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ABSTRACT

Steviol glycosides (SVglys) are a group of diterpenoids mainly present in the leaves of stevia (*Stevia rebaudiana* Bertoni). An experiment was conducted to find the functional role of SVglys compounds in stevia affected by drought stress. In this study, a liquid blend of SVglys (200 ppm) was sprayed on stevia plants grown in well-watered (90% field capacity) and drought stress conditions (45% field capacity) and then the morphological traits and metabolites were evaluated. It was observed that leaf losses caused by drought stress were stopped through external application of SVglys and consequently the harvest index (HI) of stevia was increased. Metabolite analysis of stevia leaves showed that the total SVglys content was significantly decreased due to drought stress, but was compensated by external application of SVglys. Among the SVglys, Rebaudioside A responded more to external SVglys. A slight promotion in total antioxidant activity of stevia leaves was observed when external SVglys was applied. The glucose availability in stevia leaves was increased by external application of SVglys but only in well-watered plants. According to our findings, it can be concluded that in stevia, SVglys may have a positive function in drought stress tolerance by exerting a protective role under such conditions.

Keywords: *Antioxidant capacity, drought tolerance, Rebaudioside, Soluble sugars, Stevioside*

Abbreviations: *SVglys, Steviol glycosides; Stev, Stevioside; Reb, Rebaudioside; Dulc, Dulcoside; HI, Harvest index; TSS, Total soluble sugars*

Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a perennial plant of the *Asteraceae* family, native to Paraguay. There are high sweetening compounds in the stevia leaves which are originated from a diterpenoeid compound named steviol. In fact, steviol acts as a primal structure for steviol glycosides (SVglys) forming in the stevia leaves (Brandle & Telmer 2007; Kinghorn 2003). The SVglys are sweeter than sucrose and have been approved for use as sweeteners in many countries, including United States, Canada, Australia, New Zealand, China, Japan, and South Korea as well as in Europe. In the past decades, the SVglys consumption trend has been sharply increased in the world (Singh & Rao 2005). The total SVglys content in stevia leaves has been estimated at 4-20% of leaf dry matter (Brandle et al. 1998; Starratt et al. 2002). The SVglys are found to include Steviolmonoside, Steviolbioside, Stevioside (Stev), Rebaudiosides (Reb) A, C, F and Dulcoside (Dulc) A. The major constituents of SVglys in stevia leaves are Reb A and Stev (2-4% and 5-10% of leaf dry matter, respectively) while Reb A is more potent and more pleasant-tasting than Stev (Jenner & Grenby 1989). There are three glucose molecules in the Stev structure while four glucoses are jointed to Reb A (Humphrey et al. 2006).

It has been reported that secondary metabolites could take part in stress alleviation and may play a protective role for plants under stressful conditions (Edreva et al. 2008; Jaleel et al. 2009; Oh et al. 2009). Nevertheless, there is a little information related to SVglys behavior under stress condition, particularly in drought stress condition. It has been demonstrated that drought stress (created by polyethylene

glycol) reduced the *ent-KO* transcription in stevia leaves (Hajihashemi et al. 2012), which consequently can be affect the SVglys biosynthesis. The relationship between drought stress induction and SVglys behavior may be too important for stevia because there are many glucose molecules in SVglys structure which can be participated in osmotic adjustment, as comfortably as other carbohydrates involving in osmotic adjustment (Hsiao et al. 1976; Kameli & Lösel 1995; Morgan 1984).

Induction of the antioxidant systems is one of the most important and efficient mechanism for plants to overcome the drought stress (Farooq et al. 2009; Reddy et al. 2004; Sairam et al. 1998; Sairam et al. 1997). With regard to this, stevia extract has also shown a high antioxidant activity (Gopalakrishnan et al. 2006; Kim et al. 2011; Tavarini & Angelini 2013) and has potential to ameliorate the drought stress tolerance in stevia. It has been shown that drought stress (originating from PEG) significantly increased the total antioxidant capacity of stevia leaves and it was also observed that oxidant compound scavenging was increased under drought stress (Hajihashemi & Ehsanpour 2014; Hajihashemi, et al. 2012). However, the antioxidant properties of SVglys and their functional behavior under drought stress condition have not been fully studied.

