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ORIGINAL ARTICLE

Gibberellin reverses the negative effect of paclobutrazol but not of chlorocholine chloride on the expression of SGs/GAs biosynthesis-related genes and increases the levels of relevant metabolites in *Stevia rebaudiana*

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Abstract
Steviol glycosides (SGs) and gibberellins (GAs) share the same molecular basis. However, the coordination of their respective biosynthetic pathways is very intriguing. Thus, the present study aimed to investigate the role of plant growth regulators, gibberellic acid (GA_3), chlorocholine chloride (CCC), and paclobutrazol (PBZ), on the metabolism of *Stevia rebaudiana* and identify possible ameliorates of the evaluated parameters when CCC and PBZ-treated plants were subsequently treated with GA_3. For this, explants were cultured in the absence or presence of 2 mg L^{-1} GA_3, CCC, or PBZ (Step 1). After 20 days, half explants incubated with CCC and PBZ were treated with 2 mg L^{-1} GA_3 and the other half, as well as the rest of the explants, were sub-cultured in their respective initial conditions for 20 days (Step 2). GA_3-treated plants showed increased stevioside and phenolic compounds content, as well as a downregulation of most of the SGs/GAs biosynthesis-related genes, with a more pronounced effect upstream of steviol. Following this trend, CCC downregulated some MEP pathway genes, including *SrDXS, SrDXR, SrCDPS*, and *SrKS*, and upregulated *SrUGT6G1*. PBZ also upregulated *SrUGT76G1* and inhibited five genes of the MEP pathway and all genes coding for kaurenoid pathway enzymes. The obtained results highlight the capability of GA_3 to reverse the negative effects of PBZ on the pattern of many transcripts and to additionally increase the stevioside content to levels comparable to those found in field-grown plants.

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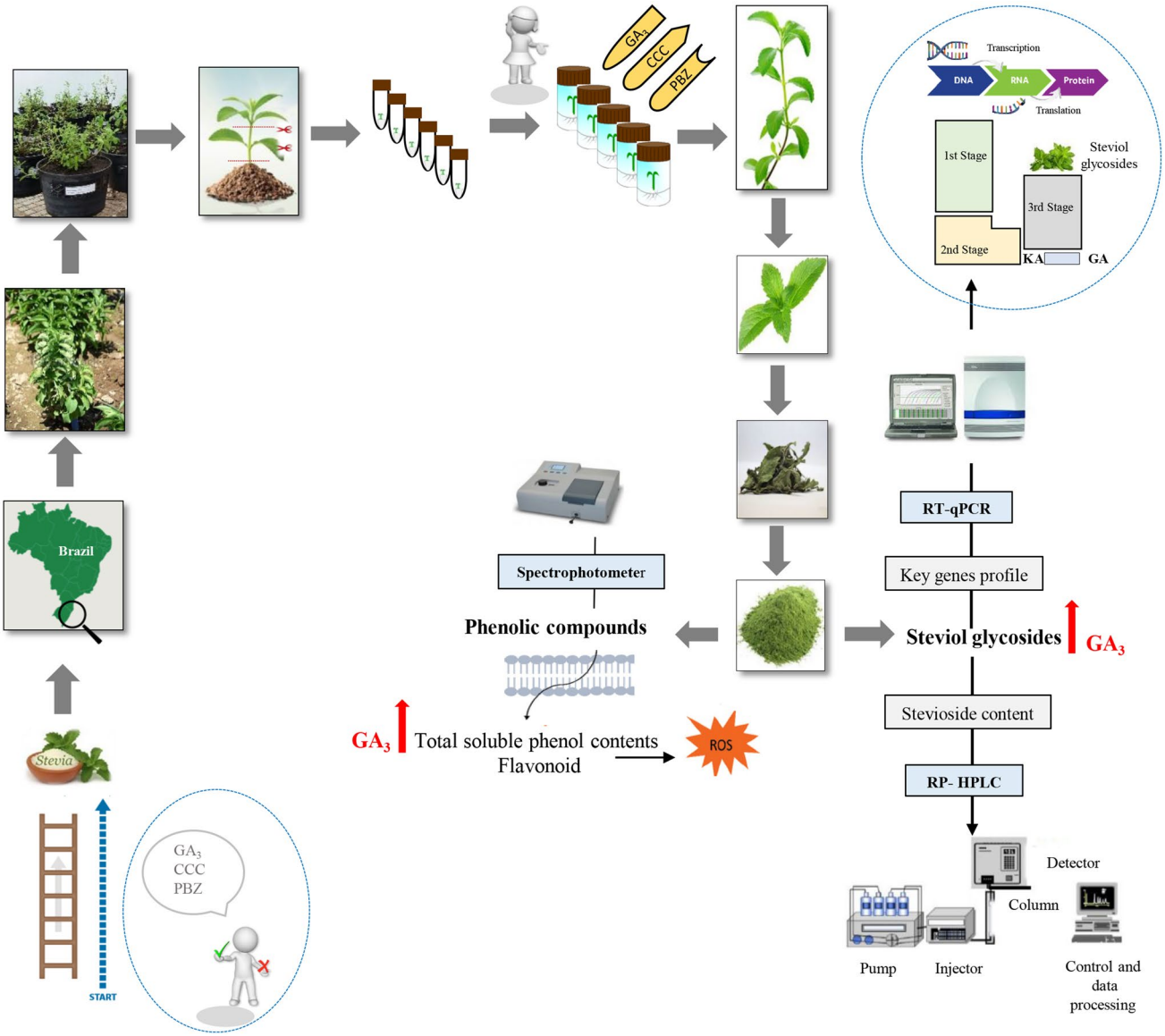
Graphic abstract



Key message

A feedback loop in in vitro cultured stevia plants in response to GA3, CCC and PBZ was demonstrated. GA3-treated plants showed increases in stevioside and phenolic compound contents.

Keywords Biosynthesis inhibitors · Gibberellic acid · Morphological traits · Specialised metabolites · Stevia · Transcriptional regulation

Abbreviations

ANOVA	Analysis of variance
CCC	Chlorocholine chloride
CDPS	Copalyl diphosphate synthase
CMK	4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase
CMS	4-Diphosphocytidyl-2-C-methyl-D-erythritol synthase
DMAPP	Dimethylallyl diphosphate
DXR	Deoxyxylulose-5-phosphate reductase
DXS	Deoxyxylulose-5-phosphate synthase
FLA	Total soluble flavonoids

GAs	Gibberellins
GA_3	Gibberellic acid
GA2ox	Gibberellin 2-oxidase
GA20ox	Gibberellin 20-oxidase
GA3ox	Gibberellin 3-oxidase
GGDP	Geranylgeranyl diphosphate
GGDPS	Geranylgeranyl diphosphate synthase
HDR	1-Hydroxy-2-methyl-2(E)-butenyl-4-diphosphate reductase
HDS	1-Hydroxy-2-methyl-2(E)–butenyl-4-diphosphate synthase
IPP	Isopentenyl diphosphate
KAH	Kaurenoic acid 13-hydroxylase
KAO	Kaurenoic acid oxidase
KO	Kaurene oxidase
KS	Kaurene synthase
MCS	4-Diphosphocytidyl-2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase
MEP	2-C-methyl-D-erythritol-4 phosphate
MVA	Mevalonate
PBZ	Paclobutrazol
PGRs	Plant growth regulators
Reb-A	Rebaudioside-A
Reb-D	Rebaudioside-D
Reb-M	Rebaudioside-M
SGs	Steviol glycosides
STEV	Stevioside
TPC	Total soluble phenol content
UGT73E1	UDP glucosyltransferase-73E1
UGT74G1	UDP glucosyltransferase-74G1
UGT76G1	UDP glucosyltransferase-76G1
UGT85C2	UDP glucosyltransferase-85C2
UGT91D2	UDP glucosyltransferase-91D2

Introduction

Stevia rebaudiana Bertoni, commonly referred as sweet leaf, is a herbaceous perennial plant of the Asteraceae family native to the South American region (Paraguay and Brazil) and has a great importance due to its sweet glycosides, called steviol glycosides (SGs) (Geuns 2003). Steviol glycosides are a group of tetracyclic diterpenes, having 100–300 times the sweetness of sucrose (Karimi et al. 2019) and a wide spectrum for many therapeutic applications (Ullah et al. 2019). Additionally, evidence has shown that stevia is safe for human consumption and does not show side effects or cause health risks (Chughtai et al. 2020).

