Plant Physiology and Biochemistry 115 (2017) 484–496

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Salt-tolerance mechanisms induced in *Stevia rebaudiana* Bertoni: Effects on mineral nutrition, antioxidative metabolism and steviol glycoside content

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ARTICLE INFO

Article history:
Received 3 February 2017
Received in revised form
24 April 2017
Accepted 25 April 2017
Available online 26 April 2017

Keywords:
Chlorophyll fluorescence
Mineral nutrition
Physiological response
Stevia plants
NaCl stress
Oxidative stress
Steviol glycosides

ABSTRACT

In order to cope with challenges linked to climate change such as salinity, plants must develop a wide spectrum of physiological and molecular mechanisms to rapidly adapt. *Stevia rebaudiana* Bertoni plants are a case in point. According to our findings, salt stress has no significant effect on plant growth in these plants, which accumulate sodium (Na^+) in their roots, thus avoiding excessive Na^+ accumulation in leaves. Furthermore, salt stress (NaCl stress) increases the potassium (K^+), calcium (Ca^{2+}), chloride ion (Cl^-) and proline concentrations in Stevia leaves, which could contribute to osmotic adjustment. We also found that long-term NaCl stress does not produce changes in chlorophyll concentrations in Stevia leaves, reflecting a mechanism to protect the photosynthesis process. Interestingly, an increase in chlorophyll *b* (Chlb) content occured in the oldest plants studied. In addition, we found that NaCl induced reactive oxygen species (ROS) accumulation in Stevia leaves and that this accumulation was more evident in the presence of 5 g/L NaCl, the highest concentration used in the study. Nevertheless, Stevia plants are able to induce (16 d) or maintain (25 d) antioxidant enzymes to cope with NaCl-induced oxidative stress. Low salt levels did not affect steviolbioside and rebaudioside A contents. Our results suggest that Stevia plants induce tolerance mechanisms in order to minimize the deleterious effects of salt stress. We can thus conclude that saline waters can be used to grow Stevia plants and for Steviol glycosides (SGs) production.

1. Introduction

Environmental stress factors, including salt stress, are an important threat to agriculture in the context of climate change (Ahuja et al., 2010). Salinity is considered to be the major environmental factor limiting plant growth, particularly in arid and semi-arid regions such as Mediterranean areas, where water availability is very restricted. The presence of NaCl in soils induces osmotic stress as well as ion toxicity in all plant organs due to excessive accumulation of the phytotoxic ions sodium (Na^+) and chloride (Cl^-). This in turn leads to nutritional imbalance due to a decrease in other important nutrients, such as calcium (Ca^{+2}), magnesium (Mg^{+2}) and potassium (K^+) (Munns and Tester, 2008; Parida and Das, 2005). The accumulation of toxic ions can be a positive mechanism for dealing with osmotic stress, but only if plants have the ability to compartmentalize these ions inside the vacuole (Acosta-Motos et al., 2015b). In addition, plants can induce the synthesis of osmolytes such as amino acids or nitrogen-containing molecules, including proline or glycine-betaine, which also contributes to osmotic adjustment, maintaining plant turgor (Ashraf and Foolad, 2007).

The inhibition of plant growth induced by salinity is associated with a decrease in the photosynthesis rate. It is known that salt

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate reduced form; CAT, catalase; DHA, ascorbate oxidized form; DHAR, dehydreascorbate reductase; DW, dry weight; FW, fresh weight; GR, glutathione reductase; GSH, glutathione reduced form; GSSG, glutathione oxidized form; gs, stomatal conductance; H_2O_2, hydrogen peroxide; LWC, leaf water content; LP, lipid peroxidation; MDHAR, monodehydroascorbate reductase; MEP, 2-C-methyl-D-erythritol-4-phospate; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; O_2^-, superoxide anion; $^{\cdot}OH$, hydroxyl radicals; PAR, photosynthetically active radiation; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SG, steviol glycosides; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive-substances; UGTs, UDP-glycosyltransferases.

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http://dx.doi.org/10.1016/j.plaphy.2017.04.023
0981-9428/

stress (NaCl stress) affects the photosynthetic process due to stomatal closure that limits CO_2 fixation (Arbona et al., 2013) and reduces chlorophyll (Chl) content. Certain plants, however, have developed mechanisms to counteract the deleterious effects of salinity, acquiring tolerance to such stress. Furthermore, salt stress also causes oxidative stress mediated by the enhanced generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (Demidchik, 2015; Hernández et al., 1993). The overproduction of these radicals could be explained as an imbalance of the electron transport chain caused by a stressful condition. The role of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and the ascorbate-gluthatione (ASC-GSH) cycle enzymes [ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and gluthatione reductase (GR)] as ROS scavengers during salt stress is another important tolerance mechanism against oxidative stress, and, therefore, against salt stress (Acosta-Motos et al., 2015a, 2015b; Gill and Tuteja, 2010; Hernández et al., 2000, 2001). In fact, numerous authors have reported that NaCl-tolerant plant species either induce or are able to show a much higher antioxidant capacity than NaCl-sensitive species (Hernández et al., 2000, 2003; López-Gómez et al., 2007; Mittova et al., 2003).

Few authors have focused their attention, however, on the response to salt stress in the *Asteraceae* family, and studies concerning the effect of such stress on Stevia are scarce. *Stevia rebaudiana* Bertoni is a perennial shrub belonging to the *Asteraceae* family. The leaves of *S. rebaudiana* contain a high concentration of steviol glycosides (SGs). Stevioside and Rebaudioside A are the major SGs (Zeng et al., 2013). These molecules are glucosylated derivatives of diterpenoid steviol, and they are used as non-caloric sweeteners in many countries due to the fact that steviol glycosides are sweeter than sucrose (Lorenzo et al., 2014). Moreover, SGs have also shown multiple therapeutic properties, most notably their antihyperglycemic and antihypertensive effects (Abudula et al., 2004; Mishra et al., 2010).

SGs are biosynthetised by the plastidial 2-C-methyl-D-erythritol-4-phospate pathway, also known as the MEP pathway, which is a common pathway with gibberelins. The final step of SGs biosynthesis occurs in cytosol catalysed by 4 UDP-glycosyltransferases (UGTs). UGT85C2 attaches one molecule of D-glucose to C-13 of steviol to produce steviolmonoside. The next reaction of glycosylation leads to the formation of steviolbioside, catalysed by a UGT that has not yet been characterised (Modi et al., 2012). UGT74G1 is involved in the formation of stevioside by the glycosylation of C-4 carboxilic acid of steviolbioside. Rebaudioside A is formed by the addition of a D-glucose molecule of the C-3' of the C-13 glucose of stevioside, catalysed by UGT76G1 (Geuns and Ceunen, 2013). It has previously been suggested that salinity might affect the transcription of some genes involved in the biosynthetic pathway of SGs (Zeng et al., 2013). These authors used adult plants grown in hydroponic cultures to study the effect of NaCl (0–120 mM) on chlorophyll and Pro levels; the activity of some antioxidant enzymes (SOD, CAT and POX); the K, Na, Mg and Ca concentration in different plant organs; and the glycoside content (Zeng et al., 2013).

In this study, we used *S. rebaudiana* Bertoni plants acclimatised to *ex*-vitro conditions to analyse the effects of different NaCl treatments on several more parameters, including plant growth; chlorophyll content and chlorophyll fluorescence parameters; mineral nutrition; proline content; antioxidant metabolism (the ASC-GSH cycle enzymes, SOD, CAT, POX); oxidative stress parameters (lipid peroxidation (LP) and ROS accumulation); and steviol glycoside content (steviolbioside, stevioside and rebaudioside A). We also analysed the distribution of Na^+ and Cl^- in leaves and roots.

2. Material and methods

2.1. Plant material and experimental design

The plants were obtained from micropropagated Stevia shoot cultures (MS medium supplemented with 0.8 mg/L of metatopolin, 6 mg/L of adenenine sulphate and 0.040 mg/L of indole butyric acid, 3% sucrose and 7% agar). For elongation and rooting, shoots with three internodes were transferred to 1/2 MS medium without growth regulators. Under these conditions, the shoots elongated and rooted in 6 weeks. All cultures were maintained at 25 ± 2 °C in a growth chamber with a 16 h photoperiod (80 μmol m^{-2} s^{-1} PAR). The rooted shoots were taken out of the medium and washed with distilled water to remove medium attached to the roots. The shoots were then acclimatised in pots containing a mixture of perlite and peat (1:2) in a controlled growth chamber. The temperature in the chamber was set at 24/18 °C (light/darkness), and there was 80% relative humidity and 350 μmol m^{-2} s^{-1} PAR, supplied by cold white fluorescent lamps with a 16 h photoperiod.

