Plant growth retardants (PGRs) affect growth and secondary metabolite biosynthesis in *Stevia rebaudiana* Bertoni under drought stress

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ABSTRACT

Beyond the inhibitory action against the gibberellin biosynthesis, some plant growth retardants (PGRs) can play an important role in regulating plant responses to abiotic stress through the induction of different tolerance mechanisms. The aim of the present study was the exploitation of the potential of PGRs in enhancing the resistance to drought stress in *Stevia rebaudiana* Bert. Therefore, the effects of three PGRs on stevia plants grown under drought stress condition were investigated. Stevia plants were first subjected to water stress and, second, treated with PGRs to detect PGRs effect on biometric, productive and phytochemical characteristics of drought stressed-plants. The control plants were uniformly irrigated at 3-day intervals, while water-stress conditions were imposed by watering the plants at 12-day intervals. Subsequently, the Chlorocholine chloride (CCC, as Copalyl diphosphate synthase inhibitor and Kaurene synthase inhibitor), Paclobutrazol (PBZ, as Kaurene oxidase inhibitor) and Daminozide (DAM, as anti-gibberellins) were applied in drought stressed-plants. The CCC and DAM were sprayed on stevia shoots, while PBZ was drenched. The obtained results showed that leaf dry weight of stevia plants was significantly reduced by drought stress, but this parameter increased as a consequence of CCC and PBZ treatments. Drought stress also caused a significant reduction in total steviol glycoside (SVglys) content. This reduction was more pronounced in drought stressed-plants treated with CCC, while PBZ was able to counteract the SVglys reduction, with SVgly content similar to that observed in the control. Similarly, PBZ was able to increase the soluble sugar production and total antioxidant capacity in the leaves of stressed-stevia plants. These findings suggested that CCC and, in particular, PBZ had a protective effect on stevia growth under drought stress by induction of antioxidant defenses and soluble sugar production. CCC seems to inhibit gibberellin biosynthesis, preventing the SVglys production, while DAM and PBZ, as gibberellin inhibitors, didn't have a negative effect on SVglys production in drought stressed-plants. This observation seems to emphasize their role in limiting the rate of target enzymes of CCC in SVglys biosynthetic pathway. Moreover, the induction of glucose production, as a substrate for SVglys biosynthesis, could be a convincing evidence for SVglys promotion in PBZ treated-plants.

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1. Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a perennial plant belonging to *Asteraceae* family that produces sweet compounds well-known as steviol glycosides (SVglys). The main SVglys present in stevia leaves are Steviolmonoside, Steviolbioside, Stevioside (Stev), Rebaudioside (Reb) A, B, C, F and Dulcoside (Dulc) A (Tavarini et al., 2015). Generally, Stev and Reb A are the two major compounds (Richman et al., 1999). Stev is the most abundant, while Reb A exhibits a more pronounced sweetness and a lesser licorice-like aftertaste and bitterness than Stev. Accordingly, Reb A to Stev ratio could be considered a good qualitative measure of sweetness (Sharma et al., 2009). The SVglys are diterpene compounds and are produced in a shared biosynthetic pathway with gibberellins, as presented in Fig. 1 (Brandle and Telmer, 2007). The divergence between SVglys and gibberellin begins from (−)-kaurenoic acid and it is dependent on the hydroxylation site of (−)-kaurenoic acid (Richman et al., 1999). The hydroxylation of (−)-kaurenoic acid
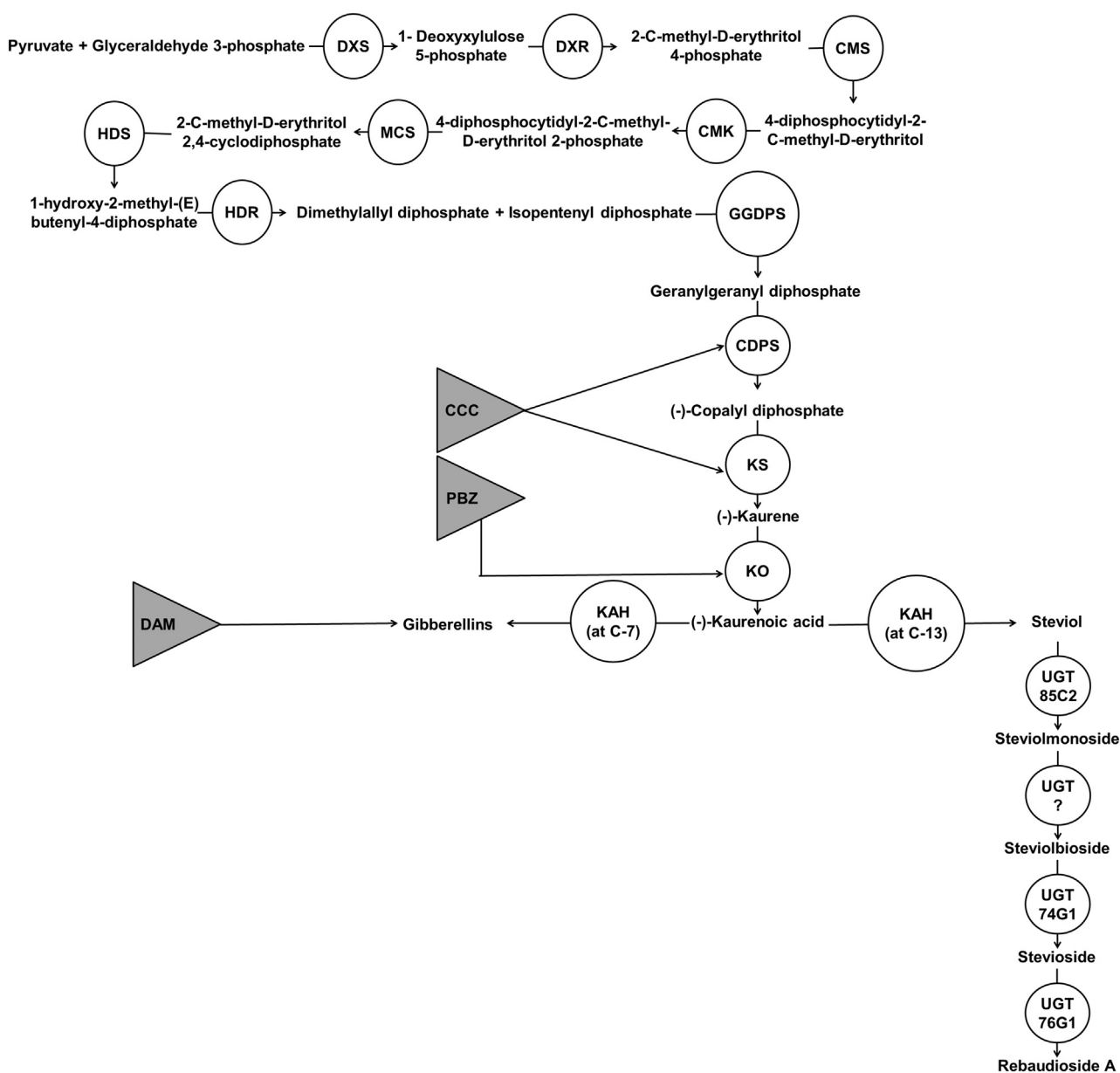
Fig. 1. PGRs (CCC, Chlorocholine chloride; PBZ, Paclobutrazol; DAM, Daminozide) effect on the biosynthesis of SVglys and gibberellins in Stevia (DXS, Deoxyxyulose-5-phosphate synthase; DXR, Deoxyxyulose-5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2-C-methylD-erythritol synthase; CMK, 4-diphosphocytidyl-2-C-methylD-erythritol kinase; MCS, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2 (E)-butenyl 4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2 (E)-butenyl 4-diphosphate reductase; GGDPS, Geranylgeranyl diphosphate synthase; CDPS, Copalyl diphosphate synthase; KS, Kaurene synthase; KO, Kaurene oxidase; KAH, Kaurenoic acid hydroxylase; UGT, UDP-glucose transferase).