The aim of this study was to assess the SVglys role in stevia under drought stress condition. Moreover, the effect of SVglys on stevia growth and its metabolites was also looked into.

Materials and methods

Experimental conditions

The experiment was carried out at the Agricultural Biotechnology Research Institute of Iran (ABRII- Central of Iran, Isfahan) using completely randomized designs. Greenhouse temperature, humidity and air CO_2 concentration were 25/22 °C, 60/40% and 400/500 ppm, during day/night, respectively.

Plant material, cultivation and treatments

Stevia (*Stevia rebaudiana* Bertoni) was propagated through tissue culture. The plants are getting by tissue culture cultivated in a peat moss medium to come about the acclimation procedure. Thereafter, three uniformly plants were taken and transplanted into the pots containing loam soil (50% sand, 15% clay; field capacity 20.2%; wilting point 10.5%; bulk density 1.38 $g.cm^{-3}$). The 20L pots were filled with soil up to 2 cm below the surface and three seedlings were cultivated in each pot. Soil moisture content was held at near the field capacity for the first two weeks and then the treatments were imposed. Before applying irrigation, a soil sample of each pot was picked out and then its moisture content was determined by gravimetric method (Gardner & Klute 1986). It was tried to keep the soil moisture content near the 90 and 45% of field capacity as normal and drought stress treatments, respectively (Karimi et al. 2015). The soil moisture variation and treatments properties have been presented (Table 1). In another part of experiment, a new set of stevia plants was grown under well watered and drought stress conditions and the plants were sprayed with an external SVglys blend (as experimental treatment) and distilled water (as a control). For each treatment, a solution (1 L volume) containing 200 ppm SVglys blend (60% Rebaudioside A, 30% Stevioside and 10% other SVglys, purity>99%) was prepared

by dissolving pure SVglys in distilled water. The plants were sprayed with 100 ml of SVglys solution or distilled water, at repeat intervals of 5 days (10 times in total). The plants were harvested at 64 days after transplanting and were dried by hot oven at 65 °C for 48 h. Then, stem and leaves were separated, weighted, powdered and kept in -4 °C to further assessments. The HI was calculated as the ratio of leaf yield to the total cumulative dry biomass at harvest (Lavini et al. 2010). Before harvesting, the fresh leaves were sampled and were kept in -20 °C in order to antioxidant assay.

Determination of SVglys

The SVglys extraction and assessment were conducted based on Karimi et al (2014a; 2014c) and Ceunen and Geuns (2013a) methods.

I. Extraction and purification of SVglys

The dry leaves of stevia were powdered to a particle size of less than 0.10 mm and then 0.1 g of powdered leaves was transferred to tubes and 3 mL distilled water were added. The solutions were kept in a water bath for 30 min at 80°C and then centrifuged at 12,000 g for 5 min. The supernatant of solutions were recovered and 3 mL distilled water was added to the pellet. This procedure was repeated 3 times and the supernatant from each process was pooled in a test tube. The pooled supernatant was centrifuged (12,000 g for 5 min) again, the resultant supernatants were transferred to new tubes and 1 mL of distilled water was added to the pellet, centrifuged again (12,000 g for 5 min) and its supernatant was added to prior. The volume of supernatant was diluted to 10 mL using distilled water and filtrated with a filter

attached to a syringe (0.45 µm). Thereafter, a C_{18} cartridge was used for SVglys purification. The C_{18} cartridge was firstly washed with 3 mL methanol and then conditioned with 3 mL of distilled water. In order to sample purification, 0.5 mL of filtrate supernatant was loaded into C_{18} cartridge and afterward the cartridge was washed with acetonitrile-water mixture (20:80; v/v) to remove the interfering substances. Finally, SVglys were eluted from C_{18} cartridge with a mixture of acetonitrile/water (8:2 v/v) (1 mL) and kept in 1.5 mL tubes at -20 °C.