After 8 weeks of acclimatisation to *ex-vitro* conditions, the *S. rebaudiana* plants were transplanted to pots (2 L) containing the same proportion of substrate used for acclimatisation. Seven days later, the plant tips were cut in order to standarise the size of all plants. The plants were then exposed to one of three different irrigation treatments for 16 and 25 d. The control plants (8 plants) were watered with 250 mL of distilled water, while the other plants were irrigated with the same volume of distilled water containing either 2 or 5 g/L NaCl (8 and 9 plants, respectively), corresponding to 34 and 90 mM, respectively. All plants (control and NaCl-treated plants) were watered twice a week with these solutions at the beginning of and throughout the experimental period.

2.2. Growth parameters and nutrient content analysis

At the end of the salinity treatment, the substrate was gently washed from the roots of four plants per treatment, and each plant was divided into leaves, stems and roots. The leaf water content (LWC) was determined in leaves from control and NaCl-treated plants. We first measured the fresh weight (FW) of the leaves, which were then oven-dried at 80 °C until they reached a constant weight in order to measure their dry weight (DW). LWC was determined using the following formula: LWC = [(FW−DW)/FW] ×100.

The measurement of different macronutrients [Ca, K, nitrogen (N), carbon (C), phosphorus (P), sulphur (S) and Mg)], micronutrients [cupper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), thallium (Tl), zinc (Zn) and borum (B)], and the Na and Cl levels was carried out in an ICAP 6000SERIES spectrometer (Ionomic Services of CEBAS-CSIC; Thermo Scientific, Madrid, Spain). To measure Cl content, an aqueous extraction (0.1g/10 mL miliQ water) was shaken for 24 h, and the results were analysed by ion chromatography (Ionomic Services of CEBAS-CSIC; Metrohm Ltd., Herisau, Switzerland). Total C and N contents were determined by combustion at 950 °C. Total C was analysed by infrared detection and N by termic conductivity in the Ionomic Services of CEBAS-CSIC (Murcia, Spain).

2.3. Proline determination

The proline in leaf samples was analysed at the end of the saline treatment (after 25 d of NaCl exposure). Briefly, 0.1 g of frozen (in liquid nitrogen) plant tissue (leaves) was homogenised in a mortar with 5 mL of 3% sulfosalicylic acid. After extraction, homogenates

were centrifuged at 4 °C at 12000 g for 10 min. A 1 mL aliquot of the supernatant was combined with an equal volume of glacial acetic acid and nihydrin reagent. This mixture was incubated at 100 °C for 1 h in a thermoblock and then cooled in an ice bath for at least 5 min. The solution was partitioned against 2 mL of toluene, and the absorbance of the organic phase was determined at 520 nm (Bates et al., 1973). A calibration curve was created using commercial proline as a standard.

2.4. Chlorophyll determination and chlorophyll fluorescence

At the end of each treatment (16 d and 25 d), 0.2 g of leaves were sampled and incubated in 25 mL of 80% acetone (v/v) for 48 h under darkness. The total chlorophyll (Chl), chlorophyll *a* (Chla) and chlorophyll *a* (Chlb) content was analysed by measuring the absorbance at 663 and 645 nm. The Chl content was calculated using the equations described by Arnon (1949).

The chlorophyll fluorescence was measured in detached leaves from control and salt-treated plants at 16 d and 25 d of NaCl treatment with a chlorophyll fluorimeter (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany). After a dark incubation period (15 min), the plants' minimum and maximum fluorescence yields were monitored. Kinetic analyses were carried out with actinic light (81 μmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 μmol quanta m^{-2} s^{-1} PAR for 0.8 s at intervals of 20 s. We also analysed the effective PSII quantum yield [Y(II)], the quantum yield of regulated energy dissipation [(Y(NPQ)], the non-photochemical quenching (NPQ), the maximal PSII quantum yield (F_v/F_m), the coefficients of non-photochemical quenching (qN) and the photochemical quenching (qP) (Faize et al., 2011).

2.5. Antioxidative metabolism

2.5.1. Enzyme extraction and analysis

All operations were performed at 4 °C. For enzymatic determination, plants were sampled at 16 d and 25 d of the NaCl treatments. Leaf samples (0.5 g) were homogenised with an extraction medium (1/3, w/v) containing 50 mM Tris-acetate buffer (pH 6.0), 0.1 mM EDTA, 2 mM cysteine and 0.2% (v/v) Triton X-100. For the APX activity, 20 mM of sodium ascorbate was added to the extraction buffer. The extracts were centrifuged at 10000 g for 15 min. The supernatant fraction was filtered on Sephadex NAP-10 columns (GE Healthcare) equilibrated with the same buffer used for homogenisation and used for the enzymatic determination. For the APX activity, 2 mM of sodium ascorbate was added to the equilibration buffer. APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), GR (EC 1.6.4.2), SOD (EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase activities (POX, EC 1.11.1.7) were all analysed following protocols used in our laboratory (Acosta-Motos et al., 2015a, 2015b; Díaz-Vivancos et al., 2013; Faize et al., 2011; Hernández et al., 2000).

2.5.2. Oxidative stress parameters

2.5.2.1. Lipid peroxidation. The extent of lipid peroxidation (LP) was estimated by determining the concentration of thiobarbituric acid-reative substances (TBARS). Leaf material (200 mg) was homogenised in 1 M perchloric acid solution (1/3, w/v), and the homogenate was centrifuged at 15000 g for 10 min. Then 0.5 mL of supernatant was added to 1.5 mL 0.5% TBA in 1 M perchloric acid. The mixture was incubated at 90 °C for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. The samples were then centrifuged at 10000 g for 5 min, and absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of TBARS was calculated from the extinction molar coefficient 155 mM^{-1} cm^{-1} (Cakmak and Horst, 1991).

2.5.2.2. Histochemical determination of ROS generation. The superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) staining in *S. rebaudiana* leaves treated with 0 (control), 2 and 5 g/L of NaCl during 16 d and 25 d was determinated by vacuum infiltration with 0.1 mg/mL Nitroblue tetrazolium (NBT) and 0.1 mg/mL 3,3'-diaminobenzidine (DAB) during 2 and 3 h, respectively. To assess the specificity of the staining, controls were performed with either 100 U mL^{-1} SOD (Cu, Zn-SOD, Sigma) or 10 mM ascorbate (Hernández et al., 2001). In both cases, leaves were rinsed with 80% ethanol to 65 °C for 10–15 min. Leaves were photographed with an Olympus BX40 microscope.

2.6. Extraction and determination of SGs content

The stevioside, steviolbioside and rebaudioside A concentrations were determined in leaf samples after 16 d and 25 d in the control and in the 2 and 5 g/L NaCl treatments. Four different biological samples were analysed for each treatment. First, plant material (0.5 g of fresh leaves) was extracted with 1.5 mL of a solution of methanol: water (70/30, v/v) at 4 °C. The mixture was then centrifuged at 10000 g for 10 min, and the supernatant was filtered through PTFE filters with a pore size of 0.45 μm. The stevioside, steviolbioside and rebaudioside A concentrations were measured by LC-MS (Metabolomic Service of CEBAS-CSIC, Murcia, Spain).

2.7. Statistical analyses of data

The data were analysed by one-way or two-way ANOVA using SPSS 22 software (SPSS, Inc, 2002). In this test, the term "Salinity" represents the concentrations of NaCl used in this experiment (2 and 5 g/L), and "Time" represents the time of exposure to those treatments (16 and 25 d). The means were separated with Duncan's Multiple Range Test at the 5% probability level ($P < 0.05$) to compare significant differences among treatments.

3. Results

In this work, we studied the response to NaCl stress in *Stevia rebaudiana* Bertoni plants acclimatised to *ex-vitro* conditions. Previously, the Stevia plants had been micropropagated in order to ensure the production of clonal, uniform and true-to-type plant material. In addition, *S. rebaudiana* shows a low rate of seed germination and great variation in the levels of steviol glycosides among seedlings.

3.1. Growth parameters, mineral nutrition and proline determinations

By 16 d, the NaCl treatment did not produce changes in the leaf FW of *S. rebaudiana* plants, whereas the leaf DW showed a significant decrease (F value = 64.48; $P < 0.001$) (data not shown). As a consequence, a concentration-dependent increase in LWC was observed. This increase was about 7.1% and 8.8% in the 2 g/L and 5 g/L NaCl treatments, respectively, when compared with control leaves (Fig. 1). At the end of the salinity period, no effect of NaCl stress on plant biomass was observed in Stevia plants, and, in fact, a significant increase in leaf DW was even detected ($F = 4.18$; $P < 0.01$) (Table 1). This increase in leaf DW produced a significant decrease in LWC in plants treated with 5 g/L NaCl, whereas no statistical differences were recorded in plants treated with the lower NaCl level (Fig. 1).

