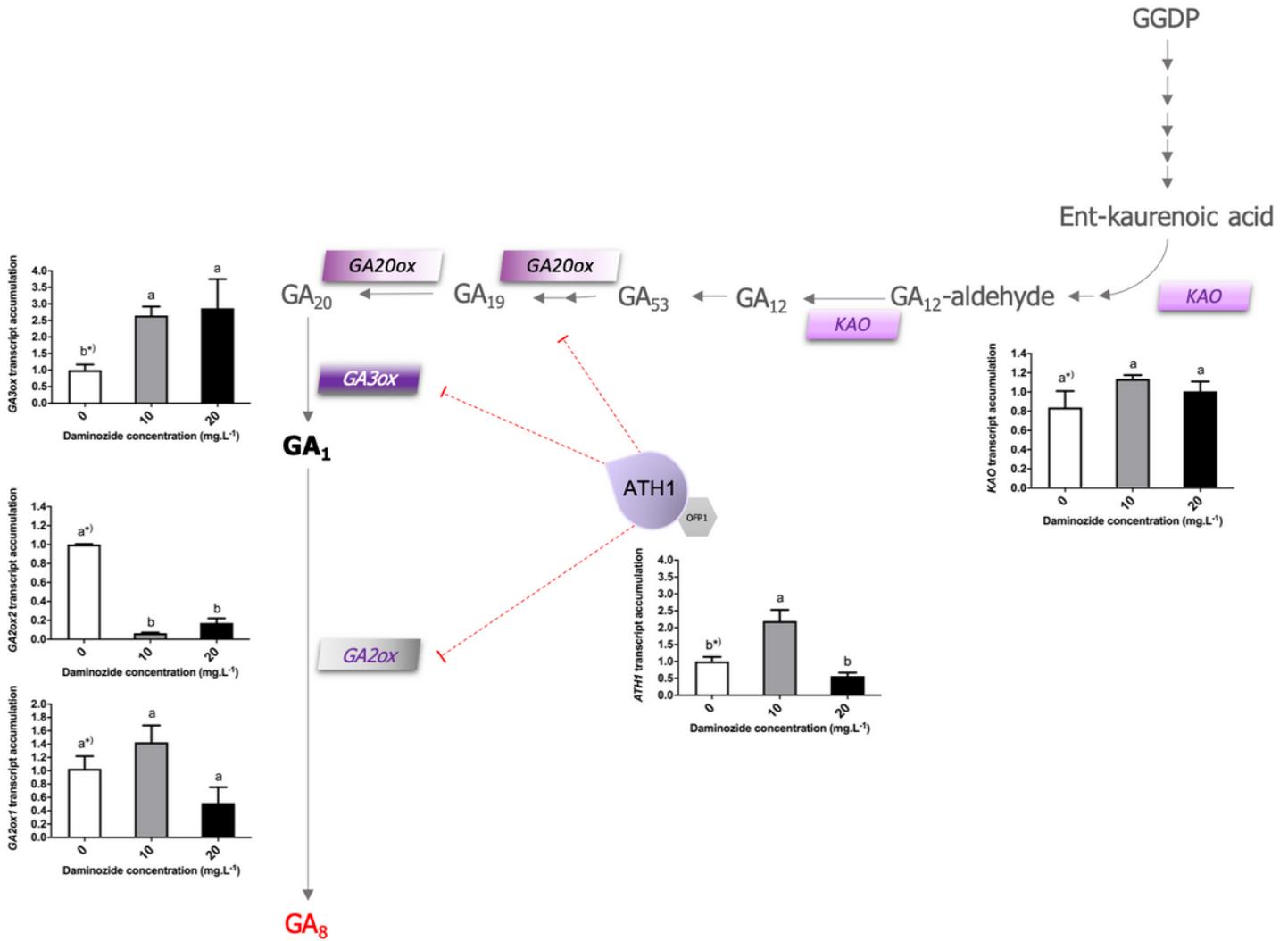


Figure 6

Gibberellin-related genes transcript accumulations from stevia after six weeks of incubation in TIB with Daminozide treatments. Different letters in each parameter indicate means are significantly different according to Tukey's post hoc test at $\alpha = 0.05$, $n = 3$.