II. High-performance liquid chromatography (HPLC)

For the chromatographic analysis, two reverse-phase C_{18} columns were connected in series and a UV-Vis detector was used to detect the SVglys at 202 nm. A gradient of acetonitrile and water (50-80%, v/v) with a flow rate of 0.5 mL min^{-1} were used as mobile phases in HPLC system. The acetonitrile ratio was increased at 50, 65, 80, 80 and 50% during 0-10, 10-18, 18-22, 22-24 and 24-30 minutes, respectively. To SVglys analysis, 40 µL of the extract was injected in HPLC pump injector and five SVglys were detected which were included: Rebaudioside A (Reb A), Stevioside (Stev), Rebaudioside F (Reb F), Rebaudioside C (Reb C) and Dulcoside A (Dulc A). For quantification purposes, pure Stevioside and Rebaudioside A (purity>99%) were used as external standards. The other SVglys such as Reb F, Reb C and Dulc A were quantified by their molecular weight ratio to Reb A, since previously, it has been shown that all SVglys have similar molar extinction coefficients (Geuns & Struyf 2009; Geuns 2010; Geuns et al. 2009). The peak area was calculated by Chromstar 7.0 software and converted to concentration (ppm). The results were expressed as percentage of SVglys in the leaf dry matter (W/W), using the calibration curves

getting from the relationship between external standards (ppm) and their relative peak area.

Total antioxidant capacity

The total antioxidant capacity was determined by the DPPH (2, 2 diphenyl-1-picrylhydrazyl) assay according to Karimi et al (2014b) and Thaipong et al (2006). The fresh leaves were powdered in liquid nitrogen with a laboratory grinding mill and 0.3 g of powdered leaves was dissolved in 3 mL methanol solution. The solution was homogenized using a laboratory Homogenizer and homogenates were kept at -4 °C for 12 h. After that, the homogenates centrifuged at 23.000 g for 20 min and their supernatants were recovered and kept at -20 °C until further analysis. A stock solution of DPPH was prepared by dissolving of 2.5 g of DPPH in 4 mL of methanol. Working solution of DPPH was prepared by dissolving different concentrations of stock solution in 5 mL methanol to obtain an absorbance of 1±0.02 units at 517 nm. The HPLC grade methanol was used as blank sample. The reaction solution was included: 422.5 µL methanol, 200 µL DPPH solution and 2.5 µL leaf extract. The reaction solution was kept in the dark condition under room temperature for 24 h and the absorbance was taken at 517 nm. The IC_{50} value was calculated as the sample concentration necessary to decrease the initial absorbance of DPPH by 50% and IC_{50}^{-1} was used as an index of total antioxidant capacity (Hasperué et al. 2011).

Soluble sugars quantification

Soluble sugars were quantified as described by Tobias et al. (1992). The dry leaves were powdered and 0.04 g of leaf samples was dissolved in 1 mL $HClO_4$ 5.5% and kept in ice for 1 h. The solutions were centrifuged (14000 rpm, 10 min) and their supernatants were transferred to new tubes. The supernatants were treated by 100 µL of K_2CO_3, kept in ice for 1 h and centrifuged again as above. The resultant supernatants were harvested and kept in -20 °C until further analysis. Soluble sugars were assayed by coupled enzymatic assay methods (Guglielminetti et al. 1995) and measuring the increase in A_{340}. A known amount of glucose were used as an external standard. The incubations of samples and standards were carried out at 37°C for 30 min. The reaction mixtures (1 mL) were included:100 mM Tris-HCl, pH 7.6; 3 mM $MgCl_2$; 2 mM ATP; 0.6 mM NADP; 1 unit Glucose 6-P dehydrogenase and 1 unit hexokinase. The fructose was assayed as described for glucose plus the addition of 2 units of Phosphoglucose isomerase and measuring the increase in A_{340} as above. Sucrose was first broken down into glucose and fructose using 85 units of invertase (in 15 mM Na-acetate, pH 4.6) and was assayed through its resulting glucose as described above. The concentrations of the standards added were similar to those estimated to be present in the tissues in preliminary experiments. Data were expressed as µmoles hexoses equivalent g^{-1} dry weight.

