



# Physiochemical Responses of *Stevia rebaudiana* Bertoni Subjected to Sodium Chloride (NaCl) Salinity and Exogenous Salicylic Acid Application

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## Abstract

Soil salinity is one of the ecological problems limiting plant growth and development, especially in semi-arid and arid regions where water availability is very limited. *Stevia rebaudiana* Bertoni (Asteraceae) is known for its sweetness and non-caloric products (steviol glycosides). It can be considered as a plant for the future sugar industry and human health nutrition. The present work aims to study the modulation of salt stress (NaCl: 80 mM) effects on *Stevia rebaudiana* Bertoni plants by an exogenous application of salicylic acid (0 mM, 0.1 mM and 0.5 mM). The results obtained show that saline stress influences the growth and development of the plant through several metabolism aspects. The exogenous application of salicylic acid to plants subjected to saline stress has improved plant biomass, chlorophyll content, chlorophyll fluorescence, stomatal conductance, antioxidant enzyme activity, MDA content and electrolyte leakage. All these results show a potential role of salicylic acid in strengthening tolerance mechanisms and mitigating physiological and biochemical damage caused by saline stress on *Stevia rebaudiana* Bertoni plant.

**Keywords** Salicylic acid · Salt stress · *Stevia rebaudiana* Bertoni · Growth · Physiological attributes

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# Physiochemische Reaktionen von *Stevia rebaudiana* Bertoni unter Natriumchlorid (NaCl)-Salinität und exogener Salicylsäureapplikation

## Zusammenfassung

Die Versalzung des Bodens ist eines der ökologischen Probleme, die das Wachstum und die Entwicklung von Pflanzen einschränken, besonders in semi-ariden und ariden Regionen, in denen die Wasserverfügbarkeit sehr begrenzt ist. *Stevia rebaudiana* Bertoni (Asteraceae) ist bekannt für ihre Süße und nichtkalorischen Produkte (Steviolglykoside). Sie kann als Pflanze für die künftige Zuckerindustrie und die gesunde Ernährung betrachtet werden. Die vorliegende Arbeit zielt darauf ab, die Modulation der Effekte von Salzstress (NaCl: 80 mM) auf *Stevia rebaudiana* Bertoni-Pflanzen durch eine exogene Applikation von Salicylsäure (0 mM, 0,1 mM und 0,5 mM) zu untersuchen. Die erzielten Ergebnisse zeigen, dass Salzstress das Wachstum und die Entwicklung der Pflanze durch verschiedene Stoffwechsellaspekte beeinflusst. Die exogene Anwendung von Salicylsäure auf Pflanzen, die Salzstress ausgesetzt waren, hat die Pflanzenbiomasse, den Chlorophyllgehalt, die Chlorophyllfluoreszenz, die stomatäre Leitfähigkeit, die antioxidative Enzymaktivität, den MDA-Gehalt und den Elektrolytverlust verbessert. All diese Ergebnisse zeigen eine potenzielle Rolle von Salicylsäure bei der Stärkung von Toleranzmechanismen und der Abschwächung von physiologischen und biochemischen Schäden, die durch Salzstress an der *Stevia rebaudiana* Bertoni-Pflanze verursacht werden.

**Schlüsselwörter** Salicylsäure · Salzstress · *Stevia rebaudiana* Bertoni · Wachstum · Physiologische Eigenschaften

## Introduction

Nowadays, the problem of salinity is considered one of the most important abiotic factors hindering plant growth and productivity, especially in arid and semi-arid regions (Zhu 2001; Rasool et al. 2013; Soliman and El Shaieny 2014). The excess of NaCl in soil or in irrigation water leads to massive production of reactive oxygen species (ROS) in plants (Arif et al. 2020), which generally causes photosynthetic malfunction and other metabolic disorders (Kalhor et al. 2018; Kapoor and Hasanuzzaman 2020). Salt stress affects all physiological processes of the plant; its effects vary according to the type of salinity, salt concentration, species, variety, plant organ and stage of development (Radhouane 2008; Hniličková et al. 2019).

The tolerance to salt stress is therefore a widely sought-after characteristic in plants of agronomic and medicinal interest to expand their cultivation in saline and water deficit regions. Some molecules such as salicylic acid (SA) have recently attracted the attention of the scientific community for their positive effects on the response of plants to abiotic stress. Salicylic acid is generally synthesized by the plant itself and seems to be involved in establishing resistance mechanisms to several environmental stresses (Mutlu et al. 2016; Shakirova et al. 2016; Sharma et al. 2018; Batista et al. 2019). The exogenous application of SA to plants under different stresses has been studied by several researchers and its role in activating germination, growth and development under saline stress has been reported in beans (Ahmad et al. 2018), wheat (Fardus et al. 2018) and tomato (Jahan et al. 2019). Moreover, the use of salicylic acid improves photosynthetic activity, chlorophyll content antioxidant enzymes and mineral nutrition uptake (N, P, K<sup>+</sup>, Ca<sup>2+</sup> and

Mg<sup>2+</sup>) (Anaya et al. 2017; Karlidag et al. 2009). According to Nazar et al. (2011), this phytohormone plays a key role in mitigating damage due to saline stress by regulating certain metabolic and physiological pathways in plants.