We observed a significant decrease in the total C content in Stevia plant leaves in parallel with the increase in NaCl

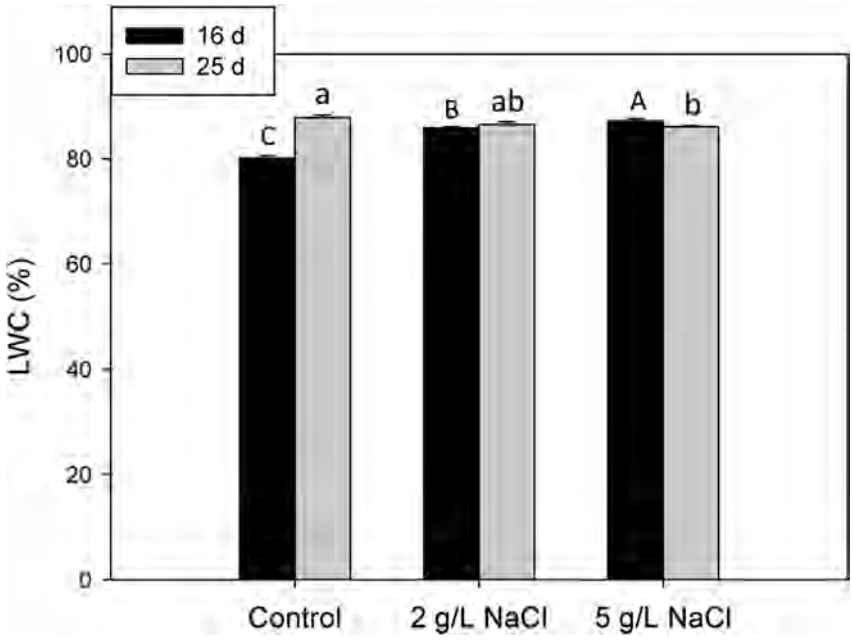


Fig. 1. Effect of short-and long-term NaCl stress on the leaf water content (LWC) of Stevia leaves. Data represent the mean ± SE from 5 different biological samples. Different letters in the same column indicate significant differences according to Duncan's test (P = 0.05). Significant differences between treatments at 16 d of NaCl stress are represented in capital letters, while the differences observed at 25 d of NaCl stress are indicate in lower case letters.

Table 1
Effects of the different NaCl treatments at the end of the stress period (25 d) on the growth of *S. rebaudiana* Bertoni. Leaf, stem and root fresh weight (FW) and dry weight (DW) are given in g plant^{-1}. Different letters in the same row denote significant differences according to Duncan's Multiple Comparisons Robust Test (p = 0.05).

	Control	2 g/L NaCl	5 g/L NaCl
Leaf FW	3.92 ± 0.09 a	3.95 ± 0.04 a	4.07 ± 0.08 a
Leaf DW	0.48 ± 0.03 b	0.54 ± 0.02 ab	0.57 ± 0.02 a
Stem FW	27.00 ± 1.29 a	26.25 ± 3.70 a	27.40 ± 2.46 a
Stem DW	4.90 ± 0.17 a	4.78 ± 0.81 a	5.2 ± 0.35 a
Root FW	6.51 ± 0.81 a	6.88 ± 0.83 a	6.54 ± 1.03 a
Root DW	0.69 ± 0.15 a	0.84 ± 0.10 a	0.82 ± 0.16 a
Root/Canopy FW	0.21 ± 0.02 a	0.23 ± 0.03 a	0.17 ± 0.02 a
Root/Canopy DW	0.13 ± 0.03 a	0.16 ± 0.04 a	0.15 ± 0.04 a

concentrations after 16 d of salt treatment, and a 13% increase in total N content was recorded in plants subjected to 5 g/L NaCl (Table 1). Similarly, after 25 d of treatment, the highest NaCl concentration resulted in a 7% decrease in C content in Stevia plant leaves (Table 2). Moreover, N accumulation in leaves dropped significantly (about 20%) due to the NaCl treatments (Table 2). No significant changes in root N content were recorded, whereas a significant decrease in root C was observed in plants treated with 5 g/L NaCl (Table 2).

Regarding the nutrient content, we observed that Na levels were very low in Stevia leaves in comparison with other plant species (Acosta-Motos et al., 2015a, b; Hernández et al., 1995). In the short term (16 d), 5 g/L NaCl increased the leaf Na concentration nearly 2.7-fold. NaCl stress also produced an increase in leaf Cl^- levels in a concentration-dependent manner (Table 3). The Cl^- values increased 3.7- and 4.8-fold with respect to control plants under the 2 and 5 g/L NaCl treatments, respectively. Furthermore, greater K^+ and Ca^{2+} concentrations were also recorded in salt-stressed plants than in control plants (Table 3). Indeed, in plants subjected to 5 g/L NaCl, K^+ and Ca^{2+} concentrations increased by about 38% and 71%, respectively. Salinity also induced an important increase in Mg^{2+} levels in Stevia leaves in relation to control plants (Table 3). On the other hand, the 2 and 5 g/L NaCl treatments did not affect P levels in *Stevia* plants after 16 d (Table 3).

After 25 d of stress, a salt-dependent increase in leaf Na was again observed. At this sampling date, we recorded 2-fold and 5-fold increases in Na^+ concentrations in plants treated with 2 and 5 g/L NaCl, respectively (Table 3). Moreover, both saline treatments produced a significant increase in Cl^- concentrations in leaves, although this rise was slightly lower than that observed at 16 d of stress (Table 3). A significant increase in K^+ content (29% when compared with control plants) was recorded in both NaCl treatments (Table 3). The leaf Ca^{2+} levels did not show statistically significant changes, although the Ca^{2+} content was statistically higher in the leaves of plants subjected to 5 g/L than in plants treated with 2 g/L (Table 3). The Mg^{2+} content fell by 20% in plants treated with 2 g/L NaCl, whereas a significant decrease in S content was recorded in all salt-treated plants (Table 3).

After 16 d of treatment, NaCl-concentration dependent increases in Mn and Zn levels were recorded (Table 3). In contrast, salinity reduced Cu and Mo contents by 21% and 44%, respectively, compared to non-treated plants (Table 3). While salt treatments did not affect Fe content (Table 3), a concentration-dependent decrease in B was recorded (Table 3). In the long term (25 d of stress), an NaCl-dependent increase in Zn^{2+}, Fe and Cu was observed (Table 3).

Macronutrient and micronutrient levels as well as Na^+ and Cl^- concentrations were also determined in roots after 25 d of stress in order to elucidate the nutrient distribution pattern between leaves and roots (Table 4). No significant changes in Cl^- levels were observed in roots, whereas root Na^+ increased significantly in conjunction with the increase in stress severity (by 88% and 138% under 2 g/L and 5 g/L NaCl, respectively, compared to control plants). Such Na^+ accumulation was accompanied by decreases in K^+ levels in response to both salt concentrations, although the only statistically significant decrease was observed under 5 g/L NaCl (Table 4). Ca^{2+}, Mg^{2+}, P and S and micronutrients did not show statistically significant changes in response to NaCl stress in Stevia roots (Table 4).

Table 2
Effect of increased NaCl levels on leaf and root Carbon (C) (g/100g DW) and Nitrogen (N) (g/100g DW) content after 16 and 25 d of salt stress. Data represents the mean ± SE from 4 plants. Different letters in the same experimental period indicate significant differences according to Duncan's Multiple Range Test (P < 0.05).

NaCl (g/L)	Leaves 16 d		Leaves 25 d		Roots 25 d	
	N	C	N	C	N	C
0	1.5 ± 0.0 b	46.4 ± 0.2 a	4.1 ± 0.0 a	43.1 ± 0.3 a	2.2 ± 0.2	40.5 ± 0.3 a
2	1.6 ± 0.0 ab	44.4 ± 0.2 b	3.2 ± 0.1 b	42.2 ± 0.3 b	1.9 ± 0.1	39.3 ± 0.2 ab
5	1.7 ± 0.1 a	43.3 ± 0.1 c	3.3 ± 0.1 b	40.2 ± 0.2 c	2.1 ± 0.1	38.7 ± 0.6 b

Table 3
Effect of increased NaCl levels on sodium (Na), chloride ions (Cl) and macro- and micronutrient concentrations in the leaves of *S. rebaudiana* Bertoni plants after 16 and 25 d of treatment. Data represent the mean from at least three measurements. Different letters in the same column indicate significant differences according to Duncan's test (P < 0.05). nd: not detected.