at C-7 position results in gibberellins (GAs) (Hedden and Kamiya, 1997), while hydroxylation at C-13 position results in steviol production (Kim et al., 1996). In plants, GAs formation starts with GA_{12} biosynthesis (Hedden and Kamiya, 1997; Pimenta Lange and Lange, 2006). After steviol biosynthesis, the glucose units are transferred to steviol by several glycosiltransferases (UGTase), which result in various SVglys (Shibata et al., 1991) that are stored in the vacuoles (Brandle et al., 1998; Starratt et al., 2002).

Plant growth retardants (PGRs) are chemical compounds able to inhibit gibberellin biosynthesis and, consequently, inhibit plant growth. Chlorocholine chloride (CCC) is a quaternary ammonium compound which inhibits the Copalyl diphosphate synthase (CDPS) and Kaurene synthase (KS) in the GAs biosynthetic pathway. Triazoles group – such as Paclobutrazol (PBZ) – blocks Kaurene oxidase (KO). In stevia, the CDPS, KS and KO are held in common between gibberellins and SVglys biosynthetic pathway and they are specific targets for CCC and PBZ. In

our previous work (Karimi et al., 2014a), carried out on stevia plants grown under optimal water conditions with the aim to preliminary test the effect of PGRs, we observed that CCC (1000 ppm) reduced stem length and SVglys biosynthesis, while PBZ (12 ppm) improved plant growth and SVglys production. Daminozide (DAM) inhibits the formation of active gibberellins in plant and GAs function (Rademacher, 2000). Gibberellins have the potential to be a competitor for SVglys production in stevia, using common substrates. Accordingly, GAs variation may alter the SVglys biosynthesis as well as stevia biomass production.

Although PGRs inhibit plant growth, some beneficial effects on plants have been observed, especially when plants are faced with abiotic stresses. For example, it has been shown that the activity of antioxidant enzymes was increased by CCC application in potato (Wang et al., 2010). Similarly, the methanolic extracts of stevia calli and leaves treated with CCC showed interesting level of antioxidant activity (Dey

et al., 2013). Since oxidative stress is one of the major causes of plant injury under drought conditions (Munne-Bosch and Penuelas, 2003), an enhancement of enzymatic and non-enzymatic antioxidants and, consequently, an improved free radical scavenging activity represent useful physiological strategies for plants to cope with drought. Moreover, triazoles can protect plants against various stresses, acting as multi-protectants thanks to their ability to induce tolerance under abiotic stresses (Fletcher et al., 2000). The alleviation of drought stress effects by PBZ application has also been reported in triticale (Berova and Zlatev, 2003), *Phillyrea angustifolia* L. (Fernandez et al., 2006) and groundnut (Sankar et al., 2007). It has also been shown that PBZ reduced the negative effect of drought stress on lipid peroxidation in stevia leaf which resulted in lower electrolyte leakage (Hajihashemi and Ehsanpour, 2013).

