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ORIGINAL ARTICLE

The effect of polyethylene glycol-induced drought stress on photosynthesis, carbohydrates and cell membrane in *Stevia rebaudiana* grown in greenhouse

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Abstract
Drought stress is one of the major environmental stresses that limit crop production in arid regions. A greenhouse culture experiment was conducted to evaluate the response of an agronomically and economically important sweet medical herb (*Stevia rebaudiana*) to polyethylene glycol (PEG 6000)-induced drought stress (5, 10, and 15% (w/v) PEG, equivalent to leaf water potentials of − 0.49, − 1.40 and − 2.93 MPa, respectively) for 1 month. Plant mass, a major determinant of *Stevia* yield, showed a reduction after PEG treatments. PEG-reduced photosynthesis traits included the maximal quantum yield of photosystem II (F_v/F_m), efficiency of photosystems I and II (PI_{abs}), intercellular CO_2, net photosynthesis, chlorophylls, carotenoids and water use efficiency, followed by the reduction of carbohydrates. Under PEG treatment, the reactive oxygen species (ROS) accumulation occurred and plants exhibited an increase in H_2O_2 generation. Consequently, an increase in malondialdehyde and electrolyte leakage was evident in PEG treatment, indicating membrane lipid peroxidation. In PEG-treated plants, the ROS accumulation was accompanied by an increase in activity of some enzymatic and non-enzymatic antioxidants. Leaf extracts of PEG-treated plants showed lower superoxide anion, hydroxyl and nitric oxide radical scavenging activity than control plants. Drought stress also caused the accumulation of the compatible solutes proline and glycine betaine. Collectively, the results demonstrated that PEG-induced oxidative stress, due to insufficient antioxidant mechanisms, provoked damages to cell membrane and photosynthetic apparatus, with consequently reduced carbohydrates and plant growth. These results are of basic importance as vegetative growth is the major determining criterion for *Stevia* crops and adequate irrigation is crucial for obtaining higher yield.

Keywords Antioxidants · Carbohydrates · Lipid peroxidation · Photosynthesis · Oxidative stress · *Stevia rebaudiana*

Introduction

One of the biggest threats posed by climate change is the availability of water around the world, whose scarcity results in agricultural constraints. The plant responses to water stress include adaptive and/or deleterious changes: drought stress has a negative effect on carbon assimilation through reduction in photosynthesis rate, which results in plant growth reduction (Chaves et al. 2002). The analysis of plant growth, photosynthetic traits and antioxidant capacity in plants exposed to drought stress appears to be a promising approach to identify the deleterious effects of water deficit.

Chlorophyll (Chl) fluorescence is an important technique in evaluating damages to the photosynthetic system under environmental stresses. The maximum quantum efficiency of PS II photochemistry (F_v/F_m) and the efficiency of both photosystems I and II, characterized by photosystems

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performance index PI_{abs}, provide information on the function of plant photosynthetic apparatus in response to drought stress (Živčák et al. 2008). Indeed, drought stress alters the values of Chl fluorescence and damages the oxygen-evolving center coupled with PSII and D1 protein (Ashraf and Harris 2013). These changes lead to the reactive oxygen species (ROS) generation, which causes the photoinhibition and oxidative injury of cellular components, such as the photosynthesis pigments (Ashraf and Harris 2013). Excess levels of ROS (superoxide, $O_2^{\cdot-}$; hydrogen peroxide, H_2O_2; hydroxyl radical, $OH^{\cdot}$) production under drought stress leads to oxidative damage in cells, with consequent inhibition of photosynthesis, damage of cellular structures, growth reduction and premature senescence of plants (Krasensky and Jonak 2012). Lipid peroxidation, an important criterion to evaluate negative effects of stress on cell membrane, can be indirectly measured by malondialdehyde (MDA) content and electrolyte leakage (Campos et al. 2003).

The responses of plants to environmental stresses are activated at all levels of organization, including redox metabolism to remove excess levels of ROS and re-establish the cellular redox balance, and production of compatible solutes (e.g., proline, glycine betaine, and soluble carbohydrates) able to maintain cell turgor by osmotic adjustment (Anjum et al. 2012; Farooq et al. 2009; Krasensky and Jonak 2012). The improvement of the antioxidant defense system is of key relevance for proficiently scavenging ROS with non-enzymatic antioxidants, such as ascorbate, glutathione, carotenoids and α-tocopherol, and by the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Anjum et al. 2012). SOD is the key enzyme involved in the first step of ROS detoxification processes, while the enzymes CAT, POD and APX are indispensable for ROS detoxification, catalyzing the dismutation of H_2O_2 into H_2O.