Steviol glycosides and gibberellins (GAs) share the same biosynthetic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids that culminates with the formation of the IPP and DMAPP (Totté et al. 2003). After the formation of these two basic isoprene units, geranylgeranyl diphosphate (GGDP) is synthesised in a reaction catalysed by geranylgeranyl diphosphate synthase (GGDPS). In sequence, GGDP is cyclised and rearranged into (−)-kaurene by the consecutive action of two enzymes namely copalyl diphosphate synthase (CPPS) and kaurene synthase (KS). Downstream of this, (−)-kaurene is oxidised in the next step by kaurene oxidase (KO) to form kaurenoic acid. From this point, divergence of the steviol and gibberellin biosynthesis pathways occurs (Humphrey et al. 2006). 13α-Hydroxylation of kaurenoic acid catalysed by kaurenoic acid 13-hydroxylase (KAH) leads to the formation of steviol, the 'backbone' of SGs, whereas the oxidation of kaurenoic acid by kaurenoic acid oxidase (KAO) forms gibberellic $acid_{12}$ (GA_{12}), which, in turn, is the precursor for the synthesis of all kinds of GAs (Richman et al. 2005; Brandle and Telmer 2007). Finally, glycosylation of steviol governed by cytosolic UDP-glucosyltransferases (UGTs), SrUGT74G1, SrUGT76G1, SrUGT85C2 (Richman et al. 2005), SrUGT91D2 (Wang et al. 2016), and SrUGT93E1 (Li et al. 2018) results in the diversity of SGs depending on the bond type and number of added glucose units (Richman et al. 2005).

Gibberellins are plant hormones that control growth and development of the plant (Hernández-García et al. 2020). Different steps of the GAs biosynthesis pathway can be inhibited by plant growth retardants (Karimi et al. 2019). Chlorocholine chloride (CCC), which has the trade name Cycocel®, and paclobutrazol (PBZ) are two growth retardants known for their anti-GA effects. In addition, CCC and PBZ are molecules with a recognised role in protecting against stress and by their ability to promote secondary metabolism changes. Some experiments have been performed to evaluate the effect of PBZ (Karimi et al. 2014; Lucho et al. 2018a; Hajihashemi 2018) and CCC (Dey et al. 2013a, b; Karimi et al. 2014; Kundu et al. 2014) on the phytochemicals of stevia plants with encouraging results. As far as we know, information concerning the effects of CCC on a molecular level in stevia and others plants is very limited, although this compound is considered the growth retardant most widely used on a global scale (Rademacher 2016). Another question that remains open is about the effect of PBZ on both the synthesis of SGs and the expression of SG-related genes (in the three stages of the SGs biosynthesis pathway) in in vitro cultured stevia plants. Unlike CCC, with only one study that evaluated its effect on the expression of the *SrKAH* gene (Kundu et al. 2014), at least three studies have been conducted with PBZ in stevia plants (Hajihashemi et al. 2013; Hajihashemi and Geuns 2017; Lucho et al. 2018a).

The effect of GA_3 on SG-related gene expression in stevia plants has also been studied, showing increases in the expression of key genes (Kumar et al. 2012; Hajihashemi et al. 2013), although decreased expression would be expected given the possible negative feedback processes.

As GAs and SGs compete for the same substrates, a possible downregulation of the *SrKAO* gene (coding for the first specific GAs synthesis enzyme) would trigger an accumulation of kaurenoic acid (KA). With increased KA availability, the enzyme upstream from steviol biosynthesis (*SrKAH*) would be ready to catalyse the first step of the SG-specific pathway, increasing the metabolic flux towards the formation of SGs. Thus, starting from the point where GA_3, CCC, and PBZ have the potential to manipulate part of the diterpenoids pathway, including SGs formation, we hypothesised that these compounds could improve specialised metabolites accumulation in stevia plants. Therefore, the objective of the present study was to investigate the physiological, biochemical, and molecular responses to GA_3, CCC, and PBZ and identify possible ameliorates on the evaluated parameters when CCC- and PBZ-treated plants were subsequently treated with GA_3.

Material and methods

Plant material and in vitro culture of *Stevia rebaudiana*

Stevia rebaudiana Bertoni plants were collected in the Pelotas region, south of Brazil (31°42′43.2″S 52°10′28.7″W). This specie was previously identified, and then, a voucher was deposited in the Federal University of Pelotas herbarium under number #26,649, as well as registration (A3AF671), in the National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) was carried out. Plants were cultivated for four months in a greenhouse (28 ± 2 °C temperature and 40–60% relative humidity). For in vitro establishment, aerial branches were rinsed several times under running tap water for 10 min (rustic washing), and then they were taken to the laminar air flow chamber, where they were shaken for 15 min in a calcium hypochlorite 1.5% (v/v) solution containing three drops of Tween® 20 surfactant per litre. The stem portions were rinsed thrice with sterilised distilled water, and then, the explants were pre-treated with a modified Murashige and Skoog medium (MS; Murashige and Skoog 1962) with macronutrients at half-strength (MS/2) containing 4000 µL L^{-1} Plant Preservative Mixture® for 90 min. After disinfection, nodal explants were excised and placed in test tubes containing growth regulator-free MS/2 medium, supplemented with 250 mg L^{-1} casein hydrolysate, 30 g L^{-1} sucrose, 1 g L^{-1} charcoal, and 7.3 g L^{-1} agar and the pH adjusted to 5.8 before autoclaving (1 atm, 120 °C, 20 min). Cultures were incubated in a growth room at 25 ± 2 °C under a photon flux density of 48 µmol m^{-2} s^{1} (16 h light / 8 h dark). After in vitro establishment, plants were in vitro propagated using glass flasks under the same conditions as described above. Aseptic and healthy in vitro plantlets were used for further studies.

Treatments and growth determinations

Node explants (2.0 cm long) were cultured in MS/2 nutrient medium in the absence (control, CON) or presence of 2 mg L^{-1} GA_3, CCC, or PBZ (Step 1). After 20 days, half explants cultured in MS/2 media containing CCC and PBZ were sub-cultured in media containing 2 mg L^{-1} GA_3 and the rest of explants, including those from GA_3, were sub-cultured in the same conditions as step 1 (Step 2). The experiment was therefore comprised of the following treatments (Step 1 medium + Step 2 medium): $\mathbf{T_1}$- Control (CON + CON); $\mathbf{T_2}$- CCC (CCC + CCC); $\mathbf{T_3}$- PBZ (PBZ + PBZ); $\mathbf{T_4}$- GA_3 (GA_3 + GA_3); $\mathbf{T_5}$- CCC + GA_3, and $\mathbf{T_6}$- PBZ + GA_3 (Supplementary Fig. 1). Flasks covered with aluminium foil were incubated under the same light and temperature conditions described above. The number of nodal segments, shoots, leaves, and roots, as well as root length and shoot height, from every explant were assessed in a non-destructive way at the end of each of the two steps, 20 days (Step 1) and 40 days (Step 2) after the beginning of the experiment. The experiment was repeated twice.