Plants can exhibit a range of physiological and biochemical adaptive responses to withstand drought stress. Among plant chemical signals, the accumulation of compatible solutes and the enhancement of the total antioxidant capacity have been reported as mechanisms that allow plants to tolerate drought stress (Smirnoff, 1998). Soluble sugar accumulation is an important strategy in plant faced with drought stress (Chaves et al., 2003; Kerepesi and Galiba, 2000) because it improves water saving in tissues through an osmotic adjustment process. It is notable that soluble sugars in stevia, especially glucose and sucrose, participate in SVglys production. However, it is not clear if, in drought-stressed stevia plants, soluble sugars shift to osmotic adjustment or remain in SVglys production systems. To the best of our knowledge, no comprehensive studies have been carried out in stevia in order to define the role of PGRs application in regulating the response of this species to drought stress. Consequently, the present study aims to investigate the overall effect of three PGRs (CCC, PBZ and DAM) on drought resistance/tolerance of *Stevia rebaudiana* Bert. Therefore, (i) stevia growth parameters and biomass yield, (ii) SVglys concentration and profile, (iii) soluble sugar content, and (iv) total antioxidant capacity have been evaluated in plants grown under water stress conditions treated with PGRs in comparison with well-watered plants as a control.

2. Materials and methods

2.1. Growing conditions and treatments

The trial was carried out in greenhouse conditions at the Agricultural Biotechnology Research Institute of Iran (ABRII, Central of Iran, Isfahan), adopting a completely randomized design with five treatments and three replications per treatment. *Stevia rebaudiana* Bertoni plants were propagated through tissue culture (Handro et al., 1977). The plantlets were initially cultivated in peat moss medium to select the well-established ones. After three weeks, the uniform plantlets were transplanted into pots containing loam soil (50% sand, 15% clay; field capacity 20.2%; wilting point 10.5%; bulk density 1.38 g cm^{-3}). The pots were filled with soil up to 2 cm below the surface and three seedlings were cultivated in each pot (3 plants in each pot and 3 pots per each treatments). Drought stress was imposed to stevia plantlets from two weeks after transplanting. During the growth period, the greenhouse temperature, humidity and CO_2 ranged between 25 and 22 °C, 40%–60% and 400–500 ppm, on day–night, respectively. The irrigation treatments were applied as a control (fully irrigated, every 3 days) and drought stress (12-days irrigation interval), according to Karimi et al. (2015a). Thereafter, three PGRs - including Chlorocholine chloride (CCC, 1000 ppm), Paclobutrazol (PBZ, 12 ppm), and Daminozide (DAM, 15 ppm) – were applied on stressed plants. Formerly, two studies have been done to select the most appropriate soil moisture level for inducing drought stress in stevia plants (Karimi et al., 2015b) and PGRs concentrations (Karimi et al., 2014a).

The Chlorocholine chloride, (2-Chloroethyl trimethylammonium chloride, CCC, CAS number: 999–81-5, from Sigma–Aldrich) was dissolved in distilled water, and a solution containing 1000 ppm was prepared. The Paclobutrazol ([(2 RS, 3 RS)-1-(4-chlorophenyl)-4,4-di-methyl-2-(1-H-1,2,4-trizol-1-yl)-pentan-3-ol], PBZ, CAS number: 76738–62-0, from DUCHEFA Biochemie B.V) was firstly solubilized in pure ethanol. Then, a 12 ppm PBZ solution was obtained by diluting stock with distilled water. The Daminozide (N-Dimethylamino-succinamic acid, DAM, CAS number: 1596-84-5, from Merck Millipore) was dissolved in distilled water to obtain 15 ppm solution. The CCC and DAM were sprayed on stevia shoot while PBZ was drenched into the soil because it is absorbed through xylem (Rademacher, 1997). The PGRs were applied to stressed-plants 7 times at 5 days interval and 150 mL of each solution was used in each application. The plants were harvested after a week from the last PGRs application (64 days after transplanting). A specific sample of fresh leaves was prepared and kept at −20 °C for antioxidant capacity assessment. Leaves and stems were manually separated and then dried in a ventilated oven at 65 °C for 48 h and their weights recorded. For SVglys and soluble sugars determinations, the dried leaf samples were ground into a fine powder (particles of 0.10 mm in size).

2.2. Extraction of SVglys

Extraction and assessment of SVglys was conducted based on Karimi et al. (2016). 0.1 g of powdered leaves was suspended into 3 mL distilled water and the mixture was kept in a water bath at 80 °C for 30 min. Resultant mixture was centrifuged at 12,000g for 5 min and the supernatant was recovered. Finally, 3 mL distilled water was added to the pellet and the last process was repeated 3 times. The supernatants were pooled in a new tube and centrifuged (12,000 g for 5 min), and new supernatant was transferred to a new tube. Finally, 1 mL distilled water was added to the new pellet, centrifuged again (12,000 g for 5 min) and its supernatant was added to the previous one. The final volume of supernatant was diluted to 10 mL using distilled water and filtrated (0.45 μm filter). In order to purify SVglys, a C_{18} cartridge was used. Then, 0.5 mL of supernatant was loaded into C_{18} cartridge, and subsequently the cartridge was washed with acetonitrile/water mixture (20:80; V/V). Finally, SVglys were eluted from C_{18} cartridge with acetonitrile/water mixture (80:20 V/V) and kept in tube at −20 °C to further assessment.

2.3. High-performance liquid chromatography (HPLC)

For chromatographic analysis, two reverse-phase C18 columns were connected in series and a UV–Vis detector was used to detect the SVglys at 202 nm. Acetonitrile and water (50–80%, v/v) with a flow rate of 0.5 mL min^{-1} were used as mobile phases, using a gradient elution (Karimi et al., 2015a). For quantification purposes, pure Stevioside and Rebaudioside A (purity>99%) were used as external standards. Reb F, Reb C and Dulc A were quantified by their molecular weight ratio to Reb A (Geuns et al., 2009). Peak area was calculated by Chromstar 7.0 software, and the results were expressed as percentage of SVglys in the leaf dry matter (w/w).

2.4. Total antioxidant capacity

Total antioxidant capacity was evaluated by the DPPH (2, 2 diphenyl-1-picrylhydrazyl) free radical method, according to Karimi et al. (2014a) and Thaipong et al. (2006). The IC$_{50}$ value was calculated as the sample concentration necessary to decrease the initial absorbance of DPPH by 50%. The IC$_{50}^{-1}$ was used as an index of total antioxidant capacity (Hasperué et al., 2011).