Leaves of *Stevia rebaudiana* (Bertoni) contain steviol glycosides (SVglys) that are natural sweeteners responsible for the sweet taste. Besides this characteristic, antioxidant activities have been reported for SVglys compounds (Hajihashemi and Geuns 2013; Ahmad et al. 2010). Today, *Stevia* plantation has been spread to several regions, including Canada, Europe and Asia (Hajihashemi and Geuns 2016). Understanding how *Stevia* plants can face drought stress under greenhouse condition can be fundamental for improving the management and production of this agronomically and economically important plant, especially since the climate-change scenarios foresee an increase in aridity in many areas where this species is cultivated (Hajihashemi and Geuns 2016). Moreover, there is less information concerning the effects of drought stress on photosynthesis and chlorophyll fluorescence in *Stevia* or their correlations with oxidative stress (Hajihashemi and Ehsanpour 2013).

On this basis, the aim of this investigation was studying the effect of PEG-induced drought stress on photosynthesis, growth, carbohydrates, proteins, proline, activity of proline-metabolizing enzymes (pyrroline-5-carboxylate synthetase, proline dehydrogenase), glycine betaine, enzymatic and non-enzymatic antioxidants (SOD, CAT, POD, APX, PPO, GR, ascorbate pool, glutathione pool and α-tocopherol), free radical ($O_2^{\cdot-}$, $OH^{\cdot}$ and $NO^{\cdot}$) scavenging activity, and membrane integrity of *S. rebaudiana* under greenhouse.

Materials and methods

Plant material

Plants of *Stevia rebaudiana* were obtained from the Laboratory of Functional Biology, KULeuven University, Leuven, Belgium. Plants were propagated by tissue culture as described already by Hajihashemi and Ehsanpour (2013). The plants were grown to the seedling stage under greenhouse, with a 16-h photoperiod. Air temperature ranged from 22 to 26 °C during the day and from 15 to 18 °C during the night. The range of relative humidity was 40–60%. After 2 months, plants with similar size were grown in 1.5-L pots filled with vermiculite and perlite (1:1; v/v), irrigated daily and fertilized once a week with Hoagland nutrient solution (pH 6.0). The 6-month-old plants were irrigated with 0% (distilled water), 5, 10, and 15% (w/v) PEG (molecular weight 6000; equivalent to leaf water potentials of −0.08, −0.49, −1.40, and −2.93 MPa, respectively). PEG treatment was carried out for 1 month. 2 days after the last day of PEG treatment, the following analyses were performed. Before plant harvesting, Chl fluorescence and photosynthesis traits were measured in both control and PEF-treated plants on apical fully extended leaves. The treatment duration was chosen based on the fact that *Stevia* plants become ready for the first harvesting 5–7 months after planting (40–60 cm in height) and that plants should be cut before flowering starts (approximately 8–9 months after planting), when the SVglys content of leaves reaches the maximum value.

Growth measurement

The number of leaves emerged in PEG-treated plants were less than in control plants so, to make an absolute comparison, the growth parameters were calculated on one leaf per treatment. Six plants for every treatment were used for growth analysis. For each plant, the leaf fresh mass (LFM) was measured in apical fully extended leaves which were emerged during treatment. To calculate the fresh mass of one leaf, the value of LFM was divided by the number of leaves. The dry weight of freeze-dried leaves for each plant was measured and the obtained result was divided

by the leaves' number to achieve the dry mass of one leaf (LDM). The absolute leaf water content was calculated as a difference between leaves' fresh and dry weight, and then one leaf water content (LWC) was calculated by dividing LWC by the number of leaves. According to the results of preliminary experiments on growth analysis and our previous experiments (Hajihashemi and Geuns 2016), it was decided to continue the experiments with 10% (w/v) PEG. In 5% PEG-treated plants, a reduction in some parameters (e.g., LWC) was not observed, while in 15% PEG-treated plants a premature senescence was observed in leaves, which would bring problems in studying photosynthesis after the 1-month stress period. Therefore, the two next plant cultivation experiments were based on 0 (control) and 10% PEG-treated plants.

Photosynthetic traits and fluorescence measurement

The analysis of photosynthesis traits and fluorescence measurement was carried out on six apical fresh fully expanded leaves each from the six plants. The examined leaves had the same size, position and exposure to light. A portable Chl fluorometer (Pocket PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, England) was used to measure the chlorophyll fluorescence. After 30 min of acclimation in the dark, leaves were illuminated with saturating light (3500 µmol m^{-2} s^{-1}) and the maximum quantum yield of photosystem II (F_v/F_m) and efficiency of both photosystems I and II (PI_{abs}), that are reliable indicators of photosynthetic efficiency, were calculated according to Živčák et al. (2008).