RNA extraction, cDNA synthesis, and RT-qPCR reactions

Leaf samples (located at the 3rd and 4th nodes from the apex) were harvested after 40 days (Step 2) and immediately frozen in liquid nitrogen. Total RNA was isolated and quantified and its quality/integrity checked. cDNA synthesis was carried out as described by Lucho et al. (2018a). Cycling conditions were optimised and are listed in Supplementary Table 1, together with a detailed description of the primers used. Some of the primer sequences were obtained from Modi et al. (2014), and others were designed based on sequences from the Genbank database on the NCBI website. The specificity of the primers used for RT-qPCR reactions was checked (Supplementary Figs. 2 and 3. The housekeeping gene *Ubiquitin* (*UBQ*) was selected as an internal standard to normalise the data (Lucho et al. 2018b). Relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three technical repetitions were performed for each biological replicate, including samples for the control treatment as a template-free control.

Determination of specialised metabolites

Sample preparation and quantification of SGs via HPLC

The extraction and identification of stevioside was performed according to Lucho et al. (2019) with minor modifications. Steviol glycosides content was determined using an HPLC–DAD Jasco LC-NetII/ADC. Separations were carried out at 40 °C as described by JECFA (2010) on a Luna C-18 reverse-phase column (250 mm × 4 mm, 5 µm) supplied by Phenomenex. A mixture 10 mM phosphate buffer pH 2.6:acetonitrile (68:32, v/v) was used as a mobile phase and applied at a flow rate of 1 mL min^{-1}. Stevioside content was expressed as mg equivalents of rebaudioside A per gram dry weight.

Quantification of total soluble phenol and flavonoid content via spectrophotometry

The determination of total soluble phenol content (TPC) was carried out according to López-Orenes et al. (2013) using gallic acid (0–2000 µM) as a standard, while the flavonoid content (FLA) was determined according to Kim et al. (2003). A standard curve of rutin (quercetin-3-rutinoside) was used for calibration (0–2000 µM). Absorbance readings for TPC and FLA were determined at 765 nm and 510 nm, respectively, using a Ultrospec® 7000/7000PC UV–Visible spectrophotometer. TPC content was expressed as mg of gallic acid equivalents and FLA content as mg of rutin equivalents per gram dry weight.

Statistical analyses

The experiment was set up in a completely randomised factorial design with 6 treatments, and each treatment consisted of five flasks with four explants per flask. The results correspond to the mean ± standard deviation. Analysis of variance (ANOVA) and Tukey's Test at a 5% probability level ($P < 0.05$) were performed to calculate the significant differences among treatments using R software. Pearson correlation analysis was performed to examine whether there was a correlation between SGs content, gene transcription, and morphological characteristics. In addition, multivariate principal component analysis (PCA) was applied to detect possible groupings among all variables.

Results

Effects of GA_3 and its biosynthesis inhibitors, CCC and PBZ, on Stevia growth parameters

After 20 days of in vitro culture, CCC treatment significantly increased the number of shoots more than two-fold when compared to the control plants (Table 1). Following this trend, the number of both leaves and roots was also significantly higher in CCC-treated plants (10.33 ± 0.11 and 2.89 ± 0.50, respectively) than control plants (6.45 ± 0.23 and 0.56 ± 0.23, respectively). Regarding the number of nodal segments, the highest mean value was observed in PBZ-treated plants (4.44 ± 0.28). Both inhibitors showed a positive effect on the root system with CCC promoting the greatest number of roots and PBZ promoting their growth (4.15 ± 0.39 cm). Overall, at the end of the step 1, no reductions in growth parameters were observed when explants were treated with CCC and PBZ.

At the end of the step 2, the only growth parameter that was not significantly affected by treatment was the number of nodes (Table 2 and Supplementary Fig. 4). PBZ reduced plant height by about 25%, and the application of GA_3 was not able to reverse this effect. Notably, PBZ also had negative effects on the root system, presenting less (1.78 ± 0.12) and shorter roots (1.35 ± 0.15 cm) than control plants (5.22 ± 0.23 and 2.95 ± 0.29 cm, respectively). A similar trend was observed in plants treated with GA_3 both alone and in combination with CCC (CCC + GA_3). The latter being the treatment that provoked a significant reduction of almost all analysed growth parameters.

Table 1 Growth parameters of *Stevia rebaudiana* grown in in vitro conditions for 20 days (first subculture) in the absence (Control) or in the presence of 2 mg L^{-1} of Chlorocholine chloride (CCC), paclobutrazol (PBZ) or gibberellic acid (GA_3)

Growth parameters	Plant growth regulators (PGR)			
	Control	CCC	PBZ	GA_3
Number of shoots	1.00 ± 0.01^{b}	2.22 ± 0.17^{a}	1.66 ± 0.11^{ab}	1.88 ± 0.17^{ab}
Number of nodes	1.78 ± 0.06^{b}	3.22 ± 0.16^{ab}	4.44 ± 0.27^{a}	4.00 ± 0.55^{ab}
Number of leaves	6.44 ± 0.23^{b}	10.33 ± 0.11^{a}	8.89 ± 0.51^{ab}	7.33 ± 0.77^{ab}
Plant height (cm)	2.79 ± 0.18^{a}	3.92 ± 0.37^{a}	3.56 ± 0.44^{a}	3.56 ± 0.41^{a}
Number of roots	0.55 ± 0.23^{b}	2.89 ± 0.50^{a}	2.44 ± 0.34^{ab}	0.11 ± 0.06^{b}
Root length (cm)	1.13 ± 0.42^{b}	2.87 ± 0.57^{ab}	4.15 ± 0.39^{a}	0.50 ± 0.28^{b}

Values represent mean ± standard deviation

Means values within a line sharing the same subscript are not significantly different based on ANOVA followed by Tukey test $P \leq 0.05$

Table 2 Growth parameters evaluated in *Stevia rebaudiana* grown in vitro conditions for 40 days in the presence of gibberellic acid (GA_3) and its inhibitors Chlorocholine chloride (CCC) and paclobutrazol (PBZ)

Growth parameters	Plant growth regulators (PGR)					
	Control	CCC	PBZ	GA_3	*CCC + GA_3	*PBZ + GA_3
Number of shoots	2.88 ± 0.16^{a}	1.88 ± 0.23^{ab}	$2.22 \pm 0{,}23^{ab}$	1.33 ± 0.19^{ab}	0.88 ± 0.06^{b}	2.33 ± 0.29^{ab}
Number of nodes	6.22 ± 0.12^{a}	6.88 ± 0.44^{a}	6.22 ± 0.37^{a}	4.88 ± 0.51^{a}	4.77 ± 0.33^{a}	7.77 ± 0.57^{a}
Number of leaves	13.88 ± 0.42^{a}	12.77 ± 0.65^{ab}	12.00 ± 0.44^{ab}	9.44 ± 0.65^{b}	9.11 ± 0.12^{b}	11.77 ± 0.46^{ab}
Plant height (cm)	4.26 ± 0.19^{a}	4.48 ± 0.01^{a}	3.20 ± 0.06^{b}	3.65 ± 0.11^{ab}	3.35 ± 0.05^{b}	3.84 ± 0.11^{ab}
Number of roots	5.22 ± 0.23^{a}	3.33 ± 0.22^{ab}	1.77 ± 0.12^{b}	1.55 ± 0.12^{b}	2.33 ± 0.11^{b}	2.22 ± 0.12^{b}
Root length (cm)	2.95 ± 0.29^{a}	2.36 ± 0.23^{ab}	1.35 ± 0.15^{b}	1.27 ± 0.13^{b}	1.21 ± 0.06^{b}	1.66 ± 0.09^{ab}

Values represent mean ± standard deviation

Means values within a line sharing the same subscript are not significantly different based on ANOVA followed by Tukey test $P \leq 0.05$

*The media containing CCC and PBZ were subcultured in media containing 2 mg L^{-1} GA_3 after 20 days

Surprisingly, the CCC treatment did not have a negative effect on plant height, resulting in an average value of 4.48 ± 0.02 cm, which was not significantly different from the value obtained in control plants (4.27 ± 0.19 cm).