2.5. Soluble sugars quantification

Dried leaves (0.04 g) were ground to a powder and extracted as described by Tobias et al. (1992) with some modifications (Karimi et al.,

2015a). Samples were assayed by coupled enzymatic assay methods, and the absorption increase at 340 nm (A_{340}) was measured (Moles et al., 2016). The accuracy of the method was tested using standards with known amounts of glucose. Incubations of samples and standards were carried out at 37 °C for 30 min. Finally, glucose, fructose and sucrose were assessed, and data was expressed as µmoles hexoses equivalent g^{-1} dry weight (DW).

2.6. Data analysis

Data were analyzed by SAS 9.2 software (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27,513) in ANOVA (Analysis of variance) procedure. Means were separated using Fisher's protected least significant difference (LSD) when F tests were significant at $p ≤ .05$. The histograms were prepared by Sigma Plot 12.3 software. The standard error (SE) is presented with mean values in the tables and histograms.

3. Results

3.1. Stevia growth

Results showed that stevia growth and yield (plant height, leaf and stem dry yield, and harvest index – HI) were significantly affected by drought stress and PGRs treatments ($p ≤ .05$, Table 1). Plant height decreased in drought stressed plants and no significant effects of PGRs treatments on plant height were observed (Table 2). Drought stress significantly reduced leaf dry weight (−16% in comparison with well-watered plants), but CCC and PBZ treatments were able to suppress this negative effect with similar yields to those observed in the control, with a higher effect observed in PBZ (+19%) in comparison with CCC (+13%). On the other hand, DAM effect in leaf weight inhibition was significantly lower (+8%), in comparison with CCC and PBZ treatments (Table 2). No significant variations in stem dry weight were observed between drought stressed and well-watered plants (Table 2). Regarding the PGRs effects, only PBZ was able to significantly increase stem dry weight in drought stressed plants (Table 2). Harvest index (HI) significantly decreased (−6%) in drought stressed plants in comparison with the control, but it returned to near the initial level (in well-watered conditions) through CCC and DAM application, which caused a significant increase in the HI of stressed plants (+9 and + 8%, respectively).

3.2. SVglys content and composition

Total SVglys content was significantly affected by drought stress and PGRs treatments ($p ≤ .05$, Table 1). The total SVglys content slightly decreased under drought stress (−11% than control). On the other hand, DAM and CCC applications intensified SVglys reduction in drought stressed plants, and the lowest SVglys content was obtained in stressed plant treated with CCC (Fig. 2a). On the contrary, PBZ treatment determined an increase of these compounds, reaching similar levels to those observed in the control. Regarding SVglys composition, Stev and Reb F were negatively affected by drought stress, while no differences were observed for the other SVglys (Fig. 2b and Table 1). PGRs

treatments significantly affected only the levels of Stev and Reb A, with a positive effect of PBZ in enhancing Stev content in stressed plants (Table 1). On the contrary, the other PGRs resulted in a further reduction of Stev (CCC and DAM) and Reb A (CCC) contents (Fig. 2b). Reb A/Stev ratio showed significant variation due to drought and PGRs treatments, in comparison with the control (Table 1 and Fig. 2c). Reb A seemed to be more stable than Stev in response to both drought stress and PGRs. Consequently, Reb A/Stev ratio increased in all drought-stressed plants and PGRs treatments, in comparison with the control (Fig. 2c).

The SVglys yield significantly varied due to drought stress and PGRs treatments ($p ≤ .05$, Table 1), according to that observed for dry leaf yield and SVglys. Drought stress caused a significant reduction (−25%) in SVglys yield, but the PBZ application reestablished SVglys yields to the level of well-watered treatment (Fig. 2d). As observed for total SVglys as well as for Reb A and Stev contents, CCC intensified SVglys yield reduction (−19%, in comparison with stressed plants), while the DAM application had no effects (Fig. 2d).

3.3. Soluble sugars

Total soluble sugar (TSS) content as well as glucose, fructose and sucrose were significantly affected by drought stress and PGRs treatments ($p ≤ .05$, Table 1). The TSS content was significantly higher both in drought stressed plants and PGRs treated plants, in comparison with the control (Fig. 3a). A sharp decline in glucose content was observed when drought and PGRs treatments were applied (Fig. 3b). On the contrary, fructose and sucrose significantly increased after drought and PGRs treatments. In particular, the highest content of sucrose was observed in plants after drought, while no differences were recorded among stressed and PGRs-treated plant for fructose content (Fig. 3b).

3.4. Antioxidant capacity

Total antioxidant capacity of stevia leaves was significantly affected by drought stress and PGRs treatments ($p ≤ .05$, Table 1). Drought stress caused a slight increment in antioxidant capacity of stevia leaves, but, interestingly, a strong increase was observed after PBZ application. On the contrary, DAM caused a significant reduction of total antioxidant capacity, in comparison to the other treatments (Fig. 3c).

4. Discussion

PGRs generally act as stem growth inhibitor, especially through interference with gibberellin biosynthesis. However, in the present study, it has been clearly observed, for the first time, that, in stevia, PGRs can be used as valuable treatments in order to reduce both leaf yield and SVglys losses due to drought. In fact, it has been observed that stem dry weight reduction in drought stressed plants was compensated by PBZ application. It means that PBZ, in stevia, can induce dry matter allocation towards the stems, despite its inherent role. These observations suggest that, in stevia, PBZ was not a growth retardant, but it stimulated the dry matter allocation in its stem, confirming previous findings observed in well-watered plants (Karimi et al., 2014a).