*Stevia rebaudiana* Bertoni is a plant that is attracting a lot of interest nowadays because of its high sweetening power and non-caloricity (Samuel et al. 2018). It is considered one of the most important plants of economic interest in the world. The leaves of the plant contain diterpene-glycosides (steviosides and rebaudiosides), which are widely used as a natural non-caloric sweetener especially for diabetics (Shivanna et al. 2013). According to several researchers, this plant is very water demanding and is very sensitive to the salinisation of irrigation water (Zeng et al. 2013; Cantabella et al. 2017; Gerami et al. 2020). Its cultivation in regions with semi-arid and arid climates, which are characterized by saline water, requires control of the plant's responses to saline stress. Few studies reported the effect of salicylic acid on stevia growth and development. The aim of this work is to study the influence of a pre-treatment with salicylic acid on the morphological, physiological and biochemical behaviour of *Stevia rebaudiana* Bertoni plants subjected to saline stress.

## Materials and Methods

### Plant Growth Analysis

The plant material used in this study consists of young seedlings (2 months after sowing) of *Stevia rebaudiana* Bertoni (Fig. 1).



**Fig. 1** Two-months old *Stevia rebaudiana* Bertoni seedlings

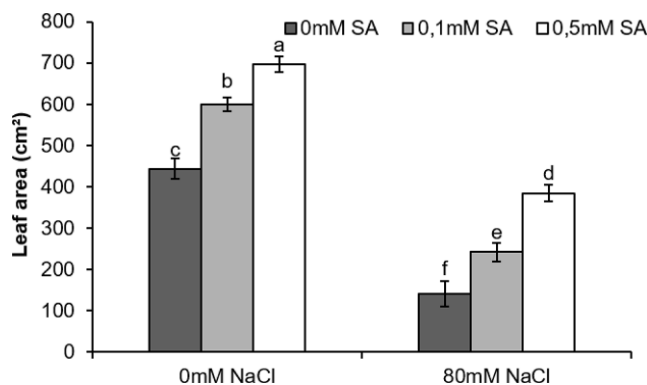
The seeds of *S. rebaudiana* (var. Bertoni) were disinfected for 5 min using NaClO and then washed five times with sterile distilled water. Healthy-looking seeds germinated in honeycombed plates contained peat previously sterilized. After 2 months of germination (Fig. 1). Seedlings of uniform size (six-leaf stage) were selected and transplanted into a 2kg plastic pots (one plant per pot) filled with sand/peat mixture (5/1). The plants were kept well irrigated (administered twice a week, field capacity of the substrate: 150 ml) for 3 weeks prior to application of the treatments. Once a week, the water is replaced by the nutrient solution (Hoagland and Arnon 1950).

The plants were treated with two levels of salicylic acid concentration (0.1 and 0.5 mM) with or without NaCl (80 mM) for 8 weeks. The SA was initially dissolved in ethanol and the concentrations of 0.1 and 0.5 mM were compensated with distilled water with 0.01% Tween-20. The SA solutions were applied exogenously with irrigation water (only once time before the saline treatment). The salt treatment began after 48 h of salicylic acid application and progressed to reach the desired concentration (to avoid osmotic shock).

#### The applied treatments were

- 0 mM NaCl/0 mM AS (T)
- 80 mM NaCl/0 mM AS (N)
- 0 mM NaCl/0.1 mM AS (S1)
- 0 mM NaCl/0.5 mM AS (S2)
- 80 mM NaCl/0.1 mM AS (C1)
- 80 mM NaCl/0.5 mM AS (C2)

The pots corresponding to the different treatments were arranged in a random pattern with twelve replicates per treatment. The experiment was carried out from March to May in a greenhouse (photoperiod of 16 h/8 h (light/dark), temperature: 25.5 °C and a light: 410  $\mu\text{m}^{-2} \text{s}^{-1}$ ) at the Faculty of Sciences Semlalia, Marrakech, Morocco.



**Fig. 2** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on leaf area of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level by using the LSD test

#### Growth Parameters

The shoot height of the plants and their fresh and dry biomass were determined after 2 months of treatment. The weight of the dry matter was measured after 48 h of drying the plant material in the oven at 70 °C with four replicates per treatment.

The leaf area was determined after processing the leaf images with the computer software “Mesurim\_Pro” with four replicates per treatment.

#### Chlorophyll Fluorescence, Stomatal Conductance and Relative Water Content of Leaves

The photochemical efficiency of photosystem II was evaluated by the Fv/Fm ratio, where Fv corresponds to variable fluorescence and Fm to maximum fluorescence. This ratio corresponds to the maximum quantum efficiency of the PSII photochemistry. Measurements were made on mature and healthy leaves using a portable fluorometer (Chlorophyll Fluorometer, OS30P+, OPTI-SCIENCES). A 20 min dark adaptation is applied before each measurement with four replicates per treatment.

The stomatal conductance is measured weekly using a portable porometer (Leaf Porometer LP1989, Decagon Device, Inc., Washington, USA). Measurements are made between 12:00 and 13:00 on two leaves per plant of the same stage with four replicates for each treatment.

Relative water content is the ratio of the water content of the sample at harvest to the maximum water content when the cells are fully turgid (with four replicates per treatment). It is calculated according to the method described by Turner and Begg (1981).

