Salt stress by NaCl alters the physiology and biochemistry of tissue culture-grown *Stevia rebaudiana* Bertoni

Rabia JAVED\(^{1,2,*}\), Ekrem GÜREL\(^{2}\)

1. Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
2. Department of Biology, Faculty of Arts and Sciences, Abant İzzet Baysal University, Bolu, Turkey

**Abstract:** This study reports the response to salinity stress (100 mM, 200 mM, and 300 mM NaCl concentration) exposure of the commercially valuable medicinal plant *Stevia rebaudiana* during micropropagation for 4 weeks. The significant enhancement of physiological parameters, steviol glycosides (SGs), i.e. rebaudioside A and stevioside, as examined by high-performance liquid chromatography, and nonenzymatic antioxidant activities, i.e. total phenolic content, total flavonoid content, total antioxidant capacity, total reducing power, and DPPH-free radical scavenging activity, was observed during the shoot formation process under up to 100 mM NaCl stress. Callus formation produced similar results regarding physiology and antioxidant assays, except that it produced merely a negligible amount of SGs. Contrarily, root formation showed marked susceptibility to 100 mM, 200 mM, and 300 mM NaCl concentrations and reduced growth parameters, sweetening compounds, and secondary metabolites. Hence, NaCl plays the role of abiotic stress elicitor, causing accumulation of reactive oxygen species and thus altering metabolic processes and physiology of *Stevia* under in vitro culture conditions.

**Key words:** Antioxidant activities, in vitro culture, NaCl stress, physiology, *Stevia rebaudiana*, steviol glycosides

1. Introduction

*Stevia rebaudiana*, commonly called “honey leaf”, is a member of the family Asteraceae (sunflower) and is indigenous to South America, specifically Brazil and Paraguay (Soejarto, 2002). It is famous for the production of natural sweeteners, i.e. steviol glycosides (SGs), including rebaudioside A (Reb A), rebaudioside C, stevioside (ST), dulcoside, and steviol bioside, which possess 300 times more sweetness than sucrose (Wölwer-Rieck, 2012). These bioactive molecules make *Stevia* protective against cancer, diabetes, high blood pressure, obesity, and inflammation (Liu et al., 2003; Dey et al., 2013). Large amounts of phenolics, flavonoids, and other metabolites have also been detected in dry leaves of *S. rebaudiana* (Tadhani et al., 2007). Due to the presence of nonmutagenic and nontoxic secondary metabolites, *S. rebaudiana* has gained paramount importance as an alternative noncaloric sweetening crop in many countries (Bopp and Price, 2001; Sekihashi et al., 2002).

Since the germination percentage of *Stevia* seeds is very low and its vegetative propagation by stem cuttings is not productive, researchers have adapted micropropagation for producing large amounts of true-to-type progenies (Khalil et al., 2014). Tissue culture has long been used to increase the SG content in the leaves of *S. rebaudiana* by employing different types of stresses (Bondarev et al., 2003; Sivaram and Mukundan, 2003; Thiyagarajan and Venkatachalam, 2012; Gupta et al., 2014; Pandey and Chikara, 2015; Gupta et al., 2016). The growth of shoots, SGs, phenolics, flavonoids, and antioxidant activities rise on exposure to abiotic stress agents such as ZnO and CuO nanoparticles, different plant growth regulators (PGRs), and hydrogen peroxide in optimized environmental conditions (Rafiq et al., 2007; Javed et al., 2017a, 2017b, 2017c). Moreover, the effect of ZnO and CuO nanoparticles on callus cultures of *S. rebaudiana* has been reported (Javed et al., 2017d). Javed et al. (2017e) studied the comparison of stimulation of physiological and biochemical parameters in shoot cultures of *S. rebaudiana* under the stress of capped ZnO and CuO nanoparticles (ZnO-PEG, CuO-PEG, ZnO-PVP, CuO-PVP) and uncapped ZnO and CuO. The stress elicitors function in accumulation of reactive oxygen species (ROS) and change of metabolism until the maximum threshold is achieved; thereafter, phytotoxicity causes damage to plant cells (Badran et al., 2015; Soufi et al., 2016; Javed et al., 2017a, 2017b, 2017c, 2017d).
Various studies of salinity stress on *S. rebaudiana* have been reported previously. For instance, Gupta et al. (2014) reported the effect of NaCl and Na₂CO₃ on the callus and suspension culture of *S. rebaudiana* for SG production. Additionally, the influence of NaCl, Na₂CO₃, proline, and PEG on SG production of *S. rebaudiana* was studied by Gupta et al. (2016). Furthermore, the expression level of vital genes in the *S. rebaudiana* biosynthetic pathway was studied along with growth parameters and SG contents, and NaCl was found as an enhancer of gene transcription of the SG biosynthetic pathway (Pandey and Chikara, 2015). The gene expression, morphological parameters, and biochemistry of compounds were also evaluated in *S. rebaudiana* by Fallah et al. (2017), producing remarkable effects.