Effects of GA_3 and its biosynthesis inhibitors, CCC and PBZ, on the transcription of SG-related genes

The evaluation of transcription patterns in the regulatory SG-related genes under the effect of GA_3 and its biosynthesis inhibitors CCC and PBZ are shown in Fig. 1

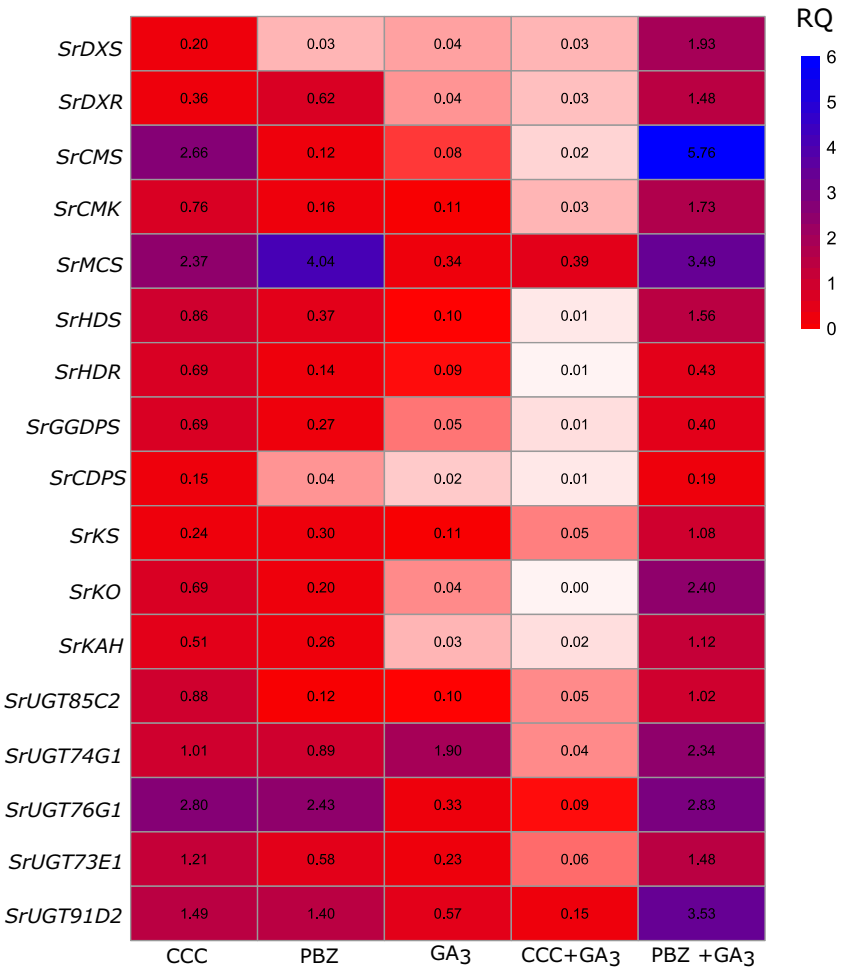


Fig. 1 Heatmap showing the relative quantification (RQ) of SG-related genes in *Stevia rebaudiana* under the effect of GA_3 and its inhibitors PBZ and CCC after 40 days of in vitro growth. *UBQ* gene was used as internal control to normalize the data of the RT-qPCR reactions

and Supplementary Table 2. The application of CCC caused downregulation of the first two genes in the MEP pathway, *SrDXS* and *SrDXR* and the last one, *SrHDR*, besides upregulation of the *SrCMS* and *SrMCS* genes. The negative effect of CCC on gene expression could not be reversed by the presence of GA_3. Stevia plants treated only with GA_3 showed downregulation of all genes in this pathway, exceto to *SrMCS*. Although PBZ provoked a noticeable increase in *SrMCS* gene relative expression, five of the seven genes that make up the MEP pathway were downregulated, including *SrDXS, SrDXR*, *SrCMK*, *SrHDS*, and *SrHDR*. Interestingly, treatment with GA_3, after exposure to PBZ during step 1 reversed this effect, except for the last gene (*SrHDR*).

Genes encoding kaurenoid enzymes, namely *SrGGDPS, SrCDPS, SrKS, and SrKO*, were downregulated by both PBZ and GA_3 treatments. Although, PBZ downregulated the expression of the four genes, treatment with GA_3 in step 2 was able to reverse the negative effects on the expression of *SrKS* and *SrKO*. Notably, *SrKO* encodes the target for the inhibitory action of PBZ. Regarding the effect of CCC on the expression of these four genes, there was a significant decrease in the *SrCDPS* and *SrKS* genes' relative expression (both genes coding for the targets of CCC inhibition). In this case, the presence of GA_3 in step 2 did not reverse the negative effect on gene expression.

Concerning results of the relative expression of the six genes involved in the last stage of SGs biosynthesis, none of the treatments positively regulated *SrKAH* gene expression relative to the control plants. In relation to the expression levels of UGT genes, GA_3 alone treatment downregulated *SrUGT85C2, SrUGT76G1, SrUGT73E1*, and *SrUGT91D2* and upregulated *SrUGT74G1* gene expression, whereas CCC and PBZ upregulated *SrUGT76G1* and *SrUGT91D2* and PBZ downregulated *SrUGT85C2* and *SrUGT73E1* gene expression. Regarding sequential treatments, CCC + GA_3 resulted in the lowest levels of expression for all UGT genes. Interestingly, PBZ + GA_3 treatment always reversed the negative effects of PBZ and even increased the UGT expression levels when the inhibitor had no apparent effect relative to the control.

Content of specialised metabolites

Effect of GA_3, CCC, and PBZ on SGs content

Stevioside content was highest (32.18 ± 0.76 mg rebaudioside A eq. g^{-1} DW) in plants treated with GA_3 alone (Fig. 2). When GA_3 was used in step 2, after the application of CCC or PBZ, stevioside content was lower than that of the control (15.56 ± 0.16, 11.03 ± 0.51, and 18.95 ± 0.59 mg rebaudioside A eq. g^{-1} DW, respectively). This same

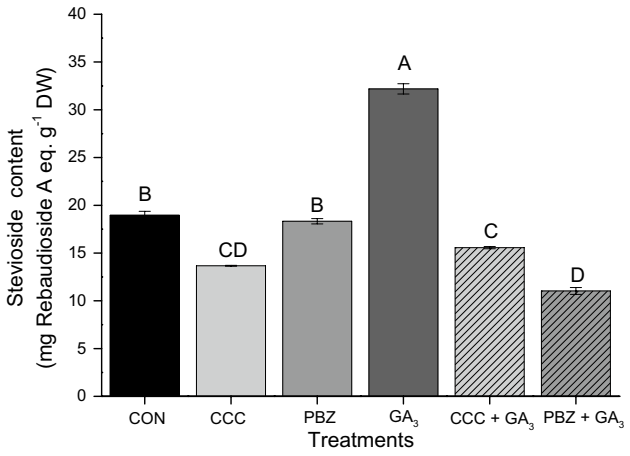


Fig. 2 Stevioside contents in extracts from *Stevia rebaudiana* under the effect of GA_3 and its inhibitors PBZ and CCC after 40 days of in vitro growth. Columns with different capital letters indicate significant differences between treatments based on ANOVA followed by Tukey test $P \leq 0.05$. Error bars indicate the standard deviation around the mean ($n = 5$)

trend was observed in stevia plants that were only treated with CCC (13.67 ± 0.06 mg rebaudioside A eq. g^{-1} DW), whereas PBZ had no influence on stevioside content, presenting values very close to those obtained in control plants (18.33 ± 0.39 mg rebaudioside A eq. g^{-1} DW). It was not possible to detect the presence of rebaudioside A in any sample analysed by HPLC.