**Table 1** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on the growth parameters of *Stevia rebaudiana* Bertoni after 8 weeks of treatment

	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Shoot height (cm)
<i>T</i>	14.85 ± 0.75b	3.03 ± 0.097b	21.185 ± 0.47c	4.86 ± 0.18b	61.5 ± 0.94b
<i>N</i>	8.8 ± 0.5e	1.95 ± 0.141d	14.43 ± 0.96e	3.01 ± 0.17d	40.5 ± 0.98e
<i>S1</i>	14.97 ± 0.68b	3.31 ± 0.088a	34.45 ± 1.06b	6.76 ± 0.4a	62.75 ± 2.3b
<i>S2</i>	17.58 ± 0.83a	3.42 ± 0.122a	37.37 ± 0.29a	6.61 ± 0.22a	71.5 ± 0.7a
<i>C1</i>	11.01 ± 0.39d	2.09 ± 0.126d	15.84 ± 0.45e	2.95 ± 0.18d	47.1 ± 1.65d
<i>C2</i>	12.52 ± 0.43c	2.45 ± 0.14c	20.5 ± 1.02d	3.82 ± 0.29c	54.75 ± 1.76c

The values represent the average of 4 replicates ± standard deviation. The values of each parameter labeled by different letters are significantly different at the 5% significance level using the LSD test

*T1* Control, *N* treated with 80 mM NaCl, *S1* treated with salicylic acid 0.1 mM, *S2* treated with salicylic acid 0.5 mM, *C1* treated with 80 mM NaCl + salicylic acid 0.1 mM, *C2* treated with 80 mM NaCl + salicylic acid 0.5 mM

## Electrolyte Leakage

The effect of salinity on membrane permeability has been assessed by the percentage of electrolyte leakage (Syeed et al. 2011). Leaf samples (100 mg) were rinsed several times with distilled water and placed in tubes containing 10 ml of distilled water. The electrical conductivities (*C1*) of the solutions were then determined using a conductivity meter after incubation for 24 h at 25 °C and stirring at 100 rpm. A second conductivity (*C2*) of the samples was determined after they were autoclaved at 120 °C for 20 min, followed by stirring for 30 min at 25 °C. The percentage of electrolyte loss was expressed as the ratio of *C1* and *C2* (with four replicates per treatment) (Electrolyte leakage (%)) = (*C1*/*C2*) \* 100.

## Assessment of Membrane Lipid Peroxidation (MDA)

Lipid peroxidation is estimated by determining the quantities of malondialdehyde according to the method of Yin et al. (2009). 100 mg of fresh leaf material is crushed and then homogenised in 0.1% Trichloroacetic acid (TCA). The homogenate is centrifuged at 10,000 g for 15 min. 500 µl of the supernatant is mixed with 1.5 ml of Thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 90 °C for 20 min in a hot-water bath. After stopping the reaction by ice, the samples are centrifuged at 10,000 g for 10 min. The optical density (OD) is read at 532 nm by using a spectrophotometer. The MDA content is determined by using the extinction coefficient 155/Mm/cm.

## Hydrogen Peroxide Content

The H<sub>2</sub>O<sub>2</sub> content of plant leaves was determined according to the method of Velikova et al. (2000). An aliquot of 100 mg of fresh material was crushed in 2 ml of TCA (20%) and then centrifuged at 15,000 g for 15 min at 4 °C. The supernatants were then recovered to determine the H<sub>2</sub>O<sub>2</sub> content of these extracts. 0.5 ml of phosphate phosphate

buffer (10 mM, pH 7) and 1 ml of iodine potassium (1 M) were added to 0.5 ml of the extract. The OD was then determined at 390 nm after 1 h incubation in the dark. The H<sub>2</sub>O<sub>2</sub> content was expressed as nmol H<sub>2</sub>O<sub>2</sub> per g of fresh weight with reference to a standard range established under the same conditions with known H<sub>2</sub>O<sub>2</sub> concentrations.

## Leaf Photosynthetic Pigments

Chlorophyll (a) and (b) concentration of the leaves was measured according to the classical method of Arnon (1949). 100 mg of fresh leaves were crushed with 5 ml of 80% acetone. After centrifugation (10 min at 5000 g) and OD reading at 663 and 645 nm, the quantification of the chlorophyll content (µg/g FM) was determined by using the following relations:

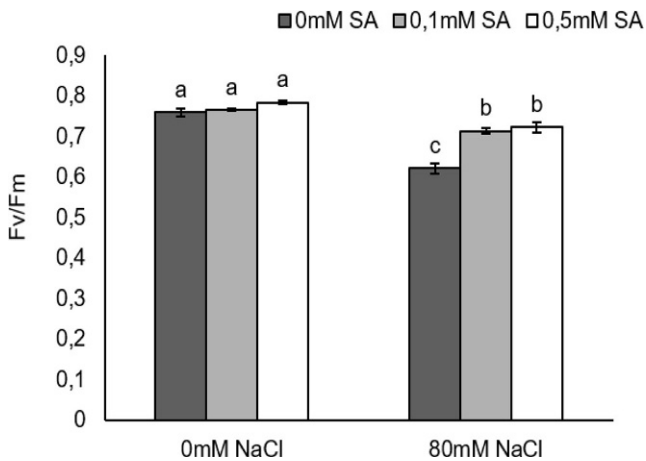
$$\text{Chl(a)}(\text{mg/gfw}) = 12.7 \times \text{DO}_{663} - 2.69 \times \text{DO}_{645}$$

$$\text{Chl(b)}(\text{mg/gfw}) = 22.9 \times \text{DO}_{645} - 4.68 \times \text{DO}_{663}$$

## Antioxidant Enzyme Assays

100 mg of fresh cold leaves are milled in 1.5 ml of phosphate buffer (0.1 M pH 7) containing 5% polyvinylpyrrolidone (pvp) and 1% EDTA. The homogenate is centrifuged at 1200 g for 15 min to recover the supernatant which is stored at -20 °C. The supernatant was used for the determination of soluble protein content and the enzymatic analysis of polyphenoloxidase (PPO), peroxidase (POX) and superoxide dismutase (SOD).