Period	NaCl (g/L)	Na (mg/100g)	Cl (mg/L)	K (g/100g)	Ca (g/100g)	Mg (g/100g)	P (g/100g)	S (g/100g)
16 d	0	4.7 ± 1.3 b	224.3 ± 10.6 c	1.80 ± 0.08 c	0.73 ± 0.03 c	0.38 ± 0.01 b	0.27 ± 0.01 a	0.11 ± 0.002 a
	2	3.3 ± 0.8 b	839.3 ± 13.8 b	2.10 ± 0.07 b	1.06 ± 0.03 b	0.69 ± 0.03 a	0.26 ± 0.01 a	0.10 ± 0.004 b
	5	12.9 ± 2.9 a	1067.5 ± 0.0 a	2.54 ± 0.06 a	1.18 ± 0.02 a	0.73 ± 0.06 a	0.29 ± 0.01 a	0.11 ± 0.002 a
25 d	0	9.0 ± 1.2 a	284.2 ± 7.8 c	1.66 ± 0.08 b	0.87 ± 0.07 ab	0.5 ± 0.0 a	0.41 ± 0.02 a	0.14 ± 0.01 a
	2	19.2 ± 4.7 b	776.0 ± 28.6 a	2.19 ± 0.06 a	0.82 ± 0.04 b	0.4 ± 0.0 b	0.35 ± 0.02 a	0.11 ± 0.03 b
	5	44.1 ± 0.3 c	552.4 ± 29.0 b	2.22 ± 0.06 a	1.0 ± 0.04 a	0.5 ± 0.0 a	0.37 ± 0.02 a	0.11 ± 0.01 b

Period	NaCl (g/L)	Cu (mg/Kg)	Zn (mg/Kg)	B (mg/Kg)	Fe (mg/Kg)	Mo (mg/Kg)	Mn (mg/Kg)
16 d	0	1.9 ± 0.1 a	22.3 ± 0.9 c	31.6 ± 1.3 a	40.8 ± 4.8 a	1.6 ± 0.1 a	116.6 ± 9.1 c
	2	1.5 ± 0.1 b	27.3 ± 0.9 b	28.0 ± 0.3 b	35.2 ± 1.4 a	0.9 ± 0.0 b	139.7 ± 5.4 b
	5	1.5 ± 0.0 b	37.0 ± 1.2 a	27 ± 0.6 c	38.3 ± 2.0 a	0.9 ± 0.1 b	168.4 ± 7.1 a
25 d	0	0.95 ± 0.11b	24.1 ± 0.3 c	nd	97.0 ± 22.6 b	nd	102.0 ± 8.2 ab
	2	6.23 ± 0.71a	28.2 ± 1.0 b	nd	133.3 ± 1.6 ab	nd	84.2 ± 1.7 b
	5	3.84 ± 1.28a	40.3 ± 3.3 a	nd	153.0 ± 6.1 a	nd	119.4 ± 2.4 a

Table 4
Effect of increased NaCl levels on sodium (Na), chloride ions (Cl) and macro- and micronutrient concentrations in the roots of *S. rebaudiana* Bertoni plants after 25 d of treatment. Data represent the mean from at least three measurements. Different letters in the same column indicate significant differences according to Duncan's test (P < 0.05).

Period	NaCl (g/L)	Na (g/100g)	Cl (mg/L)	K (g/100g)	Ca (g/100g)	Mg (g/100g)	P (g/100g)	S (g/100g)
25 d	0	0.8 ± 0.05 c	5.9 ± 0.5 a	1.9 ± 0.1 a	0.3 ± 0.05 a	0.3 ± 0.03 a	0.3 ± 0.02 a	0.4 ± 0.03 a
	2	1.5 ± 0.12 b	4.9 ± 0.9 a	1.7 ± 0.1 ab	0.3 ± 0.01 a	0.3 ± 0.03 a	0.4 ± 0.02 a	0.4 ± 0.02 a
	5	1.9 ± 0.17 a	6.3 ± 1.2 a	1.4 ± 0.1 b	0.2 ± 0.02 a	0.2 ± 0.01 a	0.3 ± 0.02 a	0.4 ± 0.05 a

Period	NaCl (g/L)	Cu (mg/Kg)	Zn (mg/Kg)	Tl (mg/Kg)	Fe (mg/Kg)	Mn (mg/Kg)
25 d	0	8.5 ± 1.7 a	29.4 ± 1.8 a	1.3 ± 0.1 a	161.6 ± 13.1 ab	33.9 ± 2.4 a
	2	16.6 ± 3.7 a	37.8 ± 0.5 a	1.5 ± 0.1 a	234.0 ± 43.4 a	33.8 ± 2.1 a
	5	8.2 ± 1.0 a	28.7 ± 1.4 a	1.6 ± 0.2 a	121.5 ± 5.22 b	26.7 ± 2.3 a

Proline content was also analysed at the end of the saline treatment (25 d) in an attempt to test the ability of *S. rebaudiana* plants to deal with the osmotic stress imposed by the salinity. No significant differences were observed in leaf proline levels under the 2 g/L NaCl treatment, but in plants treated with the highest NaCl level, a two-fold increase in proline content was recorded (Fig. 2).

3.2. Chlorophyll determinatination and chlorophyll fluorescence measurement

We analysed the effect of NaCl stress on the Chl content in Stevia leaves and found that NaCl treatments (the "Salinity" factor) had a significant effect on Chla, Chlb and total Chl content. Furthermore, only the Chlb and total Chl content were affected by the "Time" factor. However, no interaction between both factors was observed (Table 5).

After 16 d of NaCl stress, an NaCl-dependent decrease in total Chl, Chla and Chlb concentrations was observed (Table 5). The 2 g/L NaCl treatment only affected the Chla levels, whereas the 5 g/L NaCl treatment reduced both Chla and Chlb concentrations by about 20% in relation to control plants (Table 5).

After the stress period (25 d), Chlb levels were higher at 25 d than at 16 d in both control and NaCl-treated plants (Table 5). In fact, Chla represented 75% of the total Chl content at 16 d, whereas at 25 d, Chla represented 58% of the total Chl levels. On the other hand, we did not observe any effects of NaCl stress on chlorophyll content at this stage (Table 5).

In parallel to chlorophyll content determination, different photochemical and non-photochemical quenching parameters were also analysed in Stevia plants exposed to 16 d and 25 d of NaCl stress. In this case, the "Salinity" and the "Time" factors as well as the interaction between the two factors were found to have a significant effect on all the chlorophyll fluorescence parameters studied (Table 6).

After 16 d of NaCl exposure, plants exhibited an NaCl-dependent decrease in photochemical quenching parameters [Y(II) and qP] and an increase in non-photochemical quenching parameters (NPQ and qN) (Table 6, Fig. 3). At 25 d, Stevia leaves showed some degree

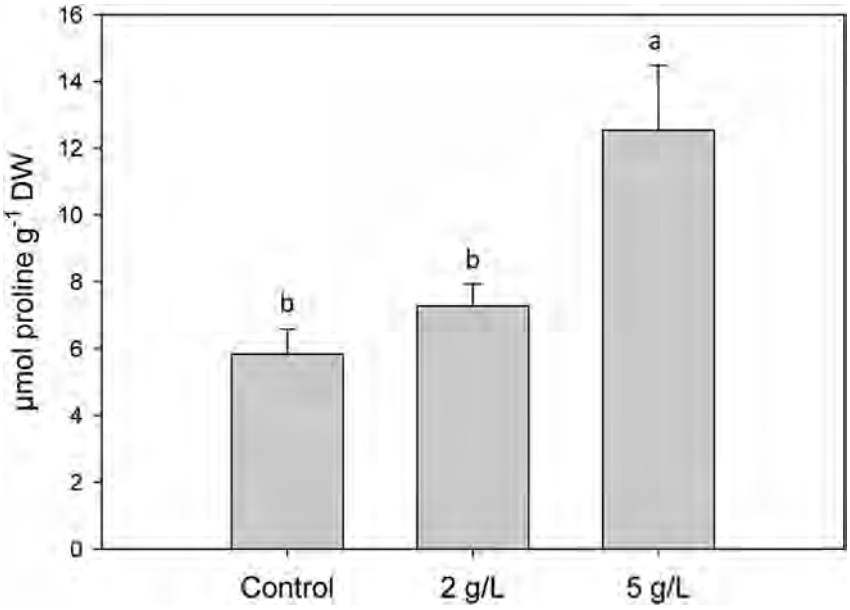


Fig. 2. Effect of salt stress on leaf proline content after 25 d of treatment. Data represent the mean ± SE from 5 different biological samples. Different letters in the same column indicate significant differences according to Duncan's test (P = 0.05).

Table 5
Effect of increased NaCl levels on total Chl, Chla and Chlb content (µg/g FW) after 16 and 25 d of salt treatment. Data represent the mean ± SE from 6 plants. Different letters in the same column indicate significant differences according to Duncan's Multiple Range Test (P < 0.05). [a]F values from two-way ANOVA for the chlorophyll content. F values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

16 d			
NaCl	**Chla**	**Chlb**	**total Chl**
0 g/L	975.8 ± 21.5 a	326.22 ± 8.5 a	1299.99 ± 29.8 a
2 g/L	835.9 ± 11.1 b	293.94 ± 12.0 a	1128.19 ± 51.7 b
5 g/L	722.8 ± 49.8 b	255.02 ± 13.2 b	976.33 ± 62.9 b
25 d			
NaCl	**Chla**	**Chlb**	**total Chl**
0 g/L	921.4 ± 44.4 a	685.87 ± 19.3 a	1558.90 ± 27.1 a
2 g/L	910.2 ± 67.6 a	653.87 ± 55.2 a	1466.02 ± 77.7 a
5 g/L	905.2 ± 38.8 a	628.05 ± 15.6 a	1531.21 ± 48.2 a
[a]F-values			
Salinity (A)	4.03*	5.52*	9.37**
Time (B)	3.10	398.73***	86.14***
(A) x (B)	3.10	0.92	2.77

of acclimatisation, particularly in plants irrigated with 5 g/L NaCl. In these plants, increases in both qP and in qN and NPQ were recorded (Table 6; Fig. 3). It is worth mentioning the fact that while Y(II) displayed a strong decrease in 5 g/L NaCl-treated plants at 16 d, no effects of salt stress were recorded after 25 d of treatment, when the 5 g/L NaCl-treated plants showed similar Y(II) values to control plants (Table 6; Fig. 3). In addition, increases in qN and NPQ were also recorded in NaCl-treated plants after 25 d of salinisation (Table 3).