Effect of GA_3, CCC, and PBZ on phenolic compound content

The results showed significant differences for both TPC and FLA content (Fig. 3 and Supplementary Table 3). Plants that were treated with only GA_3 showed an increase in TPC (18.40 ± 0.34 mg gallic acid eq. g^{-1} DW), while plants treated with PBZ showed a decrease (2.38 ± 0.18 mg gallic acid eq. g^{-1} DW) when compared to control plants (9.12 ± 0.67 mg gallic acid eq. g^{-1} DW) (Fig. 3a). Regarding FLA, the results showed that none of the treatments caused a significant increase in their content. In contrast, PBZ alone or in combination with GA_3 caused a decrease in FLA content (6.96 ± 0.20 and 6.98 ± 0.43 mg rutin eq. g^{-1} DW, respectively). Furthermore, CCC alone or in combination with GA_3 did not provoke significant changes (Fig. 3b).

Trait correlations

Pearson's test was carried out to establish the possible correlations between metabolite content (stevioside and phenolic compounds), gene transcription levels, and morphological characteristics (Fig. 4). No positive correlation was observed for any of the SG-related genes and stevioside content. However, there was a weak positive correlation between the

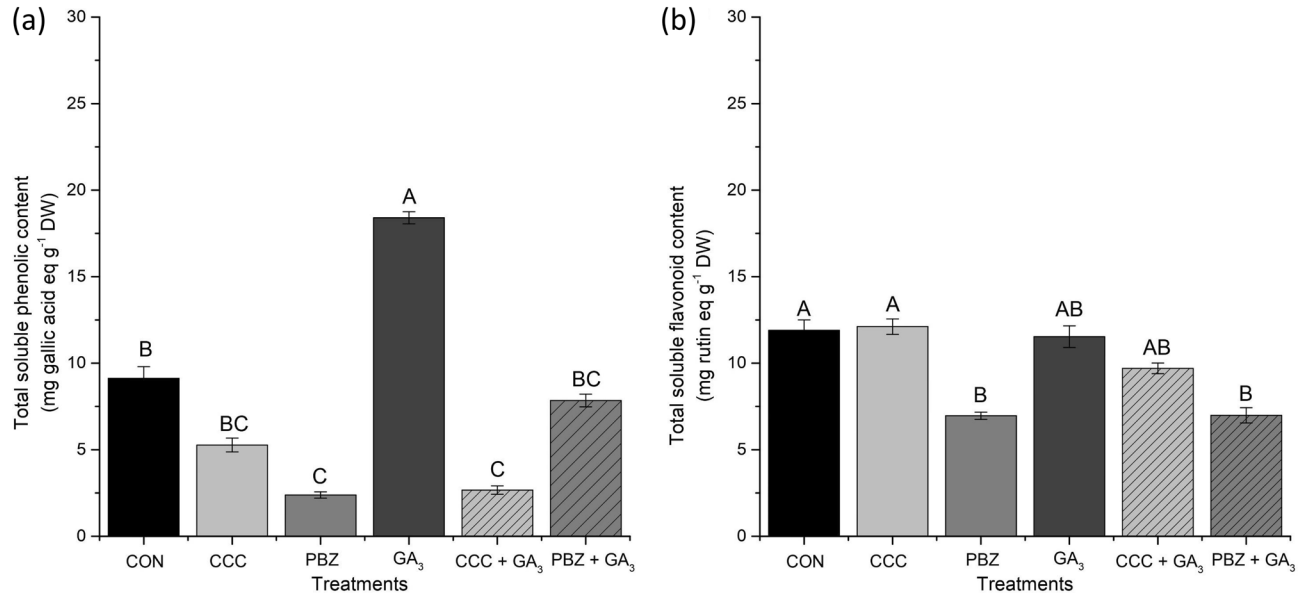


Fig. 3 Total soluble phenolic (**a**) and flavonoids (**b**) contents in extracts from *Stevia rebaudiana* under the effect of GA_3 and its inhibitors PBZ and CCC after 40 days of in vitro growth. Columns with different uppercase letters indicate significant differences between treatments based on ANOVA followed by Tukey test $P \leq 0.05$. Error bars indicate the standard deviation around the mean ($n = 5$)

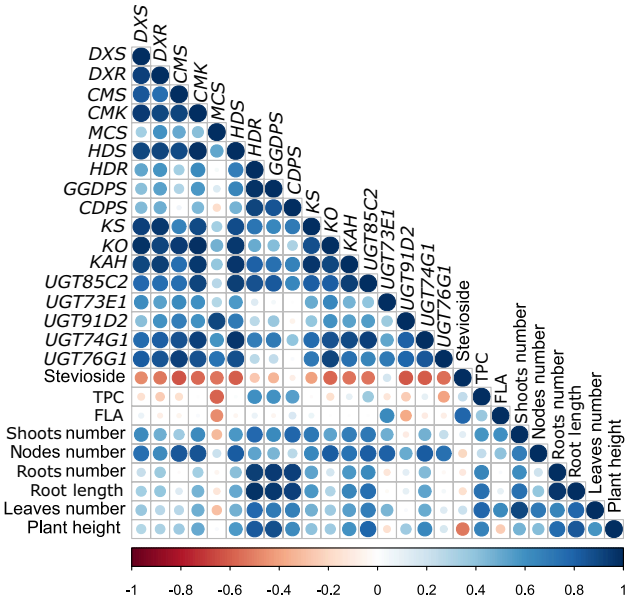


Fig. 4 Pearson correlations between SG-related genes expression, stevioside content and morphologic parameters in *Stevia rebaudiana* under the effect of GA_3 and its inhibitors PBZ and CCC after 40 days of in vitro growth. Color intensity and the size of the circle are proportional to the correlation coefficients. (Color figure online)

SrUGT74G1 gene and stevioside accumulation ($r^2 = 0.178$). Additionally, there was a positive correlation between phenolic compounds and stevioside. This correlation could be found for both FLA ($r^2 = 0.789$) and TPC ($r^2 = 0.248$). Correlations were also found between the number of shoots and both TPC and FLA content, and in the case of TPC content, also between this parameter and those related with root system development. Overall, specialised metabolite production showed a positive correlation with the analysed growth parameters.

Multivariate principal component analysis (PCA)

PCA was performed with all evaluated parameters, accounting for 83.3% of the total variance in the data set, with 67.2% in the first principal component (PC1) and 16.1% in the second one (PC2). Based on these results, a biplot graph was constructed (Fig. 5). The variables that most contributed to the distinction among treatments in PC1 were the genes encoding *SrCMK* and *SrKO* with 0.06 and *SrHDS, SrUGT85C2, SrKAH, SrUGT73E1, SrHDR, SrGGDPS, SrCMS, SrCDPS, SrKS,* and *SrDXR* with 0.05. For PC2, the most important variables were TPC (0.18), the gene encoding *SrMCS* (0.13), and some growth parameters, such as number of leaves and shoots, both with 0.12. In addition, the specialised metabolites, FLA (0.12) and stevioside (0.07), also contributed. Regarding the treatments in PCA analysis, CON and CCC were in the same quadrant, which could reflect the similarities in the responses to these treatments, mainly with regard to the growth of plants in vitro and the content of phenolic compounds. Conversely, GA_3 remained alone in the first quadrant, due in part to its unique role in inducing specialised metabolites in stevia plants.