Intercellular CO_2 (C_i), net photosynthesis (P_N) and water use efficiency (WUE) were measured with a portable plant photosynthesis meter (KR8700 system; Korea Tech Inc., Korea) comprised of infrared CO_2 analyzer and a leaf chamber, with equal conditions of leaf temperature for all samples.

The same leaves used for photosynthesis and fluorescence measurements were harvested and extracted in 80% (v/v) acetone. Chlorophyll and carotenoid contents were determined spectrophotometrically according to the method of Wellburn (1994).

Carbohydrate measurement

All carbohydrate measurements were carried out on freeze-dried leaves. Water-soluble carbohydrate (WSC) determination was based on the phenol–sulfuric acid method (Dubois et al. 1956). The amount of total reducing sugar (RS) was measured according to Somogy-Nelson (1952).

Analysis of proteins and antioxidant enzyme activities

One gram of fresh leaves was homogenized in 3 mL sodium phosphate buffer (50 mM, pH 7.8) including dithiothreitol (4 mM), EDTA (1.0 mM), magnesium sulfate (5 mM), polyvinylpolypyrrolidone (2% w/v) and glycerol (10%). The homogenate was centrifuged for 40 min at 13,000 rpm at 4 °C (Hajihashemi and Ehsanpour 2014). The supernatants were used for analysis of proteins and enzyme activity assay. Proteins were determined by the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich Co., USA) as standard. Superoxide dismutase (SOD) activity was assayed according to the method of Beauchamp and Fridovich (1971). To determine catalase (CAT) activity, the absorbance of the reaction mixture containing enzyme extract was recorded at 240 nm for 3 min (Aebi 1984). Peroxidase (POD) activity was determined as described by Plewa et al. (1991). Ascorbate peroxidase (APX) and glutathione reductase (GR) activities were determined according to Asada (1999). The activity of polyphenol oxidase (PPO) activity was measured according to a method as described by Flurkey and Jen (1980).

Analysis of ascorbate and glutathione pools

The fresh leaves were extracted with metaphosphoric acid (Hajihashemi and Ehsanpour 2014). The total ascorbate (Total ASC), reduced ascorbate (ASC) and dehydroascorbate (DHA) contents of lead samples were measured according to the method of Kampfenkel et al. (1995). Total glutathione (Glu), oxidized glutathione (GSSG) and reduced glutathione (GSH) were assayed according to the method of Griffith (1985).

H_2O_2 content and scavenging activity of radicals

Fresh leaves were extracted with trichloroacetic acid (Hajihashemi and Ehsanpour 2014) and H_2O_2 were measured according to Velikova et al. (2000) method. The fresh leaves (1.0 g) were homogenized in 10 mL of phosphate buffer (0.1 M; pH 7.6) containing ethylenediaminetetraacetic acid (0.1 mM) and centrifuged at 13,000 rpm at 4 °C for 10 min. Leaf extracts were used to measure scavenging activity of superoxide ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$) radical, according to Hajihashemi and Geuns (2013).

α-Tocopherol, malondialdehyde (MDA) and electrolyte leakage measurement

α-Tocopherol was extracted from fresh leaves and determined as described by Baker et al. (1980). A protocol of the TBA assay for lipid peroxidation was used according to

Hajihashemi and Geuns (2013). The reaction mixture was injected into a HPLC with a fluorescence detector. Samples were quantified using a standard curve based on tetramethoxypropane. Leaf membrane damage was determined by recording of electrolyte leakage as described by Valentovic et al. (2006).

Proline metabolism and glycine betaine content

Free proline was extracted from fresh leaves and determined by a spectrophotometer according to Bates et al. (1973). The activity of pyrroline-5-carboxylate synthetase (P5CS; EC 1.2.1.41) was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm (Stines et al. 1999). The activity of proline dehydrogenase (PDH; EC 1.5.1.2) was assayed by following the NAD^+ (or $NADP^+$) reduction and an increase in the absorbance at 340 nm according to Rena and Splittstoesser (1975). Glycine betaine was extracted from fresh leaves and its content was determined following the method of Grieve and Grattan (1983).

Statistical analysis

All experiments were carried out using a completely randomized design with six plants per treatment. Data were analyzed using SPSS statistical (version 24) software. Treatment means were subjected to ANOVA test and significant differences between data were measured by Duncan's multiple range test ($p \leq 0.05$).