Protein determination is carried out through the Bradford method (1976). 2 ml of the protein extracts are taken from tubes and added to 2 ml of Bradford's reagent. Optical density (OD) is measured at 595 nm. Polyphenoloxidase (PPO) activity is measured according to the method of Oktay et al. (1995). The reaction medium consists of 600 µl catechol (0.1 M), 100 µl enzyme extract and 3 ml phosphate buffer (0.1 M, pH 7) while the enzyme activity of peroxidase (POX) is determined spectrophotometrically using guaia-



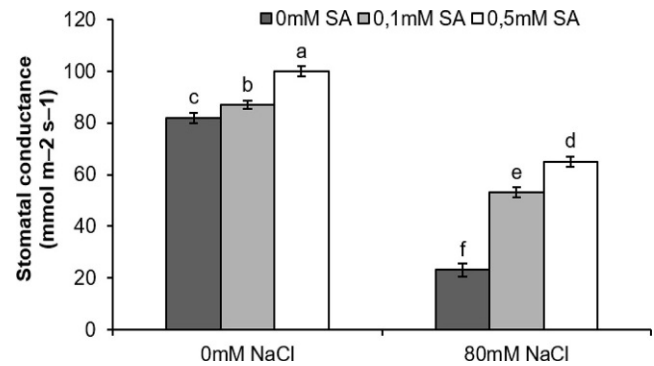
**Fig. 3** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on chlorophyll fluorescence of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

col and hydrogen peroxide ( $H_2O_2$ ) as enzyme substrates. The reaction mixture contains 100  $\mu$ l of the enzyme extract, 300  $\mu$ L of guaiacol (20 mM) and 2 ml of phosphate buffer (0.1 M, pH 6). The reaction is triggered by the addition of 200  $\mu$ l hydrogen peroxide (0.3%  $H_2O_2$ ) and then the kinetics of tetraguaiacol formation is monitored by spectrophotometry at 470 nm (Hori et al. 1997). The activity of superoxide dismutase (SOD) is determined by Beauchamp and Fridovich (1971) by measuring the capacity of this enzyme to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The absorbance of the reaction product is measured at 560 nm.

The catalase assay is carried out according to the method of Montavon et al. (2007), 100 mg of fresh leaf tissue is homogenised in 50 mmol phosphate buffer (pH 7) containing 150 mmol NaCl and 0.5 mmol EDTA. One unit of CAT activity corresponds to the amount of enzyme required to reduce 1  $\mu$ mol hydrogen peroxide ( $H_2O_2$ ) per minute.

### Statistical Analysis

Statistical analysis of the data is carried out by analysis of variance (ANOVA) to determine differences between treatments. The LSD test is used to compare the averages of the different treatments to the 5% threshold using SPSS for Windows software.



**Fig. 4** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on stomatal conductance of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

## Results

### Growth Parameters

Measurements of growth parameters of *Stevia rebaudiana* Bertoni under the different concentrations of salicylic acid and NaCl are shown in Table 1. Fresh weight of the aerial and root parts as well as the stem length of *Stevia rebaudiana* Bertoni were significantly reduced by 41, 32 and 34% respectively in conditions of stress. Furthermore, the exogenous application of salicylic acid improved significantly the biomass of plants in the presence or absence of saline stress (80 mM). It should also be noted that the 0.5 mM concentration of salicylic acid gave good results compared to the 0.1 mM concentration of salicylic acid under saline stress conditions.

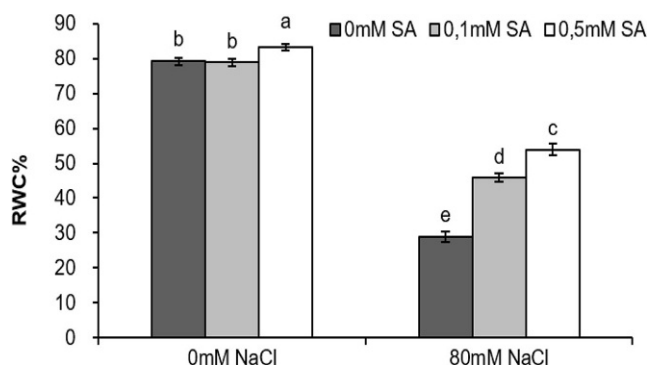
### Leaf Area

The effect of NaCl results in a significant reduction (32%) of the leaf area in plants of *Stevia rebaudiana* Bertoni (Fig. 2). Also, the addition of SA to non-stressed plants caused a significant increase in leaf area, especially the 0.5 mM concentration. In addition, the combination of NaCl (80 mM) with salicylic acid (0.1 and 0.5 mM) induces a significant improvement (41 and 63% respectively) of the leaf area compared to the stressed plants not treated with SA.

### Chlorophyll Fluorescence

To estimate the effect of salicylic acid on the photosynthetic activity of *Stevia rebaudiana* Bertoni under saline stress (80 mM), the photochemical efficiency of photosystem II (Fv/Fm) was evaluated (Fig. 3). It can be seen that this pa-





**Fig. 5** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on relative water content of *Stevia rebaudiana* Bertonii after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

parameter underwent a significant regression under the influence of salt stress. This reduction is significantly smaller in plants treated with salicylic acid in the presence of NaCl.