Zeng et al. (2013) evaluated oxidative enzyme activities (superoxide dismutase, peroxidase, and catalase), Reb A/ST ratio, and K⁺/Na⁺ ratio in field-grown *Stevia* plantlets in response to salt stress. The effects of salinity stress on *Stevia* plants grown in vitro were previously reported by Pandey and Chikara (2014) and Rathore et al. (2014). Their studies reported changes in growth, chlorophyll content, proline accumulation, and protein and sugar contents. Moreover, the influence of salt and drought stress on *S. rebaudiana* growth dynamics was revealed by Mubarak et al. (2012). A field study of NaCl stress in *S. rebaudiana* revealing effects on growth, photosynthetic pigments, diterpene glycosides, and ion content in roots and shoots was performed by Shahverdi et al. (2017). Furthermore, Cantabella et al. (2017) studied salt effects on mineral nutrition along with antioxidative metabolism and SG contents in *S. rebaudiana* under in vitro conditions. However, none of these previous studies elucidated the effect of salt stress on nonenzymatic antioxidative activities. Hence, the aim of the current study is to examine the differential effects of NaCl stress on different physiological parameters (including amount of callus; fresh weight (FW) and dry weight (DW) of callus; length of plantlets, shoots, and roots; number of nodes and leaves; DW of shoots produced by shoot formation). This was done for callus formation. However, no PGR was added in the case of shoot formation. The experiment was conducted in triplicate in a growth room chamber having a 16-h light/8-h dark photoperiod, provided by cool-white fluorescent lighting of 35 μmol m⁻¹ s⁻¹ irradiance and 24 ± 1 °C temperature at 55%–60% relative humidity. Each treatment had 15 explants that were cultivated for the required time period. The callus was harvested after 45 days while shoots were harvested after 30 days of cultivation. Finally, the physiological parameters involving amount of callus, FW of callus, and DW of callus were observed regarding callus formation. Similarly, the mean length of shoots, mean number of nodes and leaves, and FW and DW of shoots produced by shoot formation were measured. Furthermore, the developed shoots were inoculated in the root formation medium (without any PGRs) having 15 shoots per treatment and different NaCl concentrations for 4 weeks in the growth chamber under similar conditions as described above.

### 2. Materials and methods

#### 2.1. Preparation of medium containing NaCl for callus, shoot, and root formation

Murashige and Skoog (MS) medium (1962) and 3% (w/v) sucrose were used for the preparation of culture medium. A total of 4 treatments were prepared containing 0 mM, 100 mM, 200 mM, and 300 mM NaCl. The pH of the media was adjusted to 5.7–5.8 and plant agar [0.8% (w/v)] was added for solidification of media. The culture media were then autoclaved for 15 min under a pressure of 1.06 kg cm⁻² at 121 °C temperature.

#### 2.2. Growth conditions of callus, shoot, and root formation

Seeds of *S. rebaudiana* were purchased from Polisan Tarım, İstanbul, Turkey. According to the method of Javed et al. (2017b, 2017d), the seeds were cultured on basal MS medium after being disinfected with 0.1% (w/v) mercuric(II) chloride (HgCl₂) for 15 min. The leaf explants and axillary shoot nodes were excised from 4-week-old seedlings and incubated in media having PGRs: a combination of 0.5 mg/L kinetin (KN) and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) along with different concentrations of NaCl, i.e. 100, 200, and 300 mM. This was done for callus formation. However, no PGR was added in the case of shoot formation. The experiment was conducted in triplicate in a growth room chamber having a 16-h light/8-h dark photoperiod, provided by cool-white fluorescent lighting of 35 μmol m⁻¹ s⁻¹ irradiance and 24 ± 1 °C temperature at 55%–60% relative humidity. Each treatment had 15 explants that were cultivated for the required time period. The callus was harvested after 45 days while shoots were harvested after 30 days of cultivation. Finally, the physiological parameters involving amount of callus, FW of callus, and DW of callus were observed regarding callus formation. Similarly, the mean length of shoots, mean number of nodes and leaves, and FW and DW of shoots produced by shoot formation were measured. Furthermore, the developed shoots were inoculated in the root formation medium (without any PGRs) having 15 shoots per treatment and different NaCl concentrations for 4 weeks in the growth chamber under similar conditions as described above.