3.3. Antioxidative metabolism

After 16 d of NaCl treatment, no differences in LP levels were observed, whereas a slight increase in LP was observed at 25 d in a concentration-dependent manner, although differences were not statistically significant. However, LP data increased with the age of the plants (Fig. 4, F value 26.79, P < 0.001). Regarding the accumulation of H_2O_2 and $O_2^{\cdot-}$, a slight increase was observed in Stevia plant leaves with the salt treatments, and the leaves from plants irrigated with 5 g/L NaCl showed the greatest accumulation of these ROS (Fig. 5).

Table 6
Effect of increased NaCl levels on fluorescence parameters after 16 and 25 d of salt treatment in *S. rebaudiana* Bertoni plants. Data represent the mean ± SE from 30 measurements. Different letters in the same column indicate significant differences according to Duncan's test (P < 0.05). [a]F values from two-way ANOVA for the chlorophyll fluorescence parameters. F values significant at 99.9% (***) or 95% (*) levels of probability.

Period	**NaCl**	**qP**	**Y(II)**	**qN**	**NPQ**
16 d	0 g/L	0.72 ± 0.01 a	0.45 ± 0.01 a	0.68 ± 0.01 c	0.34 ± 0.05 c
	2 g/L	0.62 ± 0.01 b	0.33 ± 0.02 b	0.79 ± 0.01 b	0.52 ± 0.02 b
	5 g/L	0.54 ± 0.02 c	0.23 ± 0.01 c	0.85 ± 0.02 a	0.70 ± 0.01 a
25 d	0 g/L	0.49 ± 0.01 b	0.32 ± 0.02 a	0.58 ± 0.01 b	0.23 ± 0.01 b
	2 g/L	0.47 ± 0.04 b	0.28 ± 0.01 b	0.71 ± 0.03 a	0.36 ± 0.04 a
	5 g/L	0.56 ± 0.02 a	0.34 ± 0.01 a	0.68 ± 0.01 a	0.34 ± 0.02 a
[a]F-values					
Salinity (A)		10.03***	32.68***	120.10***	191.77***
Time (B)		87.23***	4.99*	231.44***	455.15***
(A) x (B)		31.84***	43.64***	9.60***	59.21***

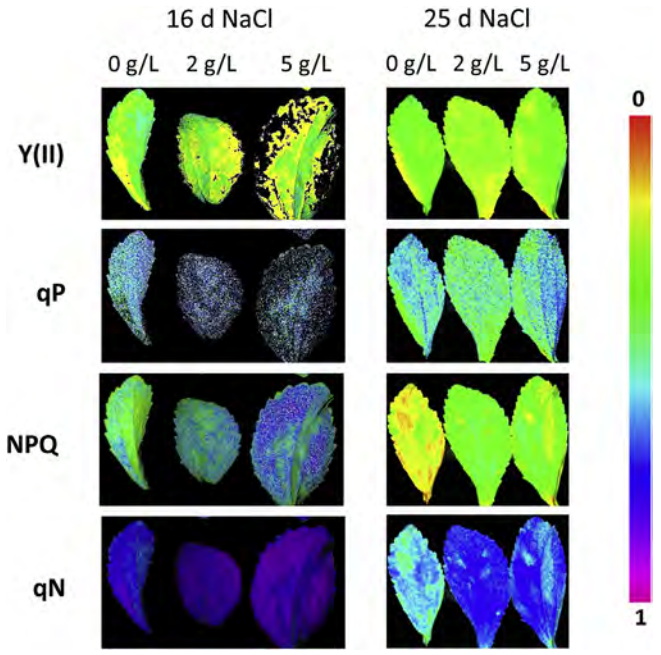


Fig. 3. Chlorophyll fluorescence parameters in Stevia leaves at 16 and 25 d of NaCl stress. Images of the coefficient of photochemical quenching (qP), the effective PSII quantum yield [Y(II)], the non-photochemical quenching coefficient (qN) and non-photochemical quenching (NPQ). 0 represents the lowest value and 1 the maximum value for each parameter.

In Stevia leaves, both the "Salinity" and "Time" factors, as well as the interaction between the two, had a significant effect on MDHAR, DHAR, GR and SOD activities. APX and POX were affected by the "Time" factor and by the "Salinity x Time" interaction, whereas both the "Salinity" and "Time" factors had a significant effect on CAT activity, but no interaction effect was observed (Table 7).

After 16 d of NaCl stress, the activity of all the antioxidant enzymes analysed (except for CAT) was significantly greater in 2 g/L NaCl-treated plants than in control plants. The ASC-GSH cycle enzymes (APX, MDHAR, DHAR and GR), for example, showed a range of nearly 2- to 3.7-fold increases. Moreover, a strong increase in SOD and POX activity (12- and 5-fold, respectively) was also observed in 2 g/L NaCl-treated plants (Table 7). In 5 g/L NaCl-treated plants, however, the response of the antioxidant enzymes was somewhat different. In these plants, an increase in SOD, CAT, POX, MDHAR and GR activities was recorded, whereas APX and DHAR activities remained unchanged (Table 7). In this treatment, the increase in antioxidant enzyme activity was thus lower than that observed in plants treated with 2 g/L NaCl. In 5 g/L NaCl-treated plants, CAT, MDHAR and GR activity increases ranged from 2- to 2.8-fold, and POX and SOD increases were 4.3- and 7.3-times higher than in control plants (Table 7).

After 25 d of NaCl treatment, the antioxidant enzymes behaved differently. The POX, CAT and SOD activities, for example, did not show statistically significant differences when compared with control plants (Table 7). APX and DHAR activities decreased by about 60% and 30%, respectively, in NaCl-stressed plants, and no differences between NaCl treatments were observed. A 45% drop in GR activity was found in plants treated with 5 g/L of NaCl. Concerning MDHAR activity, a 2-fold increase was recorded in both salt treatments. This response was similar to that observed at 16 d of stress.

3.4. Determination of SGs content

In this study, we determined the effect of 16 d and 25 d of NaCl stress on stevioside and rebaudioside A, which are the major steviol glycosides in the leaves of *S. rebaudiana* plants. Moreover, steviolbioside levels were also measured in order to obtain more information about the effect of salinity on the SGs pathway. The "Salinity" factor was found to have a significant impact on the levels of steviolbioside and stevioside, whereas "Time" and the interaction between both factors considered (Salinity × Time) significantly affected the concentrations of the three glycosides analysed (Fig. 6).

After 16 d of NaCl stress, NaCl-dependent decreases in stevioside levels were observed. The values decreased by 13% and 26% in 2 and 5 g/L NaCl-treated plants, respectively, in relation to control plants

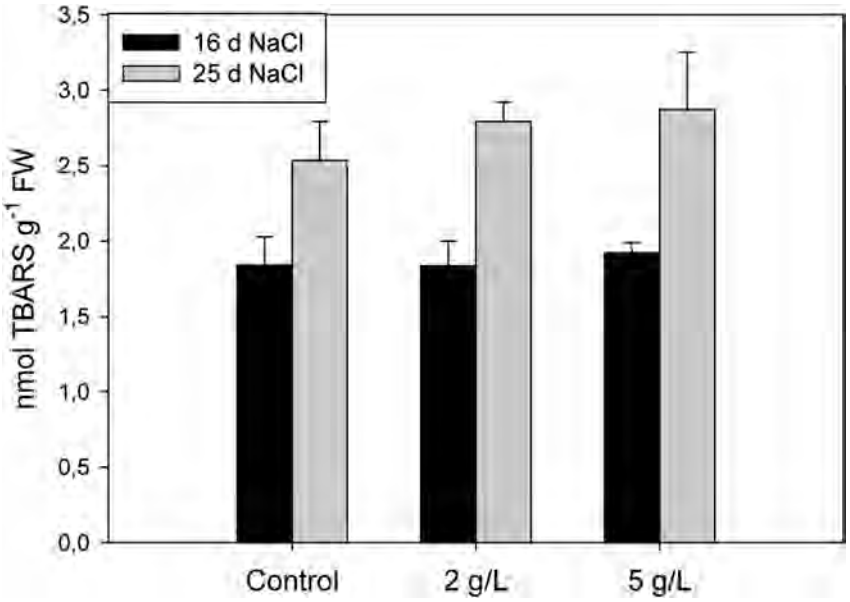


Fig. 4. Effect of salt stress on lipid peroxidation, as TBARS, in Stevia plant leaves after 16 and 25 d of treatment. Data represent the mean ± SE from 5 different biological samples. Non-statistically significant differences were found between control and NaCl treatments under both 16 and 25 d stress periods.