### Stomatal Conductance

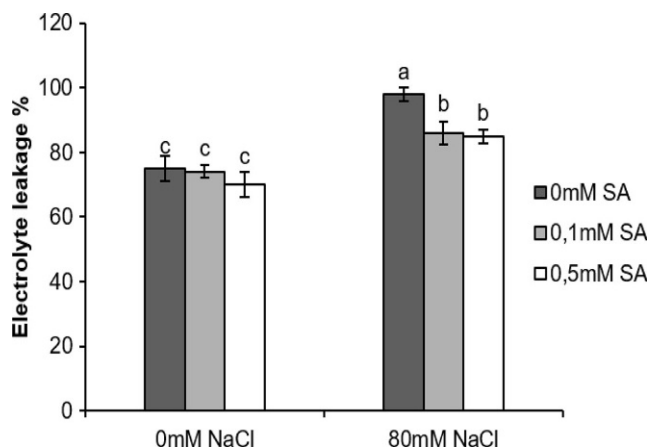
The study of stomatal conductance (Fig. 4) was significantly reduced under the effect of salt stress (72% compared to control). However, under treatment with salicylic acid (0.5 mM), non-stressed plants recorded an 18% increase compared to control plants. Yet, the combination of SA with NaCl reduced the harmful effects of saline stress on the plants gas exchanges, it was also noted that the highest values were obtained with the 0.5 mM concentration of SA.

### Relative Water Content (RWC)

According to the analysis of Fig. 5, the relative water content of the leaves of *Stevia rebaudiana* Bertonii is very high (79%) in controlled plants, while the values recorded in stressed plants with 80 mM NaCl are very low (29%). Moreover, addition of SA caused a significant increase ( $<0.05$ ) of relative water content, especially with the 0.5 mM concentration. These results show that salicylic acid positively influenced the water uptake by stressed plants.

### Electrolyte Leakage

Statistical analysis of the results in Fig. 6 shows that salt stress caused a significant increase in electrolyte leakage in all stressed plants with or without salicylic acid. Plants treated with salicylic acid and NaCl showed a low loss of electrolytes compared to plants treated with NaCl alone.



**Fig. 6** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on the electrolyte leakage of *Stevia rebaudiana* Bertonii after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

### Membrane Lipid Peroxidation

Under the salt stress conditions, the accumulation of MDA (Fig. 7) is very high (35 nmol/g fw) compared to control plants (15 nmol/g FM). The application of salicylic acid at 0.1 and 0.5 mM to the stressed plants decreased significantly ( $p < 0.05$ ) the MDA level.

### Hydrogen Peroxide Content

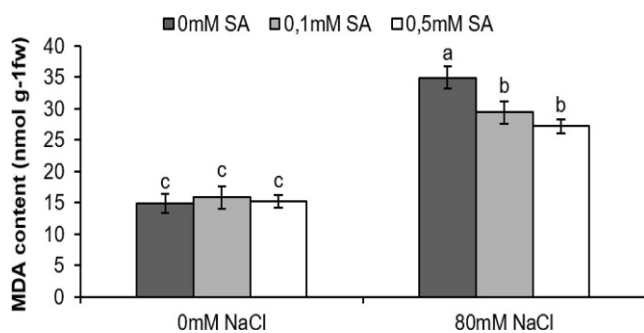
The results relative to the effect of NaCl on the  $H_2O_2$  content of the plants of *Stevia rebaudiana* Bertonii are presented in Fig. 8, the salinity of the growing medium caused an increase in the quantity of  $H_2O_2$  in the leaves, and this increase is less intense when salicylic acid is applied under salt stress. The interaction between salicylic acid and NaCl has a significant effect on the  $H_2O_2$  content.

### Leaf Photosynthetic Pigments

Analysis of the chlorophylls (a) and (b) contents (Fig. 9) shows that under salt stress, *Stevia rebaudiana* Bertonii plants experienced a remarkable and statistically significant drop compared to control plants of about 51% for chlorophyll (a) and 56% for chlorophyll (b). On the other hand, stressed plants treated with SA showed significantly higher levels of chlorophyll (a) and (b) compared to plants treated with saline stress alone.

### Protein Content

The exploitation of the results of Fig. 10 allows to underline that salicylic acid has no significant effect on the protein



**Fig. 7** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on MDA content of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

content of *Stevia rebaudiana* Bertoni, it was also detected that salinity causes a significant increase ( $p < 0.05$ ) of the foliar protein content, while the addition of salicylic acid at 0.1 and 0.5 mM to the plants under stress conditions (80 mM) induced a significant decrease ( $p < 0.05$ ) of the protein content (equal to 41 and 36% respectively).

## Antioxidant Enzymes Activity

### Polyphenoloxidases (PPO)

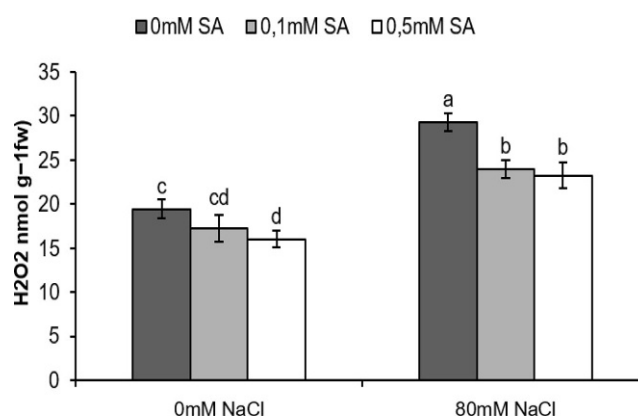
The activity of polyphenoloxidase (Fig. 11) was significantly influenced by NaCl (80 mM) and/or salicylic acid, a significant increase was observed in plants exposed to salt stress. Indeed, the application of salicylic acid at 0.1 and 0.5 mM on *Stevia rebaudiana* Bertoni plants under stress conditions increased the activity of this enzyme.