Results

In the preliminary part of this study, *Stevia* plants were treated with 0, 5, 10 and 15% PEG with the purpose of selecting one PEG level to adopt for further analysis (Table 1). PEG induced a severe drought stress leading to growth inhibition, with small leaves, which was more pronounced at 15% PEG. The LFM, LDM and LWC decreased due to smaller leaves induced by PEG treatment. At 15% PEG, the LFM, LDM and LWC showed an 81, 35 and 89% reduction, respectively. According to the obtained results from growth analysis and previous research on the same species (Hajihashemi and Geuns 2016), a concentration of 10% PEG was chosen to carry out physiological and biochemical analyses.

PEG treatment decreased the F_v/F_m and PI_{abs} values by about 30 and 70%, respectively (Table 2). Reduced values of these two parameters indicate a lower photosynthetic efficiency, with damages to the enzymatic apparatus of both photosystems I and II (Živčák et al. 2008). Parallel to Chl fluorescence trends, PEG treatment caused a reduction in of P_N, C_i and WUE by about 74, 24 and 80%, respectively (Table 2). PEG treatments caused decreases in Chl a, Chl b, total Chl and carotenoid contents compared to control plants, about 43%, 50, 45 and 30%, respectively (Table 2). Both RS and WSC showed a reduction in response to PEG treatment, by about 25 and 38%, respectively.

Data indicated that H_2O_2 content increased by about 3.2-fold in the PEG-treated plants, more than control plants (Fig. 1a). Furthermore, PEG treatment caused an increased SOD activity by about 1.5-fold higher than control plants

Table 2 The effect of polyethylene glycol (PEG 0 and 10%) on the maximal quantum yield of photosystem II (F_v/F_m), performance index (PI_{abs}), net photosynthesis (P_N), intracellular CO_2 (C_i), water use efficiency (WUE), chlorophyll a (Chl a), Chl b, total Chl, carotenoids, water soluble carbohydrates (WSC) and reducing sugars (RS), in *S. rebaudiana*

	Treatment	
	Control	PEG (10%)
F_v/F_m	78.55 ± 1.03a	54.33 ± 1.50b
PI_{abs}	1.39 ± 0.07a	0.40 ± 0.01b
P_N [μmol/(m^2 s)]	19.26 ± 0.73a	5.05 ± 0.45b
C_i (ppm)	562.5 ± 15.0a	424.5 ± 6.09b
WUE (μmol CO_2/mol H_2O)	15.51 ± 0.39a	3.10 ± 0.43b
Chl a (mg/g DW)	11.78 ± 0.07a	6.70 ± 0.64b
Chl b (mg/g DW)	3.45 ± 0.15a	1.55 ± 0.16b
Total Chl (mg/g DW)	15.24 ± 0.53a	8.26 ± 0.80b
Carotenoids (mg/g DW)	2.31 ± 0.03a	1.71 ± 0.16b
WSC (mg/g DW)	6.17 ± 0.24a	4.65 ± 0.23b
RS (mg/g DW)	2.76 ± 0.12a	1.71 ± 0.18b

Statistics like in Table 1

DW dry weight

Table 1 The effect of polyethylene glycol (PEG = 0, 5, 10 and 15%) on leaf fresh mass (LFM), leaf dry weight (LDM) and leaf water content (LWC) in *S. rebaudiana*

	Treatment			
	Control	PEG 5%	PEG 10%	PEG 15%
LFM (mg)	5.017 ± 0.191a	4.773 ± 0.180b	2.322 ± 0.120c	0.952 ± 0.118d
LDM (mg)	0.755 ± 0.033a	0.677 ± 0.022b	0.505 ± 0.020c	0.488 ± 0.008d
LWC (mg)	4.261 ± 0.174a	4.093 ± 0.168a	1.819 ± 0.104b	0.463 ± 0.135c

Values are expressed as means ± SEM of six plants per treatment. Treatments with the same lowercase letters within rows were not different based on mean comparison by Duncan's test at $p < 0.05$