Table 1
Analysis of variance for stevia traits under drought stress and PGRs treatments (CCC, Chlorocholine chloride; PBZ, Paclobutrazol; DAM, Daminozide).

Source of variation	df	MS (mean of square)																
		Plant height	Leaf dry weight	Stem dry weight	HI	Reb A	Stev	Reb F	Reb C	Dulc A	Total SVglys	Reb A/Stev	SVglys yield	1/IC$_{50}$	TSS	Glucose	Fructose	Sucrose
Treatment	4	633**	0.28**	0.5*	9.4**	0.29**	1.5**	0.02	0.01	0.0008	3.75**	0.008*	0.009**	0.29**	2376*	3914**	603**	6241**
Error	10	10.6	0.03	0.1	0.8	0.04	0.08	0.01	0.005	0.0008	0.3	0.001	0.0008	0.03	612	428	35	385
CV	–	4.7	4.2	6.8	1.9	10.4	8.3	31.4	12.7	15.6	8.3	7.3	10.06	7.1	7.9	14.1	18.9	14.5
R^2	–	0.95	0.77	0.64	0.81	0.72	0.87	0.39	0.50	0.27	0.82	0.65	0.83	0.76	0.60	0.78	0.87	0.86

** Significant at 0.01 level.
* Significant at 0.05 level.

Table 2
Means (±SE) of plant height, leaf dry weight, stem dry weight and harvest index (HI) of stevia under drought stress and PGRs treatments (CCC, Chlorocholine chloride; PBZ, Paclobutrazol; DAM, Daminozide).

Treatments	Plant height (cm)	Leaf dry weight (g.plant^{-1})	Stem dry weight (g.plant^{-1})	HI
Control	94.3 ± 2.3[a]	4.47 ± 0.09[a]	4.99 ± 0.15a[b]	47.2 ± 0.3[a]
Drought Stress	61.33 ± 0.88[b]	3.76 ± 0.13[c]	4.73 ± 0.19[b]	44.1 ± 0.7[b]
CCC (1000 ppm)	63.6 ± 2.4[b]	4.25 ± 0.02[ab]	4.57 ± 0.07[b]	48.1 ± 0.2[a]
PBZ (12 ppm)	61 ± 2.08[b]	4.74 ± 0.06[a]	5.45 ± 0.28[a]	45.1 ± 0.7[b]
DAM (15 ppm)	61.6 ± 1.2[b]	4.05 ± 0.12[bc]	4.4 ± 0.17[b]	47.9 ± 0.2[a]
LSD (p ≤ .05)	6.9	0.32	0.6	1.5

Mean values followed by different letters are significantly different according to least significant difference (LSD) test ($p \leq .05$).

The positive effect of PBZ was also observed in the leaf dry weight. Since, in stevia, the leaves represent the economically interesting yield, PBZ application could be a supportive approach to its increase, but food safety should be emphasized in PGRs application. Altogether, it can be noted that PGRs showed a protective role for stevia leaves under drought stress condition. The positive effects of CCC, PBZ and DAM on leaf yields have been also observed under normal condition (Karimi et al., 2014a; Karimi et al., 2014c). Similarly, an increase in leaf dry weight of DAM-treated *Salvia officinalis* has been previously reported (Schmiderer et al., 2010). These results suggest that anti-gibberellin compounds, such as CCC and DAM induce photo-assimilates flow into the leaves which could be an useful trait in leafy plant, such as stevia.