#### 2.3. Extraction and analysis of steviol glycosides

Steviol glycosides were extracted from the calli and leaves of in vitro regenerated shoots and plantlets grown under NaCl stress (Javed et al., 2017b). In this process, the calli, shoots, and plantlets propagated from all different treatments were carefully washed with sterile distilled water. Then the plant material was soaked on Whatman filter paper No. 1 and dried in an oven at 60 °C for 48 h. Analysis of steviol glycosides was performed by carrying out high-performance liquid chromatography (HPLC) of the samples. According to Javed et al. (2017b), the procedure involved addition of 20 mg of sample from each treatment to 1 mL of 70% (v/v) methanol in a microcentrifuge tube. After incubation in an ultrasonic bath at 55 °C for 15 min, samples were centrifuged at 25 °C.
and 12,000 rpm for 10 min. The pellet was discarded and supernatant was filtered using 0.22-µm PTFE Millipore syringe filters. Finally, HPLC analysis was performed by running all of the samples in triplicate.

According to Javed et al. (2017b), chromatography was performed using an autosampler (WPS-3000-SL Dionex Semi Prep Autosampler) injecting 10 µL of each sample, a binary pump (LPG 3400SD Dionex) solvent delivery system working at a flow rate of 0.8 mL min⁻¹, and a dual wavelength absorbance detector operating at 210 and 350 nm (MWD-3100 Dionex UV-Vis Detector). The column, an Inertsil ODS-3 (GL Sciences Inc., Japan) of 150 × 4.6 mm in length with 5 µm particle size, was kept warm at 40 °C in a column oven system (TCC-3000SD Dionex). At the end, isocratic flow was performed with an acetonitrile and 1% (w/v) phosphoric acid buffer mixture (68:32) for 20 min.

2.4. Preparation of extract and antioxidant assays

According to Javed et al. (2017b, 2017d), the calli and leaf extracts of S. rebaudiana were prepared by drying the calli and leaves, and then taking 0.1 g of their fine powder obtained under different NaCl concentrations. Methanol in an amount of 500 µL was used for the dissolution of powder. It was vortexed for 5 min and then sonicated for 30 min followed by 15 min of centrifugation at 10,000 rpm. The pellet was discarded and supernatant was stored to evaluate different antioxidant activities.

2.4.1. Determination of total phenolic content

The method of Jafri et al. (2014) was performed with slight modifications to estimate the total phenolic content in callus and leaf extracts of Stevia. The process involved transfer of an aliquot of 20 µL (4 mg/mL) of dimethyl sulfoxide (DMSO) stock solution of each sample to the respective well of a 96-well plate and then the addition of 90 µL of Folin–Ciocalteu reagent to it. The plate was incubated in darkness for a period of 1 h. All samples were run in triplicate and their absorbance was obtained at 700 nm using a Biotek ELX800 microplate reader. Ascorbic acid was used as the standard and the results were expressed as µg ascorbic acid equivalent per mg (µg AA/mg).

2.4.2. Determination of total flavonoid content

The aluminum chloride colorimetric method of Jafari et al. (2014) was performed after slight modifications to determine the total flavonoid content of different callus and leaf extracts of Stevia. First 10 µL of 10% (w/v) aluminum chloride, 10 µL of 1.0 M potassium acetate, and 160 µL of distilled water were added to an aliquot of 20 µL (4 mg/mL) of DMSO stock solution of each sample contained in the respective well of 96-well plates. It was kept at room temperature for 30 min. The samples were run in triplicate and their absorbance was measured at 415 nm using a Biotek ELX800 microplate reader. Quercetin was used as the standard and the results were expressed as µg quercetin equivalent per mg (µg QE/mg).

2.4.3. Determination of total antioxidant capacity

Total antioxidant capacity was evaluated by the procedure of Jafari et al. (2014) after slight modifications. An aliquot of 100 µL from the stock solution of each sample (4 mg/mL in DMSO) was mixed with 900 µL of reagent solutions containing 0.6 M sulfuric acid, 4 mM ammonium molybdate, and 28 mM sodium phosphate. The reaction mixture was incubated at 95 °C for 90 min and then cooled at room temperature. All samples were run in triplicate and their absorbance was measured at 695 nm using a Biotek ELX800 microplate reader. Ascorbic acid was used as the standard and the results were expressed as µg ascorbic acid equivalent per mg (µg AA/mg).