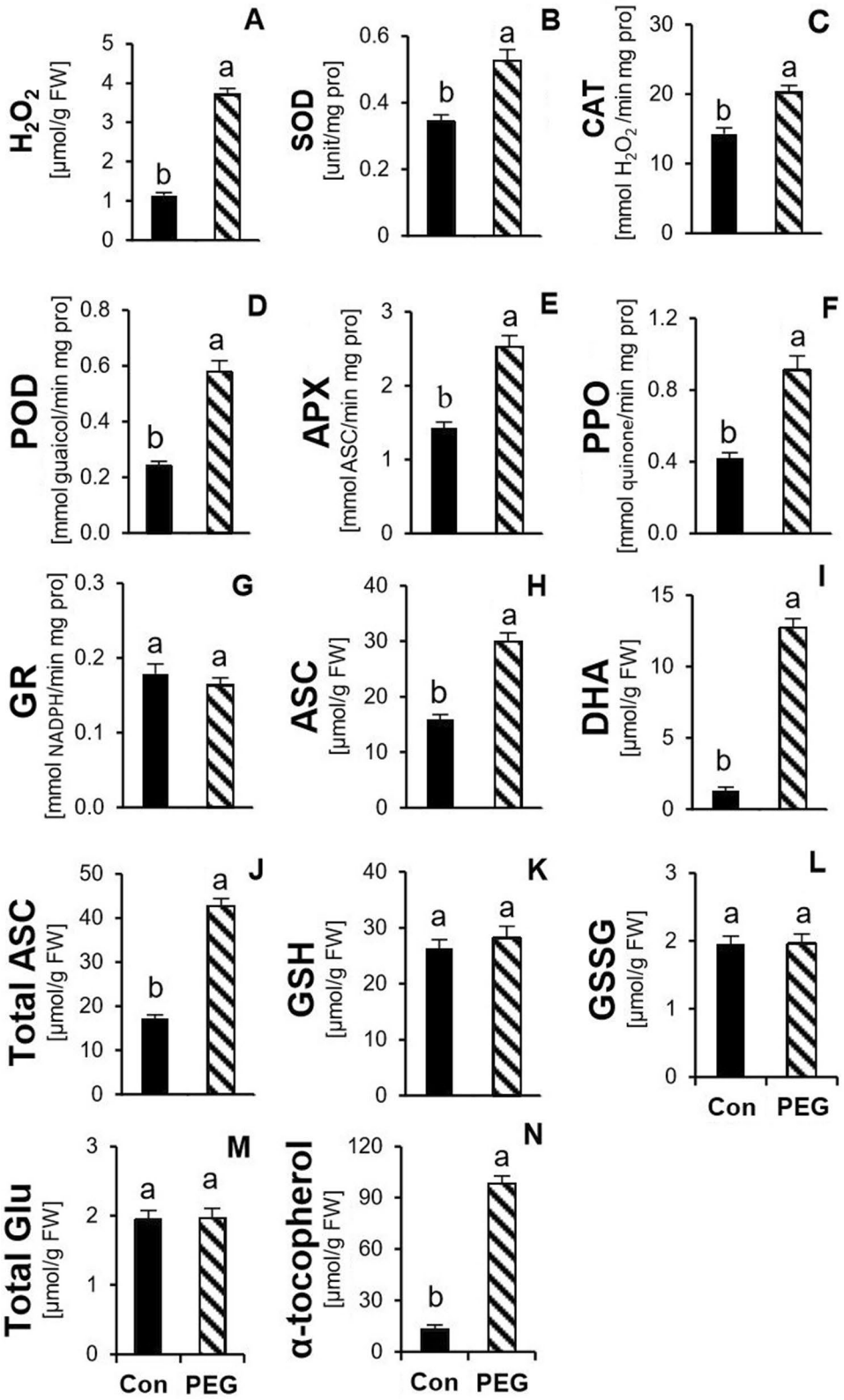

Fig. 1 The effect of polyethylene glycol (PEG = control 0%, black columns, and 10% striped columns) on H_2O_2 (**a**); the activity of superoxide dismutase (SOD; **b**), catalase (CAT; **c**), peroxidase (POD; **d**), ascorbate peroxidase (APX; **e**), polyphenol oxidase (PPO; **f**) and glutathione reductase (GR; **g**), the content of ascorbate (ASC; H), dehydroascorbate (DHA; **i**), total ascorbate (ASC; **j**), reduced glutathione (GSH; **k**), oxidized glutathione (GSSG; **l**), total glutathione (Glu; **m**), and α-tocopherol (**n**) in *S. rebaudiana*. Values are expressed as means ± SEM of six plants per each treatment. *Con* control plants, *PEG* PEG-treated plants, *Pro* protein, *FW* fresh weight

(Fig. 1b). PEG treatment also induced an increase in the activity of CAT (by about 1.4-fold; Fig. 1c), POD (by about 2.4-fold; Fig. 1d), APX (by about 1.8-fold; Fig. 1e) and PPO (by about 2.1-fold; Fig. 1f), while no changes were observed in GR activity (Fig. 1g).

In the PEG-treated plants, the amounts of ASC (Fig. 1h), DHA (Fig. 1i) and total ascorbate (Fig. 1j) contents

increased by about 1.8-, 10- and 2.5-fold, respectively, more than control plant. However, the glutathione pool was not affected by PEG treatment and the levels of GSH (Fig. 1k), GSSG (Fig. 1l) and total glutathione (Fig. 1m) did not show any changes. The quantification of liposoluble antioxidant of α-tocopherol showed an increase by about 7.6-fold higher than control plant, in response to PEG treatment (Fig. 1n).