### Peroxidases (POX)

Fig. 12 illustrates the effect of saline treatment and/or salicylic acid on the plants of *Stevia rebaudiana* Bertoni, it was found that there is an increase (34.37%) in the activity of these enzymes in the presence of saline stress compared to the non-stressed control. However, in the presence of salicylic acid, the activity of peroxidase was significantly increased in the presence or absence of saline stress.

### Superoxide Dismutase (SOD)

The data presented in the figure below (Fig. 13) show that salinity significantly increased SOD activity by approximately 32% compared to control, treatment with salicylic acid alone had no effect on the control plants, on the other hand, under saline stress, salicylic acid at 0.1 and 0.5 mM



**Fig. 8** Influence of salicylic acid (0, 0.1 and 0.5 mM) and/or NaCl (0 and 80 mM) on H<sub>2</sub>O<sub>2</sub> content of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

significantly increased SOD with percentages of 16 and 22% respectively compared to the stressed plants.

### Catalases (CAT)

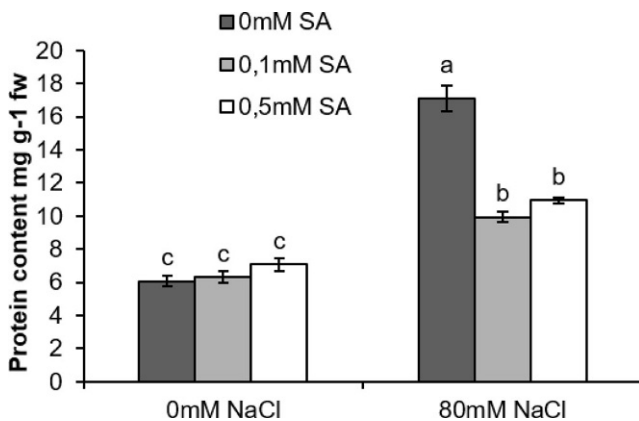
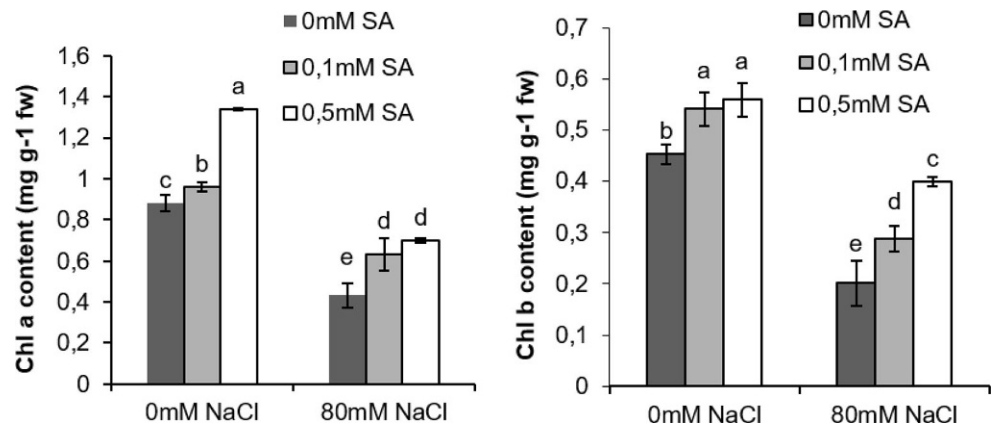
The catalase activity in the leaves is significantly affected by salinity and salicylic acid (Fig. 14), an increase in this enzyme is observed in stressed plants compared to the control plants, however, the combination of salicylic acid with NaCl can lead to a higher elevation compared to the other treatments.

## Discussion

Salicylic acid is an endogenous growth hormone, which can improve the plant's response to stress conditions (biotic and abiotic) by increasing the resistance of the plant to System Acquired Resistance (SAR) (Khan et al. 2015). Several researchers have demonstrated the interest of using this molecule to improve plant tolerance against environmental constraints such as salt stress (Simaei et al. 2012; Miao et al. 2020; Souana et al. 2020).

The results obtained show that salt affects negatively the weight of the fresh and dry matter and the stem length as well as the leaf area of the plant. These data are similar to those observed by Zhu (2001) who reported that the reduction in the growth of the aerial vegetative apparatus is an adaptive capacity necessary for the survival of plants exposed to abiotic stress. Indeed, this effect on the development allows the plant to accumulate energy and nutrient reserves to combat stress. Reduction in the number of leaves and leaf area of plants could be explained by the early senescence of leaves with a decrease in the mitotic

**Fig. 9** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on chlorophyll (a) and (b) contents of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test



**Fig. 10** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on protein content of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