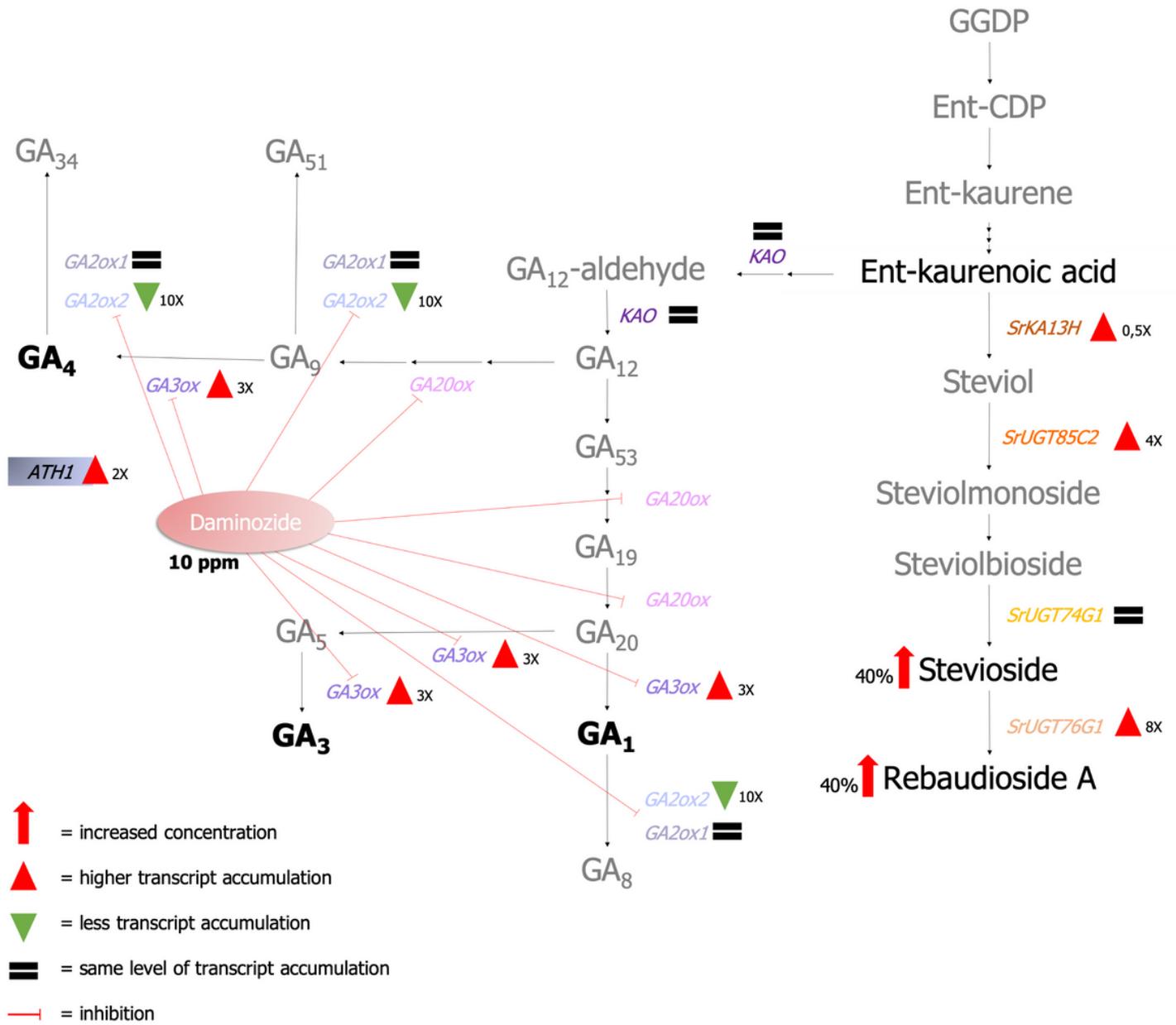


Figure 7

Illustration of 10 ppm Daminozide treatment influenced stevioside and rebaudioside A content of stevia and the transcription profile of SG and GA-related genes.