2.4.4. Determination of total reducing power

Total reducing power of samples was calculated according to the procedure of Jafari et al. (2014) after slight modifications. First 100 µL of each sample (4 mg/mL in DMSO) was mixed with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% w/v potassium ferricyanide. The resulting mixture was incubated at 50 °C for 20 min. Thereafter, the reaction was acidified with 200 µL of 10% w/v trichloroacetic acid and centrifugation was performed at 3000 rpm for 10 min. The pellet was discarded and the obtained supernatant (150 µL) was mixed with 50 µL of 0.1% w/v ferric chloride solution. All samples were run in triplicate and their absorbance was measured at 700 nm using a Biotek ELX800 microplate reader. Ascorbic acid was used as the standard and the results were expressed as µg ascorbic acid equivalent per mg (µg AA/mg).

2.4.5. Determination of DPPH-free radical scavenging activity

Since the overproduction and accumulation of free radicals is damaging to plant cells, the ability of antioxidants produced to prevent oxidative damage was elucidated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. This assay was performed according to the protocol of Haq et al. (2012) after slight modifications. First 10 µL (4 mg/mL) of Stevia callus and leaf extracts was mixed with 190 µL of DPPH (0.004% w/v in methanol). The resulting reaction mixture was incubated in darkness for a period of 1 h. All samples were run in triplicate and their absorbance was measured at 515 nm wavelength using a Biotek ELX800 microplate reader. Ascorbic acid was used as the standard and the results were expressed as µg ascorbic acid equivalent per mg (µg AA/mg).

% inhibition of test sample = % scavenging activity = (1 – A_{so}/A_{sc}) × 100

Here, A_{so} indicates the absorbance of DPPH solution with the sample, and A_{sc} is the absorbance of only DPPH solution. The IC_{50} was calculated by using Table curve software 2D Ver. 4.
2.5. Statistical analysis
The design of experiments was randomized and the statistical analysis of data was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical differences were determined using ANOVA, and the significance of differences between means ± standard error (SE) values was obtained using Duncan's multiple range tests performed at P < 0.05.

3. Results
3.1. Determination of physiological parameters
Table 1 gives the detailed comparison of amount of callus (cm), FW of callus (g), and DW of callus (g) in MS medium provided with 0 mM, 100 mM, and 200 mM NaCl. The data for 300 mM NaCl concentration are absent because the calli died at this concentration. The amount, FW, and DW of calli were revealed to be significantly higher under the control and 100 mM of NaCl stress, followed by 200 mM NaCl stress. Figure 1 clearly shows all of these physiological parameters after 4 weeks of callus formation.

Regarding shoot formation, Table 2 shows that the mean length of shoots (cm), number of nodes and leaves, FW of shoots (g), FW of leaves (g), FW of stems (g), DW of shoots (g), DW of leaves (g), and DW of stems (g) were significantly higher under 100 mM of NaCl stress, i.e. 4.1 cm, 4.6, 13, 0.43 g, 0.30 g, 0.09 g, 0.03 g, 0.02 g, and 0.02 g, respectively. These results were followed by control treatment results, i.e. 2.83 cm, 2.66, 10, 0.16 g, 0.22 g, 0.03 g, 0.02 g, 0.01 g, and 0.01 g on a respective basis. The shoots died under 200 mM and 300 mM salt stress. The data of Table 1 are clearly understandable from Figure 2.

The physiological parameters for root formation were also analyzed, and it is revealed from Table 3 that the length of plantlets and roots (17 cm and 1.83 cm); number of roots, nodes, and leaves (7.33, 12.33, and 26.6); FW of plantlets, leaves, and stems (1.02 g, 0.38 g, and 0.22 g); and DW of plantlets, leaves, and stems (0.15 g, 0.06 g, and 0.04 g) were significantly higher in the control treatment. The data of the control treatment were followed by 100 mM, 200 mM, and 300 mM NaCl concentrations, respectively. Figure 3 shows dense roots in the control treatment and then a continuous decline in root formation from 100 mM to 200 mM and 300 mM of NaCl stress.

3.2. Determination of steviol glycosides
The steviol glycosides (Reb A and ST) were not obtained from the grown calli. However, the shoots and regenerants possessed significantly greater contents of SGs, as shown in Figure 4 and 5. In the case of shoot formation, the highest amount of Reb A and ST was seen in the shoots raised under 100 mM of NaCl stress, i.e. 2.04 and 1.73, respectively. The control treatment produced 1.27 and 1 amounts of Reb A and ST, respectively. The regenerants produced higher amounts of Reb A and ST under the control treatment, i.e. 3.59 and 0.84, respectively. It was followed by the steviol glycosides obtained under 100 mM, 200 mM, and 300 mM on a respective basis.