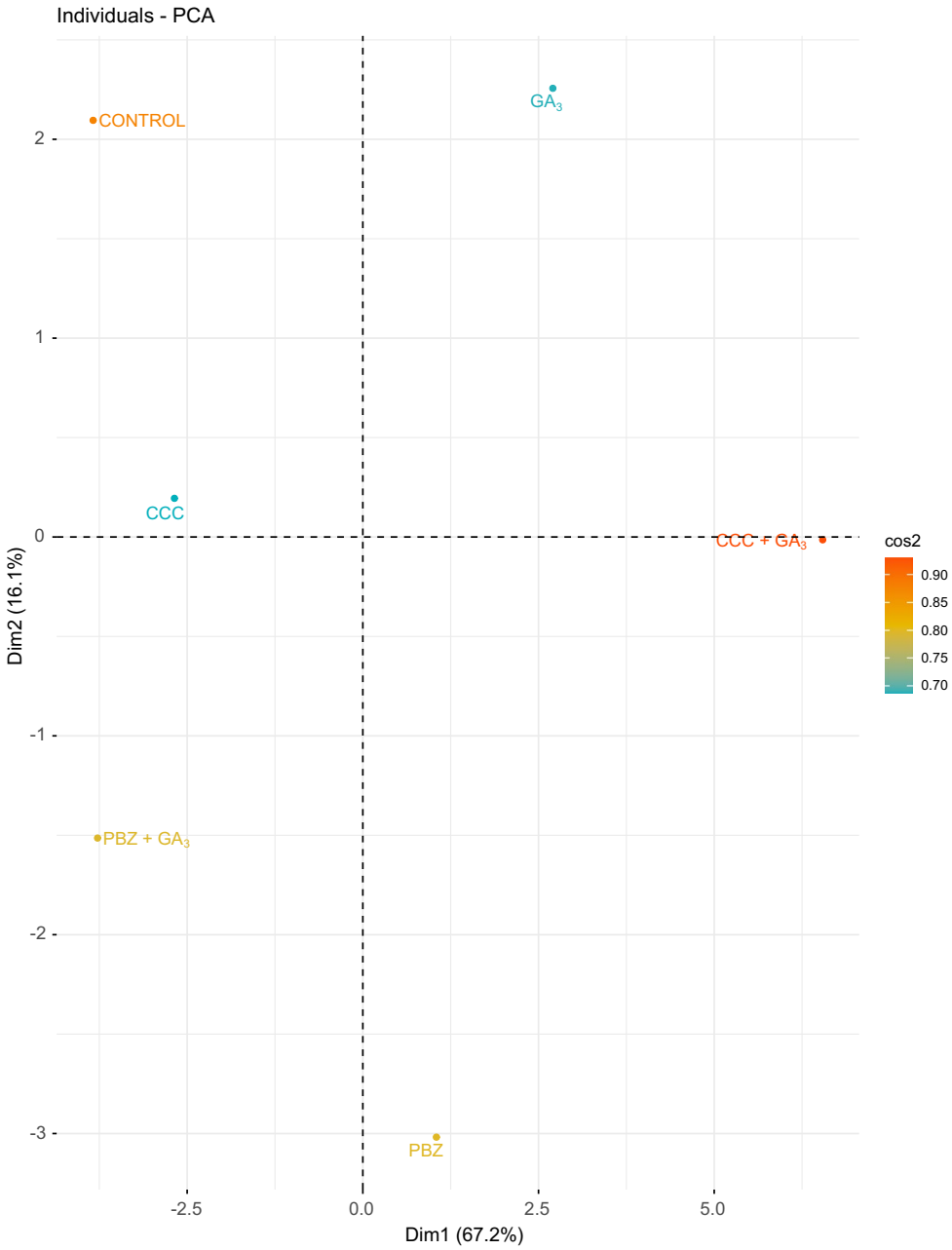


Fig. 5 Principal component analysis (PCA) based on all parameters evaluated in response to the six treatments applied to *Stevia rebaudiana*: Control (CON); chlorocholine chloride (CCC); paclobutrazol (PBZ); gibberellic acid (GA_3); chlorocholine chloride + gibberellic acid (CCC + GA_3) and paclobutrazol + gibberellic acid (PBZ + GA_3). The *cos2* values are used to estimate the quality of the representation

Discussion

The goal of this study was not just to optimise the production of specialised metabolites in stevia using PGRs but also to evaluate the possible negative side effects associated with their application. Thus, the first part of this study was aimed at assessing the effect of these molecules on growth parameters and to determine if the presence of GA_3 was able to reverse the possible deleterious effects of CCC and PBZ on stevia. Surprisingly, CCC had no significant reduction effect on the growth parameters assessed in stevia plants; instead, it promoted the largest number of shoots, leaves, and roots during step 1. Following this same trend, PBZ resulted in the highest mean values for nodal segment number and root length. According to Rogach et al. (2020), GAs biosynthesis inhibitors can inhibit and even remove apical dominance, thereby favouring the growth of lateral buds. This may partly explain the improvement in the number of shoots and nodal segments in plants treated with CCC and PBZ, respectively.

The positive effects of CCC and PBZ on some growth parameters during step 1 were not observed at the end of step 2. Conversely, PBZ was harmful, especially regarding root development (formation and growth). This result apparently disagrees with previous studies, in which application of PBZ resulted in an increased root number in in vitro cultured plants (Wang and Yao

2020). The response of plant materials to in vitro conditions can change with successive subcultures (Lajara et al. 2015; Wang and Yao 2020), which could account for the observed differences. PBZ can exert its inhibitory effect on root development through the increase of endogenous cytokinin levels that, in turn, downregulate the expression of genes involved in GAs synthesis (Fonouni-Farde et al. 2018).

Since SGs are mainly produced and accumulated in stevia leaves, the number of these organs generated in response to the different treatments applied is a growth parameter that deserves attention. Apart from CCC, no other PGR treatment resulted in clear-cut variations in leaf number at the end of step 1. Chlorocholine chloride increased the number of leaves, which is in line with results previously obtained by Dey et al. (2013b), who reported increased production of leaf biomass in in vitro-cultured stevia, and with those reported by Karimi et al. (2019) in stevia plants grown in greenhouse conditions. However, the positive effect of CCC on the number of leaves did not remain in step 2, highlighting the importance of assessing the evolution of plant material behaviour upon successive subcultures in in vitro conditions.

Biosynthesis of diterpenes, including SGs, can be initiated through two pathways: plastidial MEP and cytosolic MVA (Brandle and Telmer 2007). However, studies carried out by Wölwer-Rieck (2012) in the leaves of stevia showed that the C_5 basic building blocks, IPP and DMAPP, are predominately synthesised through the MEP pathway. Treatment with GA_3 alone downregulated almost all MEP genes (six out of seven), and all genes in stage 2 of the GAs/SGs biosynthesis pathways. Moreover, expression of the *SrKAH* gene and some UDP-glucosyltransferases (*SrUGT73E1, SrUGT85C2*, and *SrUGT76G1*) were downregulated, whereas the expression of *SrUGT74G1* was upregulated. This expression pattern could be the result of negative feedback due to an increase in the GAs pool within leaf tissues. The bioactive forms of GAs stimulate the expression of genes involved in GAs catabolism and repress the expression of biosynthetic genes (Hernández-García et al. 2020). Overall, literature is scarce regarding the effects of GA_3 on the expression profile of SG-related genes and other terpenoids, and further work that considers the expression of all GAs biosynthesis (e.g., *KAO*; *GA20ox, Ga3ox*) and catabolic (*GA2ox*) genes together with qualitative and quantitative analysis of endogenous GAs is required.