Control plants had higher antioxidant power than PEG-treated plants, as the leaf extract of control plants scavenges $O_2^{\cdot-}$ (Fig. 2a), $OH^{\cdot}$ (Fig. 2b) and $NO^{\cdot}$ (Fig. 2c) by about 1.8, 2.5 and 38%, respectively, more than PEG-treated plants. Subsequently, ROS accumulation resulted in an increase in MDA (Fig. 2d) and EL (Fig. 2e) in PEG-treated plants by about 4.5-fold and 8.5-fold, respectively, compared to the control plants.

According to the obtained results, PEG treatment resulted in no changes in protein content (Fig. 3a). *Stevia* plants accumulated 1.5-fold higher amount of proline in PEG-treated plant, compared to control plants (Fig. 3b), while no changes were observed in the activity P5CS (Fig. 3c) and PDH (Fig. 3d) enzymes which are involved in proline metabolism. Also, PEG treatment had increased glycine betaine in *Stevia*, by about fivefold more than control plants (Fig. 3e).

Discussion

Water scarcity and high temperature act synergically under field condition and this makes it difficult to define their contribution to drought stress in plants (Chaves et al. 2002). Therefore, this experiment was conducted in a greenhouse at a controlled temperature. Plant growth is considered as a foremost effect and the most drought-sensitive physiological process which is a consequence of reduced plant water potential, limited photosynthesis and increased oxidative stress (Farooq et al. 2009). Our results showed that PEG

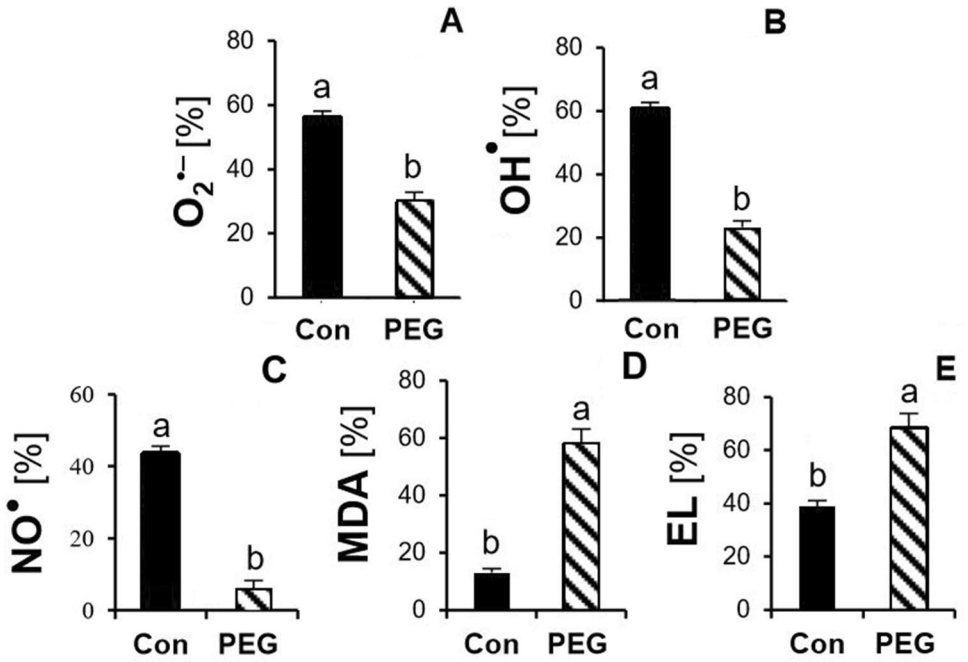

Fig. 2 The effect of polyethylene glycol (PEG = control 0%, black columns, and 10% striped columns) on leaf extract scavenging activity of superoxide radical ($O_2^{\cdot-}$; **a**), hydroxyl radical ($OH^{\cdot}$; **b**), and nitric oxide ($NO^{\cdot}$; **c**) radicals, and malondialdehyde (MDA; **d**) and electrolyte leakage (EL; **e**) in *S. rebaudiana. Con* control plants, *PEG* PEG-treated plants, *Pro* protein, *FW* fresh weight. Statistics like in Fig. 1