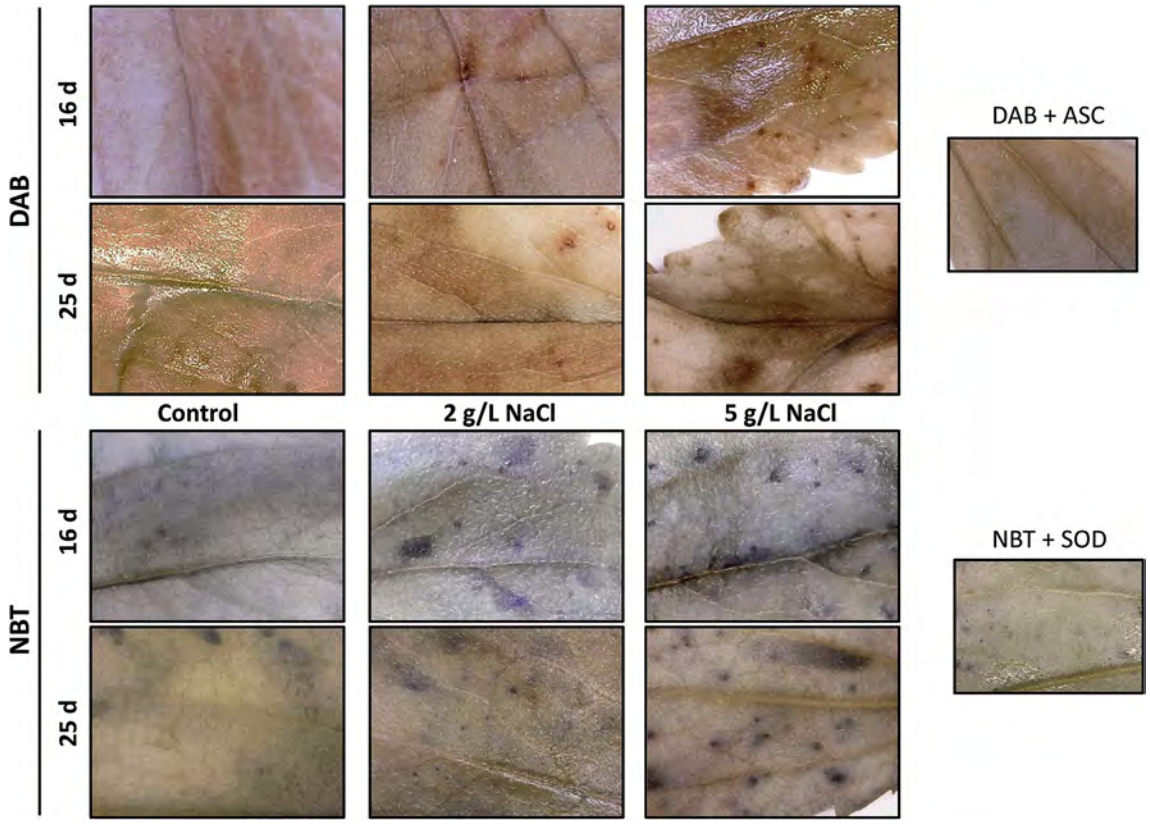


Fig. 5. Effect of NaCl on ROS accumulation in Stevia plant leaves at 16 and 25 d of treatment. Hydrogen peroxide was detected by staining with 3,3-diaminobenzidine (DAB) for 2 h, whereas superoxide accumulation was monitored by staining with nitroblue tetrazolium (NBT) for 3 h. To asses the specificity of the staining, controls using either 10 mM ascorbate or 100 U mL-1 SOD (Cu,Zn-SOD, Sigma) were performed.

Table 7
Effect of NaCl on the activity of some antioxidant enzymes in leaves from *S. rebaudiana* Bertoni plants after 16 and 25 d of salt stress. APX, MDHAR, DHAR, and GR are expressed as nmol min^{-1} mg^{-1} protein. POX is expressed as μmol min^{-1} mg^{-1} protein. CAT is expressed as mmol min^{-1} mg^{-1} protein and SOD as U mg^{-1} protein. Data represent the mean ± SE of at least four repetitions. Different letters in the same experimental period indicate significant differences according to Duncan's Multiple Range Test ($P < 0.05$). [a]F values from two-way ANOVA significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

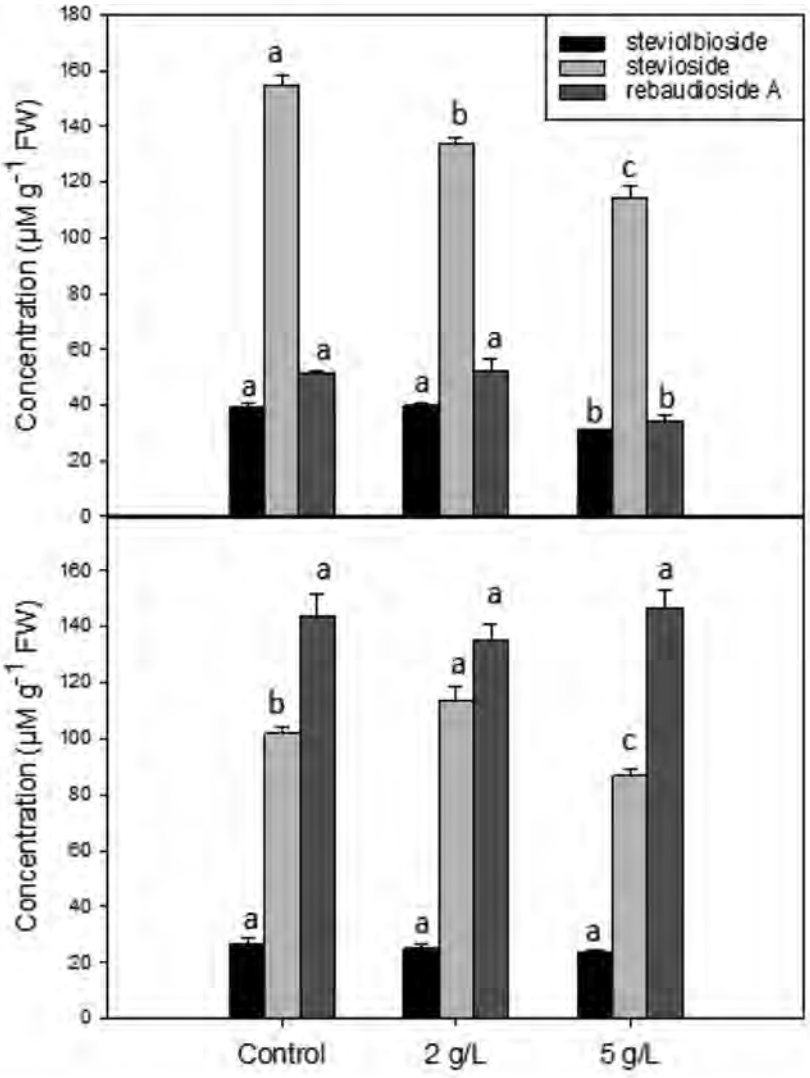
Period	NaCl	APX	MDHAR	DHAR	GR	POX	CAT	SOD
16 d	0 g/L	36.6 ± 2.9b	243.7 ± 19.1 b	30.1 ± 4.3 b	93.4 ± 9.2 b	106.5 ± 6.6 b	2.1 ± 0.2 b	2.1 ± 0.2 b
	2 g/L	67.5 ± 3.6 a	594.7 ± 38.6 a	112.1 ± 10.5 a	248.0 ± 33.9 a	566.1 ± 120.8 a	2.9 ± 0.3 ab	25.4 ± 6.6 a
	5 g/L	42.4 ± 4.1 b	521.0 ± 143.2 a	48.1 ± 6.5 b	256.6 ± 22.4 a	457.6 ± 97.3 a	4.2 ± 1.1 a	15.3 ± 2.3 a
25 d	0 g/L	82.1 ± 8.0 a	62.2 ± 6.5 b	10.5 ± 0.5 a	66.3 ± 1.7 a	207.2 ± 34.8 a	6.7 ± 0.6 a	7.3 ± 0.1 a
	2 g/L	49.4 ± 3.3 b	148.5 ± 8.0 a	7.4 ± 0.8 b	59.8 ± 4.3 a	149.0 ± 5.9 a	8.3 ± 0.4 a	6.1 ± 1.0 a
	5 g/L	54.2 ± 7.0 b	144.0 ± 30.0 a	7.4 ± 0.7b	30.5 ± 5.6 b	155.0 ± 29.9 a	7.5 ± 0.6 a	8.3 ± 1.1 a
[a]F-Values								
Salinity (A)		2.31	18.39***	40.50***	10.50**	3.61	3.88*	10.28**
Time (B)		8.12*	102.40***	201.64***	104.43***	10.67**	91.75***	11.95**
(A) x (B)		16.48***	6.4*	45.513***	18.65***	6.14*	1.50	12.20**

(Fig. 6A). On the other hand, steviolbioside and rebaudioside A levels did not show statistically significant changes in plants treated with 2 g/L NaCl, whereas 5 g/L NaCl decreased the levels of steviolbioside and rebaudioside A by 21% and 34%, respectively (Fig. 6A).