Chlorocholine chloride and PBZ are two growth retardants with sites of action in the 2nd stage of the GAs/SGs biosynthesis pathways. According to our results, CCC had a strong influence on the MEP pathway (three out of seven genes downregulated), a particular interference on genes encoding kaurenoid enzymes (downregulation of *SrCDPS* and *SrKS*), and little impact on the genes directly involved in the synthesis of SGs. According to Rademacher (2016), CCC inhibits CDPS at a lower degree than it inhibits KS. However, the results of gene expression did not allow us to forecast these differences in enzymatic activity since their relative expression was similar (0.15 ± 0.05 and 0.24 ± 0.04 for *SrCDPS* and *SrKS*, respectively).

Concerning the PBZ treatment, its negative effect on the expression of MEP genes was more pronounced than that of CCC (five out of the seven genes that make up the pathway were downregulated). These results contrasted with those of a previously published study by our group, in which we observed that PBZ had only a small effect on MEP pathway gene expression in stevia plants grown in hydroponic systems (Lucho et al. 2018a). Additionally, all genes encoding the enzymes responsible for the condensation steps leading to the formation of kaurenoic acid were downregulated, which is partially in accordance with other studies (Hajihashemi et al. 2013; Hajihashemi and Geuns 2017; Lucho et al. 2018a). Treatment of stevia with GA_3 in step 2, after PBZ treatment during step 1, was able to revert the expression of the *SrKS* gene, and even enhanced the expression of *SrKO* (the gene encoding the enzyme that constitutes the target of PBZ inhibitory action) relative to the control plants. These results contrasted with those reported by Hajihashemi et al. (2013), who found that GA_3 treatment was unable to reverse the negative effect of PBZ on *SrKO* gene expression. However, differences in experimental conditions (e.g., simultaneous *vs* sequential application of PGRs), as well as plant material origins, among other factors, may help to explain these opposing results. Interestingly, the ability of GA_3 treatment to reverse the negative effects of PBZ on gene expression was also observed in the two other stages of the SGs biosynthesis pathway (Fig. 6).

Apart from their inhibitory effect on gene expression, CCC and PBZ also upregulated the expression of some genes at the 1st and 3rd stages of the GAs/SGs biosynthesis pathways. Both inhibitors promoted increased expression of the *SrUGT76G1* gene, which is a very interesting result, since the UGT76G1 enzyme is crucial for the formation of the sweetest SGs, including tetraglucosylated rebaudioside-A (Reb-A), pentaglucosylated rebaudioside-D (Reb-D), and hexaglucosylated rebaudioside-M (Reb-M) making them a production target as high-potency natural sweeteners (Olsson et al. 2016).

This paper reports for the first time, the transcription pattern of *SrUGT73E1* and *SrUGT91D2* in stevia plants and interestingly, expression of these two genes was positively correlated with key genes of the SGs biosynthesis pathway, including *SrKAH, UGT85C2,* and *UGT76G1*. This aspect is remarkable because it has been demonstrated that *SrKA13H* and *SrUGT85C2* are regulatory genes influencing carbon flux between SGs/GAs biosynthetic routes (Guleria and Yadav 2013). Previous studies reported a correlation

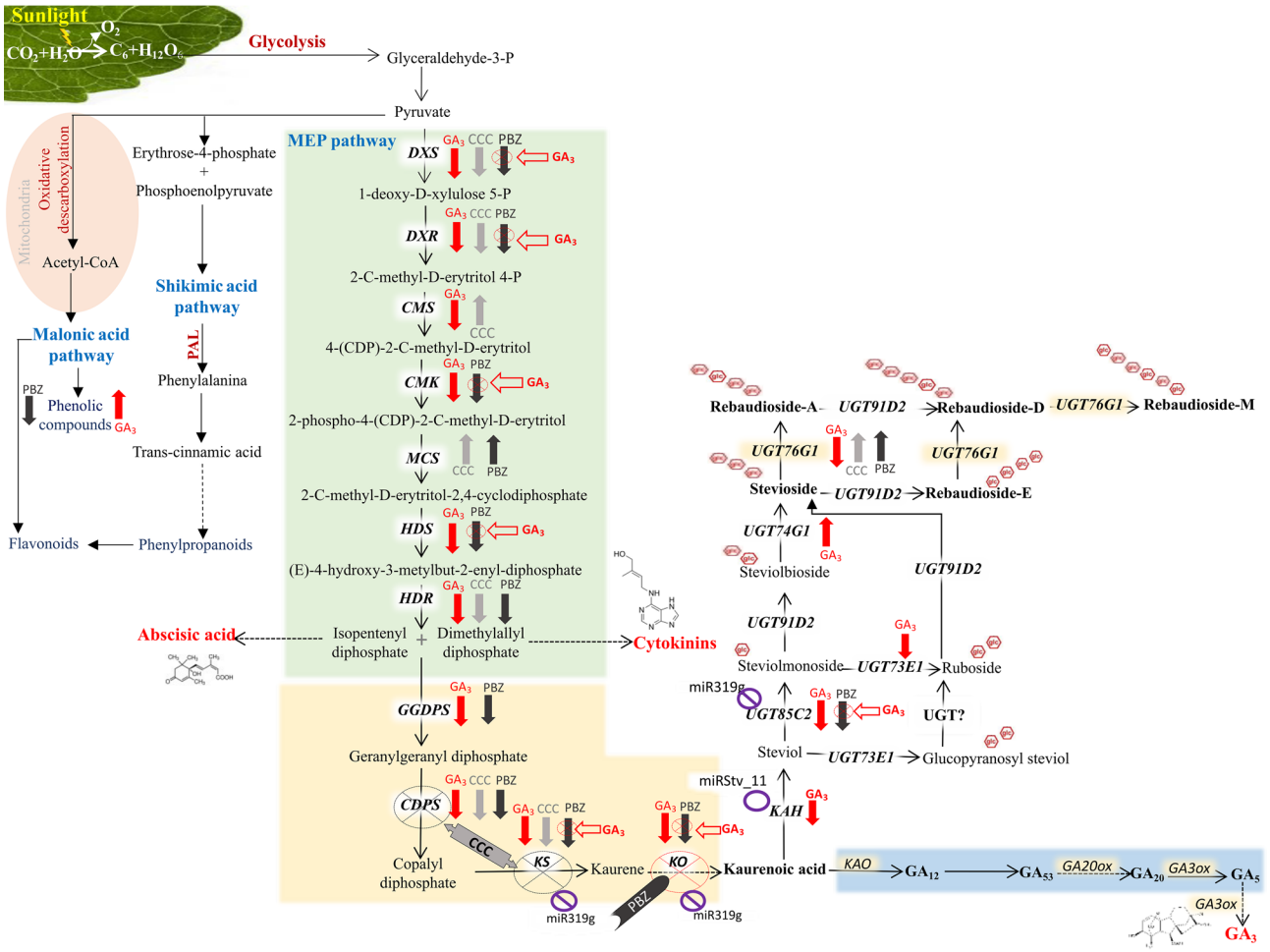


Fig. 6 Schematic representation of biosynthetic steps involved in phenolic compound biosynthesis and SGs/GAs in *Stevia rebaudiana* and points of inhibition by plant growth retardants (CCC and PBZ). Differential expression pattern of genes encoding enzymes of the 1st stage (green box), 2nd stage (cream box), and 3rd stage (white) are shown, as well as a simplified pathway of GAs biosynthesis (blue). Enzymatic flow is depicted as arrows from substrates to products. Enzymes catalyzing each step are shown into each arrow. Solid upward arrows in red (GA_3), light gray (CCC), and dark gray (PBZ) represent upregulation by the respective PGR, whereas those solid downward arrows represent downregulation and the hollow red arrows represent reversal of negative effect by GA_3 treatment. A single arrow indicates a one-step reaction, and double arrows represent multiple-step pathway

between the transcription of *SrUGT85C2* and SGs accumulation (Hajihashemi et al. 2013; Yoneda et al. 2018). Moreover, the *SrKAH* gene showed a positive correlation with 12 of the 17 genes in the pathway. The KAH enzyme is upstream from steviol and is of great interest in biotechnology (Brandle and Telmer 2007; Kundu et al. 2014).