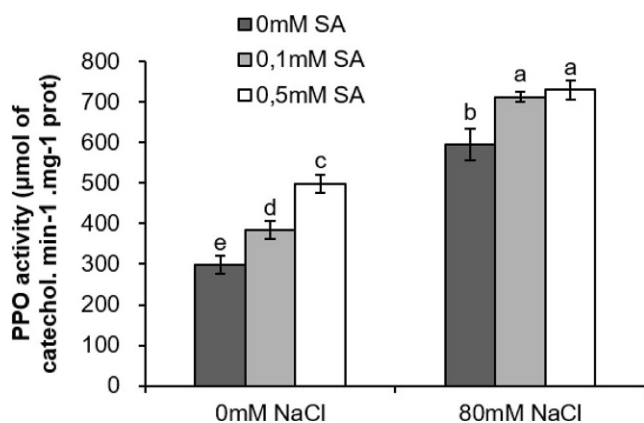
activity of epidermal cells. This poor development is generally due to the adaptive mechanism of plants that aims to limit plant leaf transpiration during adverse conditions (Ghanem et al. 2008; Santos et al. 2016). According to Ordóñez et al. (2018), stevia can tolerate water with electrical conductivity up to 2.0 mS cm<sup>-1</sup>, and that at higher salinity ( $\geq 80$  mM), the biomass yield was significantly reduced (Mahajan et al. 2020).

The exogenous application of salicylic acid improved the biomass of *Stevia rebaudiana* Bertoni seedlings in the presence or absence of NaCl (80 mM), which is consistent with the results in maize (Gunes et al. 2007), strawberry (Karlidag et al. 2009), tomato (Mimouni et al. 2016) and rice (Jini and Joseph 2017), which demonstrated that pre-treatment with salicylic acid under conditions of salt stress has positive effects on plant growth parameters. This effect might be related to the effect of salicylic acid in inhibition of Cl<sup>-</sup> and Na<sup>+</sup> accumulation and increased the K<sup>+</sup> content under salt stress (Jini and Joseph 2017).

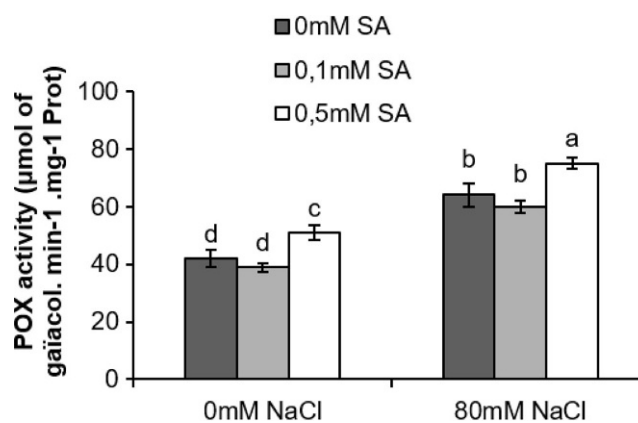
In this work, chlorophyll fluorescence, specifically the maximum efficiency of PSII (Fv/Fm), was used to evaluate the impact of salicylic acid and/or salt stress on the photosynthetic activity of *Stevia rebaudiana* Bertoni. The Fv/Fm ratio was negatively influenced by salt in this experiment. This reduction generally implies damage in the photochemical efficiency of PSII (Mehta et al. 2010). However, treatment with salicylic acid had a positive effect on the Fv/Fm ratio. Methenni et al. (2018) also reported a similar increase in photosynthetic activity antioxidant system under saline condition. Several studies have reported that pre-treatment with salicylic acid restored the functioning of the photosystem II reaction centre by restoring the activity of protein D1 and protein kinase (Zhao et al. 2011; Anaya et al. 2017).

Determination of stomatal conductance is one of the simplest and most non-destructive techniques, indicating the plant's foliar transpiration rate. In this experiment, stomatal conductance decreased significantly under the stress conditions. These results are consistent with those of Mahlooji et al. (2018) who reported that plant survival under stress conditions requires a reduction in gas exchange (with stomatal closure), which leads to a drop in the rate of CO<sub>2</sub> assimilation necessary for photosynthesis (Schulze and Hall 1982). Addition of SA improved stomatal conductance under both saline and non-saline conditions. These results are consistent with those presented by Ma et al. (2017) who reported that under saline stress, salicylic acid improves stomatal density and stomatal conductance, and this is due to the fact that salicylic acid limits ethylene formation by inhibiting the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (Lotfi et al. 2020). This improvement results in a reduction in the toxic effects of salt stress. A decrease in RWC is considered to be one of the first symptoms of water shortage in plant tissues (Valentovic et al. 2006). The results obtained showed that the relative water content of the leaves decreased significantly in stressed plants. According to Khator et al. (2020), this decrease can





**Fig. 11** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on PPO of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test



**Fig. 12** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on POX of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

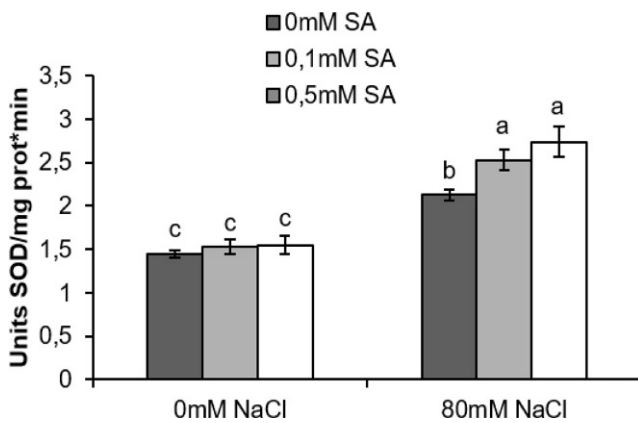
be explained by the low turgidity due to the limited availability of water for the cell extension process. These results also agree with those found by Karlidag et al. (2009) who found that salinity negatively affects the relative water content in strawberries. In the presence of salicylic acid and under salt stress conditions, the relative water content was significantly increased in the leaves of *Stevia rebaudiana* Bertoni, these results are in agreement with those found by Sabzmejdani et al. (2020) on Kentucky bluegrass.