After 25 d of stress, the stevioside and steviolbioside concentrations detected in Stevia leaves were lower than those observed at 16 d. In contrast, a time-dependent increase in rebaudioside A was recorded in both control and NaCl-treated plants. Furthermore, while steviolbioside and rebaudioside A levels remained unchanged in plants subjected to NaCl stress, we observed a 25% increase in stevioside levels in plants treated with 2 g/L NaCl and a 15% decrease in stevioside levels in plants treated with 5 g/L NaCl (Fig. 6B).

4. Discussion

In the Mediterranean region, soil salinity has increased over the last 20 years. A soil is considered to be saline when the soil solution reaches about 40 mM NaCl (Acosta-Motos et al., 2017). The salinity levels applied in this study are in concordance with those that can be found in irrigation water in semiarid regions in the Mediterranean Basin. The results of this work suggest that Stevia plants can either be irrigated with saline waters or grown in salt-affected soils



[a]F-values			
ANOVA	**Steviolbioside**	**Stevioside**	**Rebaudioside A**
NaCl (A)	10.08**	34.00***	0,95
Tiempo (B)	116.97***	129,41***	515.98***
A x B	4.98*	11.64**	4.30*

Fig. 6. Effect of different NaCl concentrations on different steviol glycosides in Stevia plant leaves after 16 and 25 d of treatment. Data represent the mean ± SE from 5 different biological samples. Different letters in the same column indicate significant differences according to Duncan's test (P = 0.05). [a]F values from two-way ANOVA for the steviol glycoside content. F values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

and that it is possible to grow these plants in adverse conditions in a context of climate change. Moreover, our data suggest that Stevia plants are able to deal with short- and long-term NaCl stress, displaying a series of mechanisms to acclimate to salinity, including ion homeostasis, modified growth parameters, photosynthesis protection, proline accumulation and antioxidative metabolism-related changes. In addition, in the long term, the levels of rebaudioside A and stevioside, the most common steviol glycosides, were not affected by 2 g/L NaCl, and rebaudioside A levels were not affected in plants irrigated with 5 g/L NaCl.

4.1. Growth parameters, mineral nutrition and proline determination

NaCl stress tolerance is a complex phenomenon that involves different processes, including osmolyte accumulation and ion

homeostasis, antioxidant metabolism and photosynthesis protection (Acosta-Motos et al., 2015a; Kumar et al., 2012; Lima Neto et al., 2014). Salinity produces several negative consequences for plants, including reduced plant growth. Different growth parameters, such as the DW and FW of shoots and roots and the leaf area and leaf number, are seriously affected by salinity in different plant species (Ahmad et al., 2012; Hernández et al., 1995; Sabra et al., 2012; Saleh, 2012). In our study, however, we did not observe significant effects of salinity on plant growth in Stevia plants. The increase observed in LWC in NaCl-treated plants after 16 d could reflect an adaptation response in Stevia plants to avoid excessive water loss. In the longer term, Stevia plants seemed to be tolerant to the imposed NaCl stress, given that no negative effects of salinity on plant growth were detected at 25 d. One of the earliest consequences of NaCl stress is the promotion of an osmotic effect in the soil, limiting water uptake by the plant (Deinlein et al., 2014). In response to this effect, Stevia plants may display a decrease in evapotranspiration and stomatal conductance during the stress period, as described in other plant species (Acosta-Motos et al., 2015a, b). However, after 25 d of stress, a significant decrease in LWC was only observed in 5 g/L NaCl-treated plants. Our findings agree with those of Zeng et al. (2013), who previously described *Stevia rebaudiana* Bertoni as a moderately salt-tolerant plant.

Nutritional imbalance is another negative effect of salinity on plants. In our study, a dramatic Cl^- accumulation was observed in Stevia leaves under both 16 and 25 d of NaCl stress. However, no evident symptoms of salinity were observed in Stevia plant growth in the presence of NaCl, suggesting that these plants are able to compartmentalise the Cl^- ions, thus contributing to the osmotic adjustment of the plants. In the roots, Stevia plants accumulated Na^+ but not Cl^- under the 2 and 5 g/L NaCl treatments. Nonetheless, the plants are able to control salt concentration, thus limiting accumulation of Na^+ ions in the aerial parts. In fact, Stevia may be able to limit the translocation of Na^+ to the aerial parts in order to avoid toxic effects, thus displaying another tolerance mechanism (Acosta-Motos et al., 2015a; Munns and Tester, 2008).

In terms of Ca^{2+} and K^+ content, a NaCl concentration-dependent increase occured in Stevia plants, mainly after 16 d of treatment. It is well known that Ca^{2+} and K^+ play an important role in the maintenance of osmotic adjustment and ion homeostasis. Furthermore, both nutrients play an important role in different physiological mechanisms, including plant growth and development, the stomatal response, cellular turgor, cell wall and membrane stability, enzyme activation and cell signaling (Acosta-Motos et al., 2015a; Marschner, 1995). Moreover, it has been reported that Ca^{2+} addition provides some protection to the membranes in loquat and anger plants (Hernández et al., 2003). In Stevia, Zeng et al. (2013) described an increase in Ca^{2+} and Mg^{2+} levels in leaves and stems subjected to 5.3 g/L (90 mM) and 7 g/L (120 mM) NaCl as a possible strategy to deal with the stressful conditions. In contrast, these authors reported a decrease in leaf K^+ levels in the presence of high NaCl levels, and they suggested that this response could be related to a negative effect of NaCl stress on the membrane systems. Moreover, high Na^+ concentrations may cause a reduction in K^+ uptake due to competition between the two ions for plasma membrane channels. However, under our experimental conditions, the increase in leaf Na^+ content in NaCl-treated plants did not affect K^+ uptake in leaves, probably due to the limited translocation of Na^+ to the aerial parts, as observed by the low Na^+ concentrations detected in the leaves. The increased leaf K^+ and Ca^{2+} levels observed in Stevia plants may be related to the fact that the levels of lipid peroxidation remained unchanged under salinity conditions.

In roots, however, salinity had no effect on the Ca^{2+} levels, and K^+ concentrations decreased. In addition, salt treatment induced important increases in leaf Mg^{2+} levels. These results do not agree with the findings of Zeng et al. (2013), who described a decrease in Mg^{2+} levels in the aerial parts of Stevia plants subjected to 3.5 g/L (60 mM) and 5.3 g/L (90 mM) of NaCl that correlated with a drop in Chla and Chlb levels.

Different authors have previously described imbalances in micronutrient concentrations under NaCl stress conditions in different plant species, including in the leaves and purified chloroplast from *Pisum sativum* L. plants, *Andrographis paniculata* and *Brassica napus* L. (Hernández et al., 1995; Talei et al., 2012; Tunçtürk et al., 2011). These cited authors have shown that the effect of salinity on micronutrient concentrations depends on the plant species, even among different cultivars belonging to the same species. In our study, an alteration in micronutrient content was reported after 16 and 25 d of NaCl stress, mainly in leaves. Micronutrients are required to complete the growth cycles and for different functions such as photosynthesis performance, enzymatic activities and secondary metabolite production. Changes in micronutrient levels may directly or indirectly influence the susceptibility of plants to environmental stresses, including salinity (Hajiboland, 2012; Talei et al., 2012).

In the long term, we also observed increases in leaf Cu and Fe. Although both Cu and Fe are constituent of different enzymes, these increases could be dangerous for plant cells. Free Fe or Cu can catalyse hydroxyl radical formation in the so-called Haber-Weiss reaction (Halliwell and Gutteridge, 2003). In Stevia plants, leaf Cu and Fe concentrations were much higher at 25 d of treatment than at 16 d, particularly in salt-treated plants. As mentioned above, NaCl had no effect on lipid peroxidation. However, the TBARS values observed after 25 d of treatment were statistically higher than those recorded at 16 d of stress ($F = 26.79$, $P < 0.001$), which correlated with the higher leaf Cu and Fe levels observed at 25 d.

Plants are able to induce the synthesis of low molecular weight organic molecules involved in osmotic adjustment to deal with NaCl stress (Arbona et al., 2013). Among the amino acids, proline is the main effector in this response. We observed that proline played a role as an osmoprotectant in *Stevia rebaudiana* Bertoni leaves after 25 d of stress, especially at the highest salt concentration, as has been previously described (Zeng et al., 2013). These cited authors described enhanced proline content with increased NaCl concentrations in Stevia plants. Increased proline levels have also been recorded in *Eugenia myrtifolia* and *Pisum sativum* subjected to NaCl stress (Acosta-Motos et al., 2015a; Ozturk et al., 2012). Enhancement of osmolytes such as proline could be an adaptative mechanism to cope with NaCl stress, maintaining cell turgor and acting as an osmoprotectant as well as an antioxidant molecule (Deinlein et al., 2014).