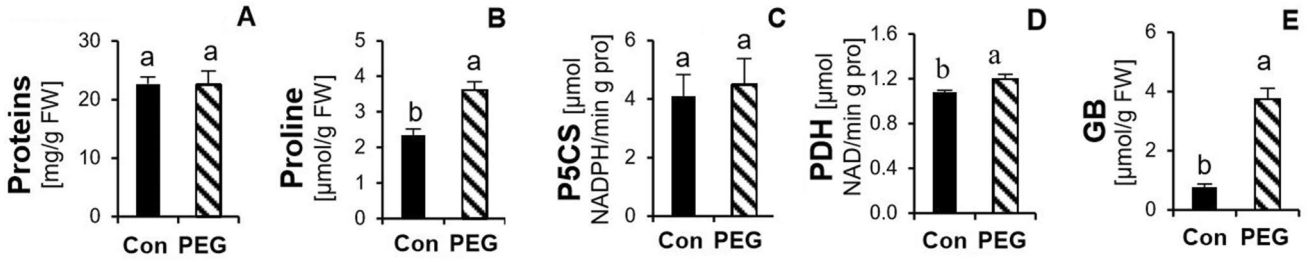

Fig. 3 The effect of polyethylene glycol (PEG = control 0%, black columns, and 10% striped columns) on proteins (**a**), proline (**b**), pyrroline-5-carboxylate synthetase (P5CS; **c**), proline dehydrogenase (PDH; **d**) and glycine betaine (GB; **e**) in *S. rebaudiana. Con* control plants, *PEG* PEG-treated plants, *Pro* protein, *FW* fresh weight. Statistics like in Fig. 1

treatment reduced LFW in *S. rebaudiana*, with the highest reduction in 15% PEG, and this was concomitant with the decrease in LWC (Table 1). The first adverse effect of drought stress is a decline in plant water content which has been observed in many plant species (Valentovic et al. 2006), and it is accompanied by changes in molecular, physiological, morphological events and their complex interaction (Farooq et al. 2009). PEG treatment decreased both LFM and LDM in *Stevia* plants, while the negative effect on LFM was greater than LDM, likely due to the reduction in LWC (Table 1). The same negative effect of PEG (0–6% w/v) on growth parameters of *Stevia* was observed in in vitro culture (Hajihashemi and Ehsanpour 2013). A critical issue in *S. rebaudiana* as an economic plant is the foliar accumulation of SVglys that are responsible for the sweet taste (Geuns 2003). Accordingly, the exposure of *Stevia* to drought stress is an unfavorable economic event, due to the reduction of leaf mass production, which is followed by reduction in SVglys yield (Hajihashemi and Geuns 2016).

Leaf water potential is an important characteristic that influences stomatal conductance and subsequently photosynthesis (Farooq et al. 2009). At the whole plant level, the ratio between plant dry mass and water consume is called water use efficiency (Monclus et al. 2006). The reductions in WUE and LDM co-occurred in PEG-treated *Stevia* (Table 2). Drought stress causes reduction in photosynthetic pigments, damages to photosynthetic apparatus, limitation to net carbon gain due to stomatal closure, which are other important reasons for lower plant growth (Farooq et al. 2009). The results of the present study confirmed the adverse effect of PEG-induced drought stress photosynthesis parameters in *Stevia*, including Chl fluorescence, net photosynthesis, CO_2 accumulation and photosynthetic pigments (Table 2). The Chl fluorescence traits, including F_v/F_m and PI_{abs}, are early important stress indicators in plants (Sharma et al. 2012). In *Stevia*, drought stress reduced F_v/F_m and PI_{abs} (Table 2).

A major adverse consequence of water deficit is the limitation of photosynthesis rate arisen by damages in photosystems, limitation in CO_2 absorption and reduction of chlorophylls (Ashraf and Harris 2013; Farooq et al. 2009). The observed reduction in C_i content in *Stevia* under water-deficit condition (Table 2) can be a consequence of restrictions to CO_2 diffusion in leaf, due in part to stomatal closure (Chaves et al. 2002). This reduction in CO_2 accumulation has also consequences on the photosynthetic rate (Ashraf and Harris 2013), and this is in accordance with our results regarding P_N reduction in PEG-treated *Stevia* (Table 2). The reduction of photosynthesis driven by lower chlorophyll contents has been observed in numerous plants in response to drought stress (Chaves et al. 2002). The result of the present research showed that PEG treatment caused a decline in the contents of Chl a and b, and carotenoids (Table 2). The reduction in chlorophyll contents due to pigment photo-oxidation and degradation occurring under drought stress is a typical symptom of oxidative stress (Farooq et al. 2009). Besides, carotenoids are known to act as efficient quenchers of triplet chlorophyll and singlet oxygen (Sultana et al. 1999). Thus, the observed decrease in carotenoids observed in *Stevia* (Table 2) can be associated with the degradation of β-carotene and to the formation of zeaxanthins that are involved in the protection against photo-inhibition (Sultana et al. 1999). Carbohydrates are the first and main products of photosynthesis, being a source of carbon during growth or acting as osmoprotectants under stress condition (Chaves et al. 2002). In addition, another important function of sugars in *S. rebaudiana* is participating in SVglys biosynthesis (Geuns 2003; Hajihashemi and Geuns 2016). In this study, PEG treatment provoked reduction in the content of water-soluble carbohydrates and reducing sugars (Table 2). This could be the consequence of the observed reduction in Chl content and photosynthetic rates (Table 2), resulting in growth inhibition (Table 1).