Statistical analysis

Data were analyzed by SAS 9.2 software (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513) through Anova procedure and means of treatment were compared by least significant difference (LSD) test at $p \leq 0.05$. The histograms were

prepared by Sigma Plot 12.3. The means of the treatments (from three replications) are presented together with their standard error (SE) values in histograms and tables.

Results

Morphological traits

Morphological traits (plant height, leaf dry weight and HI) were significantly affected by drought stress and external application of SVglys (Table 2). The plant height was decreased under drought stress (39% in comparison with control) while did not affected by external application of SVglys (Table 3). Drought stress caused a significant decline (10% in comparison with well watered condition) in leaf growth while the leaf losses were covered by external application of SVglys under drought stress condition (Table 3). Stem dry weight did not demonstrate a significant variation in any treatments. Drought stress also reduced the HI value in stevia while the HI was kept stability near the control treatment by external application of SVglys (Table 3). The highest and lowest value of HI was founded in plants grown in well watered and drought stress conditions, respectively, without external application of SVglys (43.6 and 42.9%, respectively).

SVglys content and compositions

The total SVglys content of stevia leaves was significantly affected by drought stress and external application of SVglys (Table 2). Total SVglys content was decreased by

drought stress (14% value in comparison with well watered treatment), but was corrected through external application of SVglys, so returned to SVglys content in the control treatment (Fig. 1a). Among the SVglys types, Reb A and Stev showed a significant variation while Reb F, Reb C and Dulc A were not affected by drought stress and external application of SVglys (Table 2). With respect to this, Reb A/Stev ratio was also affected by experimental treatments (Table 2). Stev showed a same trend with total SVglys content while Reb A did not thin out due to drought stress and was increased by external application of SVglys in drought stressed-plants (Fig. 1b). Regardless of external application of SVglys, Reb A was increased under drought stress condition and therefore the highest ratio of Reb A/Stev was obtained in plant treated with drought stress treatments (Fig. 1c).

Total antioxidant capacity and soluble sugars

It was observed that total antioxidant capacity of stevia was significantly affected by drought stress and external application of SVglys (Table 2). Results showed a significant induction in antioxidant capacity of stevia leaves affected by both of the drought stress and external SVglys application, but the drought stress was more efficient than the external SVglys application with respect to induction of antioxidant capacity (Fig. 1d). The maximum and minimum values of total antioxidant capacity were observed in stressed-plants treated with external SVglys and in well-watered plants, respectively. Although the effect of drought stress was larger than the external SVglys treatment regarding to total antioxidant capacity, it is noteworthy that the last treatment could increase the total antioxidant capacity in either well watered and drought stressed plants.

Total soluble sugars (TSS) and sucrose did not show changes due to experimental treatments while hexose sugars, glucose and fructose, were significantly affected by drought stress and external SVglys treatments (Table 2). A precipitous increase (69% in comparison with control) in glucose content was observed in plant treated with external SVglys, but this increment only took place in well-watered plants and not observed in drought status (Fig. 2b). Fructose was increased by drought stress treatments, in comparison with well watered conditions (Fig. 2b).

Discussion

The leaf growth improvement in drought stressed-plant coming about by external application of SVglys could be aligned with Congmin et al (2009) who observed the useful effect of SVglys in senescence postpone of Rice leaves. Moreover, our results suggest that SVglys could prevent the destructive consequence of drought stress for stevia, but their precise mechanism has not been identified until now. However, the SVglys behavior may attribute to a hormonal result because the weak hormonal effect of SVglys derivatives has been suggested by researchers (de Oliveira et al. 2008; Ruddat et al. 1963). In addition, the maintenance of high water potential in plant cell is a vital mechanism for plant in overcoming the drought stress (Farooq, et al. 2009; Ingram & Bartels 1996) and soluble compounds like SVglys could be helpful in this operation. In fact, the SVglys participation in water potential maintenance could be a probable reason for leaf growth improvement in stevia under drought stress condition. However, it has been noted that SVglys could not singly account for tolerance of higher water stresses and may help plants through energy providing during the

drought stress period (Ceunen & Geuns 2013b). The HI stability inducing by external SVglys in drought stressed-plant was due to the leaf growth improvment by external SVglys because the stem, as an unusable part, did not record any variation among the treatments while the leaf growth was increased under external application of SVglys.