4.2. Chlorophyll determinatination and chlorophyll fluorescence measurement

The adaptation of *S. rebaudiana* Bertoni to NaCl stress was also reflected in chlorophyll levels and chlorophyll fluorescence parameters in our study. Stevia plants irrigated with 5 g/L NaCl for 16 d showed a significant decrease in total Chl, Chla and Chlb concentrations when compared to non-treated plants. The decrease in chlorophyll levels was reflected in the photosynthetic machinery, such as in the decrease in the qP and Y(II) fluorescence parameters. Likewise, Zeng et al. (2013) previously described significantly lower levels of Chla and Chlb in Stevia plants treated with 5.3 g/L NaCl. A similar effect on chlorophyll content has also been recorded in other salt-sensitive species (Hernández et al., 1995; Ozturk et al., 2012).

Nevertheless, after 25 d of stress, NaCl had no effect on chlorophyll content. In fact, higher Chlb levels were observed at 25 d than at 16 d in both control and NaCl-treated plants. Chlb has been

reported to pass absorbed light to Chla for driving photosynthesis. Furthermore, Chlb moieties absorb light in the blue region, whereas Chla absorbs light in the red region. Red light plays an important role in the development of the photosynthetic machinery, and blue light is important for chlorophyll formation (Ouzounis et al., 2015). Chlorophyll levels can be seen as a biochemical marker of salt tolerance in plants. The maintenance of Chl content under NaCl stress can be considered a mechanism for protecting the photosynthesis process. According to previous studies, salt-tolerant species such as *Myrtus communis* and *Eugenia myrtifolia* (Acosta-Motos et al., 2015a, b) show increased or unchanged levels of total chlorophylls under saline conditions. Accordingly, *Stevia* plants may be considered a relatively salt-tolerant species in terms of chlorophyll content.

After 16 d of saline treatment, Stevia plants displayed a decrease in photochemical quenching parameters [qP and Y(II)]. Furthermore, an increase in non-photochemical quenching parameters (qN, NPQ) correlating with the severity of NaCl stress was recorded as an adaptative response to NaCl stress. Different authors have previously described the increase in non-photochemical parameters as an efficient mechanism for dissipating excess light energy and thus minimising ROS generation (Maxwell and Johnson, 2000). After 25 d of NaCl stress, the salt-treated plants in this study seemed to adapt to the stress-imposed conditions, especially the plants irrigated with 5 g/L NaCl. In this case, an increase in both photochemical and non-photochemical quenching parameters was observed, suggesting a recovery response in terms of chlorophyll fluorescence. The increase in qP and Y(II) values observed in plants irrigated with 5 g/L NaCl for 25 d may be related to the higher Chla and Chlb levels observed in these plants than in the 16 d NaCl-treated plants. This increase resulted in greater photosynthetic capacity at 25 d.

4.3. Antioxidative metabolism

NaCl stress induced ROS accumulation in Stevia plants that correlated both with the NaCl-treatments and the time of application. In spite of this ROS accumulation, no damage to membranes was detected, as monitored by TBARS measurements. As mentioned above, TBARS values were much higher at 25 d than at 16 d of NaCl stress, including in control plants. This behaviour could be related, among other factors, to the constitutive levels of the different antioxidant enzymes detected and to the different responses of the plants in each period, including the aforementioned increases in leaf Fe and Cu levels. In the short term, it seems that Stevia plants can tightly control ROS generation by inducing different antioxidant mechanisms. For example, the significant increases in ROS-scavenging enzymes (APX, POX, CAT, SOD) as well as in the rest of the ASC-GSH cycle enzymes observed at 16 d show that Stevia plants have a balanced antioxidant machinery at this stage. This response is clearly reflected in the ROS accumulation monitored by histochemical staining. CAT levels increased during the development of Stevia plants, and CAT seems to be involved in the removal of the H_2O_2 that is produced in peroxisomes during photorespiration (Willekens et al., 1997). Increased CAT levels could thus be indicative of higher photorespiratory activity, suggesting a possible role for this metabolic pathway in the response of Stevia plants, especially to long-term NaCl stress. These results are similar to those described in *M. communis* plants subjected to NaCl stress (Acosta-Motos et al., 2015a).

In the long term, however, an imbalance in the ASC-GSH cycle enzymes occurs, as reflected in the NaCl-induced decreases in APX, DHAR and GR, mainly in plants treated with 5 g/L NaCl. The effect of NaCl in reducing APX activity has been described in other plant species, including *Myrtus communis, Eugenia myrtiflora* and *Echinacea angustifolia* (Acosta-Motos et al., 2015a, b; Ozturk et al., 2012). APX acts as a redox modulator and seems to be crucial for plant stress tolerance as well as for plant growth and development (Shigeoka and Maruta, 2014). The long-term effect of NaCl on the antioxidative defences correlated with the higher H_2O_2 and $O_2^{\cdot-}$ accumulation as well as with the higher TBARS levels detected at this stage.

4.4. SGs content

SGs create a group of secondary metabolites derived from the mono-, di- and tetra-terpene biosynthetical pathway (Geuns and Ceunen, 2013), and information regarding the role of these kinds of metabolites under salinity conditions is very scarce. In this study, stevioside concentrations were significantly affected in plants treated with NaCl in both periods of stress. At 16 d of stress, stevioside was the major SG in Stevia leaves. In contrast, at 25 d, a greater abundance of rebaudioside A was found. These results suggest that a time-dependent modulation in the SGs pattern occurred in Stevia leaves, probably mediated by enhanced transcription of the *UGT76G1* gene. In a recent work, Zeng et al. (2013) suggested that salinity could modulate the transformation of stevioside to rebaudioside A by enhancing the transcription of the *UGT76G1* gene. In our study, however, we found that it was the age ("Time" factor) of the plant rather than the NaCl stress that had an effect on modifying the SGs pattern.

The role these metabolites play as osmoprotectant molecules under water stress conditions has been previously suggested (Geuns and Ceunen, 2013). While stevioside contains three glucose molecules in its structure, rebaudioside A contains four. Our findings could suggest a greater contribution of these metabolites to osmotic adjustment in *Stevia rebaudiana* leaves under salinity conditions; however, more research is needed to support this notion.

5. Conclusion

We observed that Stevia plants implement a series of adaptations to acclimate to salinity at the physiological level (plant growth, ion accumulation, chlorophyll fluorescence) and at the biochemical level (antioxidative metabolism and chlorophyll and proline contents) (Fig. 7). Ion accumulation (Na^+, K^+, Ca^{2+}, Cl^-) can contribute to osmotic adjustment only if plants have the ability to compartmentalise the ions into the vacuoles. In addition, proline accumulation can also contribute to osmotic adjustment. Moreover, K^+ and Ca^{2+} play important roles in ion homeostasis and plant growth and development.

In the long term, an increase in chlorophyll content was induced as a mechanism to protect the photosynthesis process. Interestingly, an increase in Chlb content occurred in the oldest plants. In addition, we found that NaCl-induced ROS accumulation in Stevia leaves was more evident in the presence of 5 g/L NaCl. However, Stevia plants are able to induce (16 d) or maintain (25 d) their antioxidant enzymes to cope with NaCl-induced oxidative stress (Fig. 7). In addition, we observed a time-dependent increase in rebaudioside A, which has a better flavor than stevioside. This increase could thus be of potential interest in agro-industrial production. Our results suggest that Stevia plants induce tolerance mechanisms in order to minimize the deleterious effects of NaCl stress and are thus of considerable interest in a context of climate change. Saline waters or salt-affected soils are therefore suitable for Stevia plant growth as well as for stevioside and rebaudioside A production.

Fig. 7. Schema showing the effect of salt stress (16 and 25 d) in *Stevia rebaudiana* Bertoni plants.

Contributions

D. Cantabella: performed the experiments, carried out statistical analysis and was involved in data interpretation and manuscript writing. Abel Piqueras was involved in the micropropagation and acclimatisation of Stevia plants. J.R. Acosta-Motos performed the Proline analysis and was also involved in data interpretation and writing. A. Bernal-Vicente was involved in the chlorophyll, steviol glycoside and lipid peroxidation analyses. JA Hernández: performed the chlorophyll fluorescence and histochemical experiments and was involved in data interpretation and manuscript writing. Pedro Díaz-Vivancos: was involved in the antioxidative enzyme measurements, data interpretation and manuscript writing.

Acknowledgments

PDV would like to thank CSIC and the Spanish Ministry of Economy and Competitiveness for his 'Ramon y Cajal' research contract (RYC-2011-09419), cofinanced by FEDER funds.

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