The disruption of photosynthesis is also due to the reduction in the content of chlorophyll pigments (a and b) under conditions of saline stress. In the presence of saline treatment, *Stevia rebaudiana* reduced the content of its chlorophyll pigments. Zhang et al. (2018) reported that NaCl causes a drop in photosynthetic pigment content in Castor bean. This reduction is attenuated by the exogenous application of salicylic acid that may have a protective effect on chloroplasts and the thylakoid membrane against the oxidative effects of salt stress as demonstrated by Bose et al. (2017).

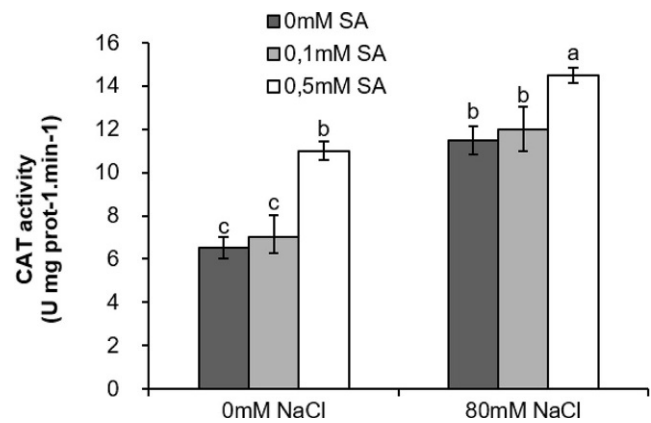
Membrane permeability and MDA content are considered among the methods for assessing oxidative effects on plants (Astaneh et al. 2019). Under salt stress, a high accumulation of Na<sup>+</sup> and Cl<sup>-</sup> ions in plant cells causes a disturbance in the membrane permeability of the cells (Kaya et al. 2018). Application of salicylic acid (0.1 or 0.5mM) on *Stevia rebaudiana* Bertoni plants, significantly reduced the loss of electrolytes and the accumulation of MDA, a marker of membrane lipid peroxidation, under stressful conditions. This is consistent with the results of Kaya et al. (2020) in peppers and El-Esawi et al. (2017) in rosemary under salt stress. On the other hand, the H<sub>2</sub>O<sub>2</sub> content was decreased by the addition of salicylic acid, which is consistent with the results found by Alsahli et al. (2019), who showed that sal-

icylic acid contributes to the neutralization of H<sub>2</sub>O<sub>2</sub> caused by the stress, which explains the potential role of salicylic acid in protecting the cell membrane against the adverse effects of salt stress.

According to the results found, *Stevia rebaudiana* Bertoni increased the activity of the antioxidant enzymes specifically peroxidase (POX), polyphenoloxidase (PPO), superoxide dismutase (SOD) and catalase (CAT) under saline stress. The increased activity of these enzymes plays a very important role in the elimination of ROS under saline conditions (Ahanger and Agarwal 2017). Induction of these enzymes in plants has also been reported in several species in response to saline stress (Vighi et al. 2017; Ghaderi et al. 2018; Srivastava and Shahi 2018; Tahjib-Ul-Arif et al. 2018). Our results also show that the treatment of plants subjected to salt stress with salicylic acid clearly helped the plants to further increase the activity of the enzymes studied compared to plants treated only with salt. Recently, a study conducted on radish (Bukhat et al. 2020), reported that the application of salicylic acid induced an increase in antioxidant enzymes with the aim of strengthening the antioxidant system against reactive oxygen species (ROS) such as superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH<sup>-</sup>), and singlet oxygen (1 O<sub>2</sub>), confirming the results found in this study. Thus, salinity tolerance in *stevia rebaudiana* was associated with the induction of an antioxidant enzyme system by the application of salicylic acid, which improved the plant performance as well as protected against various damages caused by salt stress.



**Fig. 13** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on SOD of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test



**Fig. 14** Influence of salicylic acid (0, 0.1 and 0.5mM) and/or NaCl (0 and 80mM) on CAT of *Stevia rebaudiana* Bertoni after 8 weeks of treatment. The values represent the mean of 4 replicates  $\pm$  standard deviation. Bars of each parameter labeled by different letters are significantly different at 5% significance level using the LSD test

## Conclusion

The exposure of *Stevia rebaudiana* Bertoni to salt stress influenced all the morphological, physiological and biochemical parameters studied through the reduction of the biomass, the disturbance of the photosynthetic apparatus and the deterioration of the plant's cell membrane. An exogenous pre-treatment with salicylic acid improves the plant's tolerance to saline stress. This positive effect of salicylic acid can be explained by the enhanced growth and improved pigment content, relative water content and photosynthesis. In addition, salicylic acid, especially the 0.5 mM SA concentration, plays a very important role in reducing the oxidative effects of stress through the activation of a range of antioxidant enzymes. We can therefore consider salicylic acid to be one of the techniques that can be used to improve the tolerance of this plant to saline conditions.

**Conflict of interest** I. Janah, A. Elhasnaoui, O. Issa Ali, K. Lamnai, S. Aissam and K. Loutfi declare that they have no competing interests.

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