The SVglys induction in drought stressed-plant due to external feeding of SVglys suggests that SVglys biosynthesis in stevia could be regulated as a self-induction process. Since SVglys are produced in cytosol and immediately transported to the vacuole (Humphrey, et al. 2006), the self-induction process could not actually occur in the stevia leaf cells. The possibility can also be raised that limiting factors for SVglys production in drought stressed-plant has been disqualified by external feeding of SVglys. In this regards, it can be assumed that the external application of SVglys prepared the necessary substrates for SVglys production in stevia tissues. Among the SVglys types, Reb A and Stev were more variant than others which could be an oversee for stevia breeding, because Reb A is a determinative factor for SVglys quality (Sharma et al. 2009) while Stev quantity is dominant among the SVglys compositions. Under drought stress condition, Reb A was more responding to external SVglys. It means that Reb A has been induced by extra SVglys in drought stressed-plants and it may be associated to Reb A chemical structure because Reb A has four glucose units while there are three glucose units in Stev structure. So, it takes in guaranteeing that the differences between Stev and Reb A in response to external SVglys were related to glucose units. The self-induction theorem of SVglys is most convincing about Reb A because Reb A portion (60%) in the external SVglys blend was more than others and in the same way Reb A was more induced by external application of SVglys. The results suggest that Reb A is not only stable against drought stress, even is also stimulated due to drought stress because was significantly

increased in drought stressed-plants treating with SVglys. Altogether, stevia growth and internal SVglys production were more responsive to external SVglys especially under drought stress conditions, in comparison with well-watered situation. It means that SVglys can be more efficient in drought stress situation for stevia.

The antioxidant system improvement is one of the main plant strategy to overcome abiotic stress, especially drought stress, because free radicals are created by drought stress and could destroy the molecules and membranes (Sairam, et al. 1998; Sairam, et al. 1997; Zhang & Kirkham 1994). In fact, the antioxidant induction in the drought stressed-plant is a physiological response in order to scavenge the free radicals. In our research, the antioxidant capacity of stevia leaf was stimulated by drought stress which can be taken as a confronting mechanism for stevia against drought stress disadvantages. A same result has been also reported in stevia treated with PEG (Hajihashemi & Ehsanpour 2014; Hajihashemi, et al. 2012). Based on our results, it can be outlined that the growth induction in the drought stressed-stevia causing by external application of SVglys may be mediated through induction in antioxidant systems because a slight increment was observed in antioxidant capacity of plants treated with SVglys. In this regard, it has been also reported that stevia leaf extract had a high antioxidant activity (Congmin, et al. 2009; Gopalakrishnan, et al. 2006; Sairam, et al. 1997; Tavarini & Angelini 2013) which may be originated from SVglys behaviors.

Osmotic adjustment is an another vital mechanism enabling plants under drought stress to water absorption (Cattivelli et al. 2008). During osmotic adjustment, compatible solutes are overproduced in cytosol and can be considered as prominent responses of plants to drought stress (Ashraf 2010). With this respect, monosaccharides, i.e., glucose and fructose, has been reported largely responsible for

participating in osmotic adjustment (Yakushiji et al. 1996). Accordingly, fructose increase in stevia leaf under drought stress giving in our results could be considered as an osmotic adjustment process. Since stevia leaves are reached in glucose units attaching in SVglys and the glucose is easily converted to fructose by phosphoglucose isomerase (Thomas et al. 1992), it can be argued that SVglys could act as a source of compatible solutes acting in osmo-regulation process. It was also seen that the external application of SVglys caused a remarkable increment in internal glucose in well-watered plants which was implicated on glucose enrichment obtaining from SVglys compounds. The glucose may also absorb separately from inter cellular space because the SVglys movement across plant cell membrane has not been studied until now. However, based on our finding, it can be outlined that SVglys can actively participate in osmotic adjustment through providing the hexose sugars.