Oxidative stress is the consequence of an imbalance of ROS generation in response to environmental stresses, such as drought (Farooq et al. 2009; Hajihashemi and Ehsanpour 2014). A high H_2O_2 accumulation was observed in PEG-treated *Stevia* plants (Fig. 1a), and this likely damaged the photosynthetic apparatus and reduced photosynthetic rate and products (Table 2). Also, a linear correlation was observed between SOD activity and H_2O_2 contents in PEG-treated plants (Fig. 1b). The next enzymatic step of dismutation of $O_2^{\cdot -}$ to H_2O_2 by SOD is the deactivation of H_2O_2 accompanied by enhanced activities of H_2O_2 scavenging enzymes, such as CAT, POD and APX (Anjum et al. 2012). The results of the present study showed that CAT, POD and APX activities of *Stevia* plants increased in response to PEG treatment (Fig. 1c–e). Moreover, PPO activity increased in PEG treatment (Fig. 1f) that is responsible for the oxidative browning which accompanies plant senescence and responses to pathogens (Thipyapong et al. 2004).

Non-enzymatic antioxidants have an important role in detoxifying ROS generated during drought stress (Liu et al. 2011). A major component of these defence mechanisms is the ascorbate–glutathione cycle that involves APX and GR for the removal of H_2O_2 (Torres-Franklin et al. 2008). PEG treatment increased APX activity and this was followed by an increased ascorbate pool (Fig. 1h–j), while the glutathione pool and GR activity were stable under PEG treatment and showed no changes in *Stevia* (Fig. 1k–m). Therefore, it might be suggested that the ascorbate pool is an effective ROS scavenger while the glutathione pool was not. Furthermore, α-tocopherol is another non-enzymatic antioxidant with an important role in adsorbing and neutralizing free radicals (Wang and Quinn 2000), and it increased in PEG-treated *Stevia* plants (Fig. 1n). The drought-associated production of ROS modifies membrane lipids and causes lipid peroxidation (Farooq et al. 2009), whose marker is

MDA content. In PEG-treated *Stevia* plants, MDA content was positively related to electrolyte leakage (Fig. 2d, e). This could be the result of reactive oxygen attack to membrane lipids and decline in cell membrane stability (Anjum et al. 2012). The leaf extracts of control plants removed $O_2^{\cdot-}$, $OH^{\cdot}$ and $NO^{\cdot}$ more efficiently than PEG-treated plants (Fig. 2a–c). As tolerance to water deficit is correlated with the capacity to scavenge and detoxify ROS (Liu et al. 2011), it is suggested that *Stevia* is a drought-sensitive plant.

Besides ROS detoxification, the maintenance of leaf turgor, due to accumulation of solutes such as proteins, proline and glycine betaine, can also improve drought tolerance through increasing water uptake from drying soil, reducing injury to cells, and preservation of the structure of macromolecules (McNeil et al. 1999; Anjum et al. 2012). In *Stevia*, drought stress had no effect on protein content while proline accumulation increased (Fig. 3a, b). Proline synthesis is catalyzed by glutamate dehydrogenase, P5C synthetase (P5CS), while its oxidation is mediated by proline dehydrogenase (PDH) (Chaitanya et al. 2009). The same authors reported that both P5CS and PDH activities increased in the mulberry under drought stress which was followed by an increase in proline accumulation. On the other hand, here no correlation was observed between P5CS and PDH enzyme activities and proline accumulation (Fig. 3b–d). Glycine betaine increased after PEG treatment (Fig. 3e), therefore, improving the ability of *Stevia* plant to withstand the stress.

In conclusion, the results obtained in this study support our previous in vitro experimental findings (Hajihashemi and Ehsanpour 2013), and confirm that *S. rebaudiana* is a drought-sensitive plant. Since drought stress caused a reduction in plant productivity, *Stevia* should be fully irrigated for obtaining high-yielding crop and supporting a commercial SVglys production. The comprehension of how *Stevia* plants respond to drought stress can play a vital role in enhancing the performance and cultivation of this important species.

Author contribution statement SH: manuscript writing, greenhouse trial and statistical analysis. AS: manuscript editing and laboratory analyses.

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Compliance with ethical standards

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