Regarding STEV quantification in GA_3-treated plants, the results showed twice STEV content as that in control plants. Interestingly, GA_3-treated plants showed upregulation of *SrUGT74G1* (the gene encoding the enzyme that glycosylates steviolbioside at the C-19 position, forming STEV). Kumar et al. (2012) also showed that GA_3 upregulated the *SrUGT74G1* gene in stevia plants, however they did not report the content of SGs. Other authors have reported that STEV content increased (Hajihashemi and Geuns 2017; Yoneda et al. 2018), decreased (Karimi et al. 2015), or did not show significant changes (Pazuki et al. 2019) in response to GA_3 treatment. Overall, our investigations found a higher STEV amount in GA_3-treated, in vitro-cultured stevia plants than that reported by Bondarev et al. (2001) and Ladygin et al. (2008) (less than 5 mg g^{-1} DW in in vitro leaves and about 25 mg g^{-1} DW in leaves from intact plants, respectively).

It seems paradoxical that the highest STEV levels were obtained when the expression of almost all GAs/SGs biosynthesis genes were downregulated in response to GA_3. Relatively high levels of exogenous GA_3 were expected to repress the GAs/SGs biosynthesis pathways by negative feedback, thus decreasing product accumulation. However, the levels of SGs in stevia leaves were several orders of

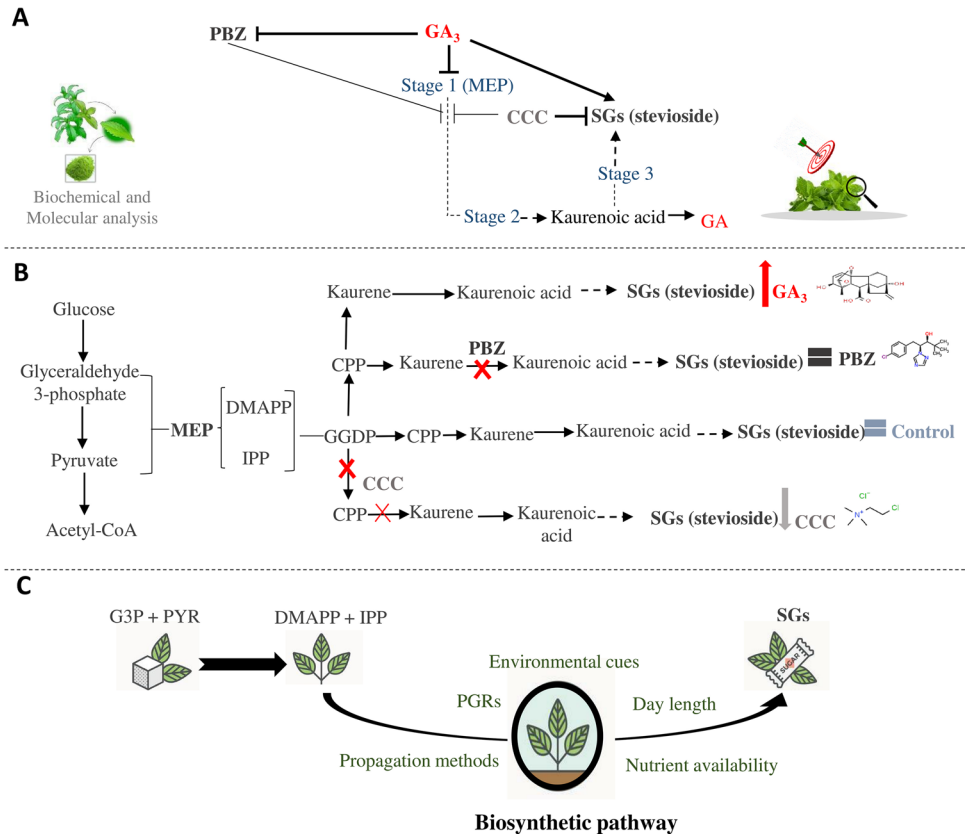


Fig. 7 Model depicting the metabolic flux in *Stevia rebaudiana* in response to GA_3, CCC and PBZ. Feedback loop (**a**), simple illustration of the biosynthetic steps of SGs (**b**) and factors that can influence their synthesis (**c**). Dashed arrows represent multistep pathways, whereas solid arrows represent only one step (X, x = major and minor inhibitory activity, respectively)

magnitude higher than those of GAs (Geuns 2003), which suggests that although biosynthesis intermediaries are common for both pathways, regulatory mechanisms might differ between biosynthesis routes as some authors have previously proposed (Karimi et al. 2015).

Additionally, PBZ-treated plants showed STEV levels very similar to those obtained in control plants (about 18 mg g^{-1} DW). Based on the statement that the *SrKO* gene has two functional copies (Humphrey et al. 2006), Karimi et al. (2019) recently suggested that one copy may be more related to SGs biosynthesis, while the other would be related to GAs biosynthesis. This might be why treatment with PBZ, even when blocking the *SrKO* gene, showed a STEV content similar to that of the control plants. Conversely, CCC-treated plants showed a decrease in STEV content. Karimi et al. (2014, 2019) observed similar trends in relation to the negative effects of CCC on the accumulation of SGs. A possible reason for this STEV decrease in CCC-treated plants may be related to its inhibitory action on two enzymes (CDPS and KS), instead of on only one (KO), as that seen for PBZ.

In the search for differences and similarities between treatments, PCA analysis was performed (Fig. 5). Based on the obtained results, the factors that had the most influence on the separation of samples were those related to gene expression in PC1 and growth parameters in PC2. Overall, the GA_3 alone treatment influenced all the evaluated parameters, unlike its inhibitors. In addition, GA_3 reversed the negative effects of PBZ, mainly at the molecular level, partially confirming our hypothesis. Collectively, ours results suggest that the application of GA_3 may be a viable way to improve the production of stevioside and phenolic compounds in stevia plants and that this compound can be considered a pivotal regulator of SGs/GAs biosynthesis-related genes. Besides, this study provides the first model for regulation of the expression of SG-related genes in stevia leaves and explores other factors that may influence its biosynthesis (Fig. 7). A better understanding of these pathways and of how external factors, including PGRs (GA_3, CCC, and PBZ), influence them will allow us to take full advantage of the richness that stevia plants offer.

Conclusion

Chlorocholine chloride and PBZ showed different effects in relation to the morphological characteristics and specialised metabolite synthesis in stevia plants. However, at the molecular level, both inhibitors upregulated the *SrUGT76G1* gene. Interestingly, among the three PGRs evaluated, GA_3 had the most negative impact on gene expression both when used alone and especially after treatment with CCC. Despite GA_3 not having a positive impact on transcription patterns,

the obtained results demonstrated that STEV and TPC contents were much higher than those observed in the control plants. Thus, our experiment confirmed that exogenous supplementation of GA_3 caused negative feedback on SGs/GAs biosynthesis genes and the negative modulator role of CCC and PBZ on the kaurenoid genes acid condensation step. Besides, we demonstrated for the first time a feedback loop in stevia plants cultured in vitro in response to GA_3 and its inhibitors CCC and PBZ.

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Author contributions Conceived and designed the experiments: SRL and EJBB. Performed the experiments: SRL, CM, LA and MNA. Analysed the data: SRL, MNA and EJBB. Wrote the paper: SRL, AAC and EJBB. Corrected the manuscript: MAF, AAC, VJB and EJBB.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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