The main agronomic effect of PGRs on stressed stevia plants was the significant increase in HI. The increase of HI by DAM and PBZ was also observed in previous studies (Karimi et al., 2014a; Karimi et al., 2014c), supporting the hypothesis that PGRs could shift the dry matter allocation into the leaves. In the present study, the low value of HI in PBZ-treated plants, in comparison with the control and the other PGRs treatments, was due to the strong increase of stem yield induced by PBZ. All these findings underlined that, among the PGRs, PBZ was the most effective in promoting drought tolerance in stevia plants in terms of maintenance and/or enhancement of plant growth. Similarly, in a study carried out on *Brassica carinata*, it was also found that PBZ treatment was responsible for significant increase in total dry matter (Setia et al., 1995). It can be argued that PBZ, as a triazole compound, could alleviate the drought stress in stevia, confirming the multi-protective properties of this substance. It is not clear how PBZ improved stevia growth under drought stress, but it is believed that PBZ effects may occur through the induction of abscisic acid, proline and stomatal
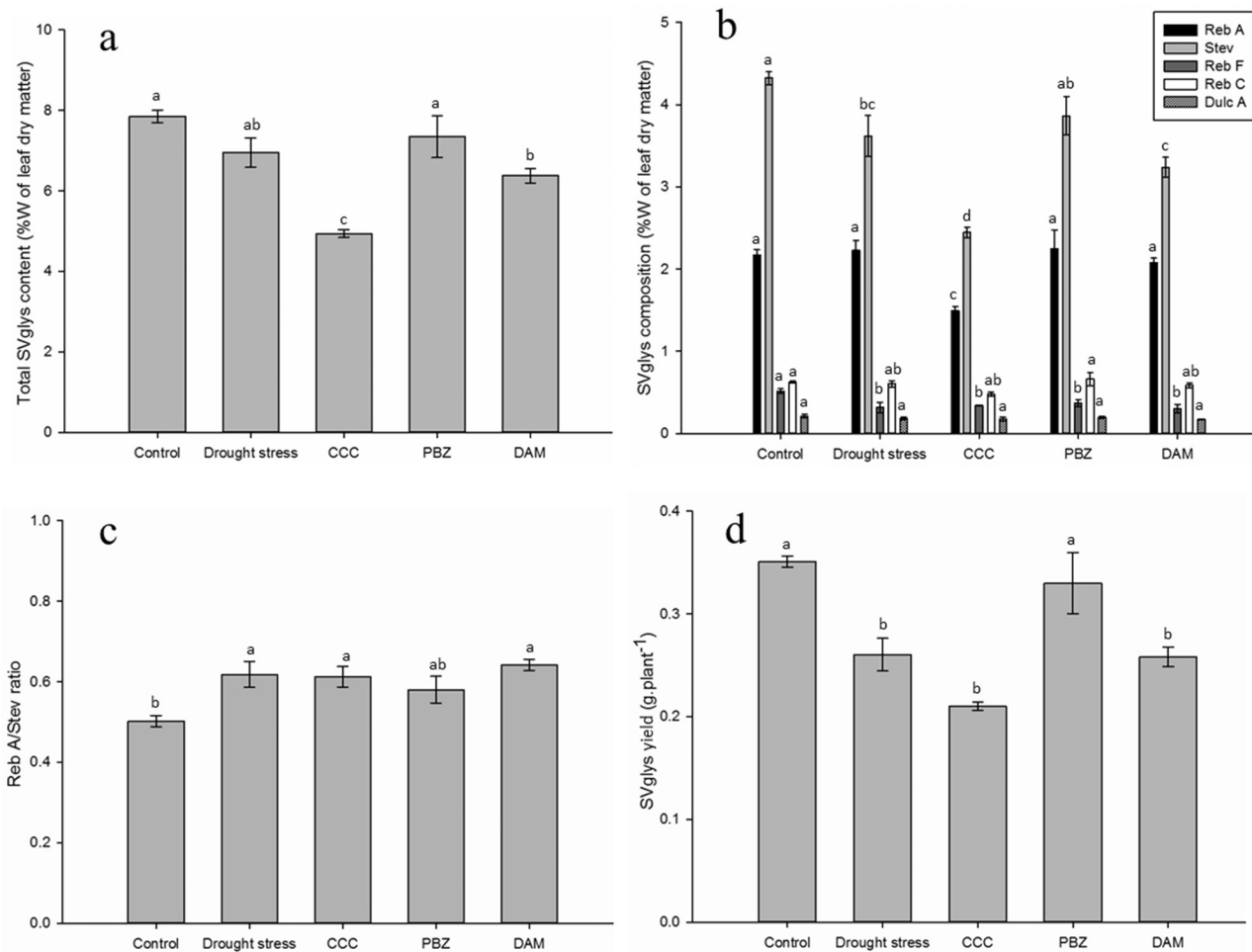

Fig. 2. Total SVglys content (a), SVglys composition (b – each compound was separately compared by LSD test among treatments and not compared with others), Reb A/Stev ratio (c) and SVglys yield (d) of stevia under drought stress and PGRs treatments (CCC, Chlorocholine chloride; PBZ, Paclobutrazol; DAM, Daminozide). Standard error (SE) for means values is presented as a vertical bar. The letters presented on histograms are based on LSD test and the different letters show the significant differences.
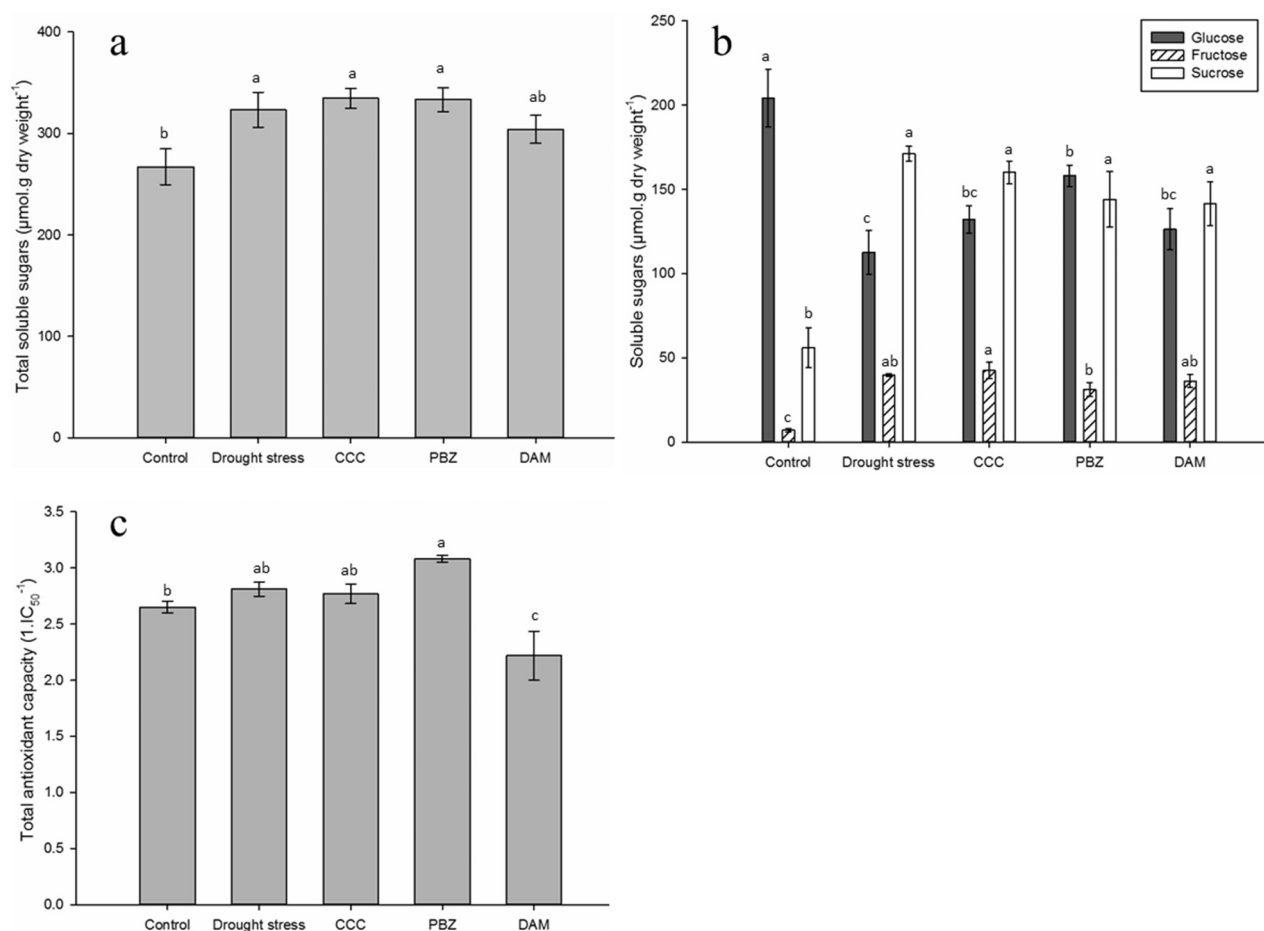
Fig. 3. Total soluble sugars (a), Soluble sugars (b – each soluble sugar was separately compared by LSD test among treatments, and not compared with others) and Total antioxidant capacity (c) of stevia under drought stress and PGRs treatments (CCC, Chlorocholine chloride; PBZ, Paclobutrazol; DAM, Daminozide). Standard error (SE) for means values is presented as a vertical bar. The letters presented on histograms are based on LSD test and the different letters show the significant differences.