Conclusion

According to the presence results, it can be concluded that SVglys producing in the stevia leaf have some useful effects for stevia in the face of drought stress. The roles of SVglys in opposing to drought stress may divided into two parts. A modest percentage of that may associated with induction of antioxidant systems which could help to preserve of plant cell organelles and molecules versus free radical originating from drought stress. The remarkable percentage of that may associated with osmolyte preparation through SVglys participating in the osmotic adjustment process. Consequently, the osmotic adjustment enabled the plant to reserve water into the cells

and tissues and finally helps plants overcome the drought stress. As a general conclusion, SVglys have a protective role in stevia facing drought stress condition.

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Table captions

Table 1. Characteristics of experimental treatments.

Table 2. Analysis of variance for stevia traits affected by experimental treatments.

Table 3. Mean comparison and standard error for morphological traits of stevia (means were obtained from three replications and standard errors presented with means).

Figure captions

Figure. 1. Total SVglys content (a), SVglys compositions (b), Reb A/Stev ratio (c) and total antioxidant capacity (d) of stevia under drought stress and external SVglys application (well watered and drought stress are 3 and 12-days irrigation intervals, respectively. The external SVglys was sprayed in 200 ppm concentration; Standard error of means within treatment is reported as a vertical bar).

Figure. 2. TSS (total soluble sugars, a) and soluble sugars (b) availability in stevia leaves under drought stress condition and external application of SVglys (well watered and drought stress are 3 and 12-days irrigation intervals, respectively. The external SVglys was sprayed in 200 ppm concentration; Standard error of means within treatment is reported as a vertical bar).

Table 1 Characteristics of experimental treatments.

Treatments	Irrigation interval (day)	Soil water content 0-30 cm (% on weight basis)	Soil water content (% FC)	Solution
Well watered	3	19.1	90	100 ml distilled water
Drought stress	12	9.1	45	100 ml distilled water
Well watered+ SVglys blend spray	3	19.1	90	100 ml SVglys blend (200 ppm)
Drought stress+ SVglys blend spray	12	9.1	45	100 ml SVglys blend (200 ppm)

Table 2 Analysis of variance for Stevia traits affected by experimental treatments.

		Mean of Squares (MS)											
Source of Variation	df	Plant height	Leaf dry weight	Stem dry weight	Harvest index	Reb A	Stev	Reb F	Reb C	Dulc A	Total SVglys	Reb A/Stev	1/
Treatment	3	1463**	0.1**	0.095	0.27*	0.073*	0.41*	0.003	0.003	0.004	0.8*	0.01**	12
Error	8	17.91	0.009	0.023	0.05	0.015	0.07	0.01	0.003	0.002	0.18	0.001	0
CV	-	5.2	2.25	2.77	0.54	5.58	6.9	26.29	8.7	20.62	5.7	6.23	1
R^2	-	0.96	0.80	0.60	0.64	0.64	0.67	0.09	0.27	0.42	0.61	0.75	0

** Significant at 0.01 level; * Significant at 0.05 level; df, degree of freedom; CV, coefficient of variation; R^2, coefficient of determination.

Table 3 Mean comparison and standard error for morphological traits of Stevia (means were obtained from three replications and standard errors presented with means)

Treatments	Plant height (cm)	Leaf dry weight (g.plant^{-1})	Stem dry weight (g.plant^{-1})	Harvest index
Well watered	102.33±3.1^{a}	4.36±0.03^{a}	5.64±0.08^{a}	43.6±0.2^{a}
Drought stress	63±2.3^{b}	3.97±0.07^{b}	5.28±0.09^{b}	42.9±0.1^{b}
Well watered + SVglys	95.33±2.8^{a}	4.33±0.04^{a}	5.64+0.07^{a}	43.45±0.07^{a}
Drought stress+ SVglys	59±0.5^{b}	4.29±0.05^{a}	5.62±0.09^{a}	43.3±0.11^{ab}
LSD ($p \leq 0.05$)	7.97	0.17	0.28	0.44