closure (Berova and Zlatev, 2003; Jiang and Fry, 1998; Still and Pill, 2004; Zhang et al., 2007), reducing water losses (Wang et al., 1986), detoxification by ROS (Kraus and Fletcher, 1994; Lin et al., 2006) and improving root growth (Bandara and Tanino, 1995; Davis et al., 1991; Jaleel et al., 2007; Yim et al., 1997). DAM effects on growth promotion were lower than PBZ. However, it can be stated that DAM also improved stevia growth under drought stress.

The SVglys biosynthesis in stevia is constituted from a complex metabolic pathway, and it is not clear which stage of this pathway can be affected by drought. In addition, individual SVglys were differently affected by drought stress. For example, Stev was negatively affected by drought stress and with a greater extent than Reb A. There is no clear reason why Reb A is more stable than Stev to drought. This different behavior may be related to their chemical structures and, consequently, to the number of the glucose units and their relative position. In this regard, in fact, Reb A is characterized by having a glucose unit more than Stev (4 glucose units in Reb A versus 3 units in Stev), and it is the final product of glycosylation process in SVglys biosynthetic pathway. Finally, it can be noted that the stability of the Reb A leads to better quality of SVglys in stressed plants, due to an improved Reb A to Stev ratio under these conditions.

Since SVglys yield depend on SVglys content and leaf yield, it can be noted that, in drought-stressed plants, the reduction in SVglys yield was mainly due to the decrease of leaf yield (Table 2). Similar to that observed for other traits, PBZ was able to maintain the SVglys yield in a reasonable range. These results emphasize the role of PBZ as a guardian compound for stressed plants. The preservation of SVglys in stressed plants is remarkable since the attainment of the highest SVglys yield is

the main purpose of stevia cultivation. On the contrary, in CC treated-plants, the SVglys yield reduction depended on the significant decrease of total SVglys content. In fact, although stevia leaf growth was promoted by CCC application, SVglys yield was reduced due to the very low quantity of SVglys in the leaves of CCC-treated plants. This result was in accordance with previous reports, under normal irrigation condition (Karimi et al., 2014b).

The main reason for SVglys reduction in CCC-treated plants could be related to CCC action mode. As previously reported (Brandle and Telmer, 2007; Totté et al., 2000), CCC acts as an inhibitor for CDPS and KS enzymes, which are directly involved in the SVglys biosynthetic pathway. In this regard, in our preliminary investigation (Karimi et al., 2014a), we observed that, under well-watered conditions, 1000 ppm CCC caused a 50% reduction in total SVglys content. Considering the SVglys biosynthetic pathways, SVglys reduction induced by CCC seems to be involved in the cyclization stages (including the activity of CDPS and KS). It seems that, in stevia, CDPS activity may be more important than KS and may be more influenced by CCC, due to the low KS inhibition by CCC (Shechter and West, 1969). Moreover, it has been reported that KS gene has been duplicated in the stevia genome (Richman et al., 1999). Consequently, most of the CCC efficacy may probably occur through CDPS inhibition and this enzyme might act as a rate limiting factor in the SVglys biosynthetic pathway. Previously, it has been reported that corn cdps mutants were not completely inactive in gibberellin biosynthesis (Hedden and Kamiya, 1997), and it can be concluded that this stage may not be a rate limiting factor for gibberellin biosynthesis. On the other hand, in our study, using CCC as an anti-gibberellin compound and as a CDPS inhibitor, it can be reasoned that

this stage could be a limiting factor at least for SVglys biosynthesis and, probably, in gibberellin biosynthetic pathway.

PBZ is a KO inhibitor and it was expected that total SVglys content will be reduced by PBZ application, due to the action mode of KO in SVglys biosynthesis. However, it was observed that the content of SVglys did not reduce in PBZ-treated plants. This result was similar to a previous experiment, carried out in a well-watered stevia plants (Karimi et al., 2014a). In stevia, it has been found that there are two functional copies for *KO*, while just one copy of the *KO* has been found in other species (Humphrey et al., 2006). Therefore, it can be assumed that the KO targeting by PBZ is not active in the SVglys biosynthesis. On the basis of our results and according to the hypothesis of Humphrey et al. (2006), and also for stevia, it might be considered that one copy of *KO* acts for gibberellin biosynthesis and the other one acts for SVglys biosynthesis. Since there was a lack of effect of PBZ on SVglys production, it can be considered that, if KO inhibition occurred in SVglys biosynthetic pathway, the process can be supplied from an alternative pathway. Due to the induction of leaf growth and total SVglys content by PBZ application, the SVglys yield was also stabled in PBZ-treated plants and it was not reduced by drought stress. Similar results have been also observed in a previous work (Karimi et al., 2014a).

DAM acts as a gibberellin inhibitor and in the post divergence point between steviol and gibberellins (Brown et al., 1997; Rademacher, 1992; Rademacher, 2000). Therefore, assuming that DAM inactivated the active gibberellins units, it can be outlined that modification in the post-divergence point in the biosynthesis pathway cannot have a significant effect on the SVglys biosynthesis in stevia. In fact, inactivation of active-gibberellin units (by DAM) did not have any effect on SVglys biosynthesis in stevia. This is involved in the high-precision regulation system of SVglys and gibberellin biosynthesis in stevia leaves. Moreover, it can also be annotated that exogenous gibberellin application could not act as a serious competitor for SVglys biosynthesis, as found in well-watered plants (Karimi et al., 2014c).

The increase in TSS under drought stress could be considered as a strategy for plant faced with drought stress (Silva and Arrabaça, 2004; Souza et al., 2004; Zeid and Shedeed, 2006). The presence of soluble sugars in the cytosol could help with water saving and water absorption from inter-cellular spaces, which help the drought-stressed tissues to be physiologically active. This process is well-known as osmotic adjustment to overcome drought stress. Moreover, it has been found that, in stevia tissues, osmotic adjustment mainly occurred through the enhancement of fructose and sucrose contents, but not of glucose, since glucose units were mainly used in SVglys biosynthesis. It was found that PBZ was more effective than other PGRs in glucose induction in stevia leaves. Since glucose is one of the main parts of the chemical structure of SVglys (Shibata et al., 1995; Shibata et al., 1991), it can be outlined that the induction of SVglys biosynthesis by PBZ may be mediated through the supply of glucose units. The glucose production as well as the improvement of the osmotic adjustment lead to the enhancement of stevia growth under drought stress conditions. Moreover, it can also be assumed that glucose shortage has occurred in stevia leaves under drought. All these findings conclude that, under drought stress condition, a competitive process between SVglys biosynthetic pathway and osmotic adjustment occurred. Under such condition, SVglys biosynthesis prevailed and the cells have been forced to use fructose and sucrose as osmolytes, while glucose was mainly used for SVglys biosynthesis.

The ROS production and the biosynthesis of active compounds are mechanisms which usually occur during drought stress in plants (Larson, 1988; Mittler, 2002; Reddy et al., 2004). In this condition, the plant is forced to induce the production of specific antioxidant compounds able to remove and scavenge the ROS. In our study, an increased antioxidant activity in drought stressed plants, except given for DAM-treated ones, was observed as plant response to overcome drought stress. Similar findings have been observed in our previous study (Karimi et al., 2015a), in which the total antioxidant activity of stevia leaf extracts increased with drought stress intensification. The induction of antioxidant activity by PBZ was remarkable, as previously observed under well-watered conditions (Karimi et al., 2014a). The positive effect of PBZ on drought stressed-plants was not only limited to improving antioxidant activity, but also in the growth traits and metabolite production. Accordingly, it can be argued that PBZ could act as a protective treatment for stevia under drought stress condition, confirming previous studies (Hajihashemi and Ehsanpour, 2013). In addition, it can be noted that the protective effects of PBZ were probably due to osmotic adjustment and to the induction of antioxidant defense mechanisms in stressed plants (Fletcher et al., 2000; Jaleel et al., 2007). Similarly, it is believed that the positive effects of PBZ in stressed-plants could be mediated through the detoxification of ROS (Kraus and Fletcher, 1994) and the reduction of oxidative damages (Lin et al., 2006). The improvement of antioxidant capacity might also be intensified in stevia because PBZ was able to induce the SVglys biosynthesis and, as previously demonstrated (Karimi et al., 2014a), steviol is able to enhance total antioxidant activity. Induction of tolerance to drought stress by PBZ in stevia seedlings has been also reported (Hajihashemi and Ehsanpour, 2013).

5. Conclusion

Our findings demonstrate that, in stevia, PGRs play an important role in regulating plant responses to drought stress by sensitizing growth, physiological and biochemical processes, such as SVglys biosynthesis, stimulation of antioxidant activity and soluble sugars production. Among the tested PGRs, PBZ was the most effective in alleviating the adverse effects of drought stress in stevia plants. The positive effects of this triazole compound might be due to the induction of antioxidant activity and to the increase of glucose production and, consequently, to osmotic adjustment. With respect to the SVglys biosynthesis, it can be assumed that CDPS, as a target of CCC, could act as rate limiting factor. On the other hand, the PBZ target (KO) in stevia could be assumed to be different from that observed in other plants. As an unexpected phenomenon, PBZ had a stimulating effect of plant growth and SVglys biosynthesis. The induction in glucose unit production can be responsible for the increased SVglys biosynthesis in stressed plants treated with PBZ. In addition, it was also found that DAM (as an inhibitor of the active-gibberellins units) had no significant effect on the SVglys biosynthesis, meaning that gibberellin biosynthesis could not act as a competitor for SVglys biosynthesis. The SVglys quality (Reb A/Stev ratio) was improved under drought stress. Moreover, it can be assumed that stevia preferred the use of soluble sugars for osmotic adjustment and, as a result, the drought stress tolerance was acquired. In stevia, osmotic adjustment probably occurred through over production of sucrose and fructose molecules, while glucose was mainly expended in SVglys production. The induction of antioxidant activity might represent another strategy to overcome the drought stress in stevia plants.

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