3.3. Determination of antioxidant activities
The antioxidant activities in calli were measured and it was found that antioxidant activities were possessed by the calli grown under control, 100 mM, and 200 mM NaCl stress as shown in Table 4. Calli were not formed under 300 mM NaCl and the leaf explants were found to be dead. A significantly higher amount of all antioxidant activities, i.e. TPC, TFC, TAC, TRP, and DPPH-free radical scavenging activity, was revealed by the calli obtained under 100 mM NaCl, followed by control treatment and then 200 mM NaCl concentration.

All antioxidant activities, i.e. TPC, TFC, TAC, TRP, and DPPH-free radical scavenging activity, were higher under the stress of 100 mM NaCl as shown in Table 5. The TPC, TFC, TAC, TRP, and DPPH-free radical scavenging activities at 100 mM of NaCl stress were 7.48 µg/mg of DW, 5.04 µg/mg of DW, 14.63 µg/mg of DW, 10.39 µg/mg of DW, and 49.48%, respectively. Antioxidant activities were also possessed by the control treatment. However, no antioxidant activity was seen under 200 mM and 300 mM NaCl concentrations as the shoot nodes could not cope with this stress and hence died.

It is revealed from Table 6 that all of the antioxidant activities, i.e. TPC, TFC, TAC, TRP, and DPPH-free radical scavenging activities, were obtained by NaCl stress of 100 mM, 200 mM, and 300 mM as well as by the control treatment. Despite the antioxidant activities revealed by all three conditions of stress, significantly higher antioxidant activities were shown by the regenerants grown under no stress, i.e. the control treatment. The data of the control treatment, 5.83 µg/mg of DW, 2.65 µg/mg of DW, 11.5 µg/mg of DW, 10.5 µg/mg of DW, and 64.04%, were followed by 100 mM, 200 mM, and 300 mM NaCl concentration treatments, respectively.

4. Discussion
The physiological parameters of callus formation and shoot formation were significantly enhanced when MS medium was supplemented with up to 100 mM NaCl concentration, whereas a decline in all parameters was observed with increasing NaCl concentration during the formation of regenerants. The best growth was obtained in the case of the control during root formation. The most probable reason for growth reduction in the case of root formation is the change in metabolic activities resulting in reduction in cell division, elongation, and differentiation of roots. Mubarak et al. (2012) obtained significantly less growth after exposure to rising salt stress. The control treatment produced the best results with respect to shoot number, shoot length (cm), node number, leaf number, root number, root length (cm), and % survival rate.
Table 1. Comparison of physiological parameters in 6-week-old calli produced from leaf explants on MS medium supplemented with different concentrations of NaCl.

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>Amount of callus</th>
<th>FW of callus (g)</th>
<th>DW of callus (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5 ± 0.20a</td>
<td>0.09 ± 0.05a</td>
<td>0.02 ± 0a</td>
</tr>
<tr>
<td>100</td>
<td>1.5 ± 0a</td>
<td>0.09 ± 0.02a</td>
<td>0.02 ± 0a</td>
</tr>
<tr>
<td>200</td>
<td>1.0 ± 0b</td>
<td>0.03 ± 0b</td>
<td>0.01 ± 0b</td>
</tr>
</tbody>
</table>

±: Standard error; means with the same letters within columns are not significantly different according to Duncan’s multiple range test at confidence level of 95%.

Figure 1. Calli grown on MS media containing PGRs, i.e. combination of 0.5 mg/L of KN and 0.5 mg/L of 2,4-D, under 0, 100, 200, and 300 mM NaCl stress after 6 weeks.

Figure 2. Shoots grown from shoot nodes raised from seedlings without using PGRs under 0, 100, 200, and 300 mM NaCl stress after 4 weeks.
Table 2. Comparison of physiological parameters in 4-week-old shoots produced from nodal stem explants on MS medium supplemented with different concentrations of NaCl.

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>Length of plantlets (cm)</th>
<th>Length of roots (cm)</th>
<th>No. of roots</th>
<th>No. of nodes</th>
<th>No. of leaves</th>
<th>FW of plantlets (g)</th>
<th>DW of plantlets (g)</th>
<th>FW of leaves (g)</th>
<th>DW of leaves (g)</th>
<th>FW of stems (g)</th>
<th>DW of stems (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17 ± 1.15a</td>
<td>1.83 ± 0.16a</td>
<td>7.33 ± 1.33a</td>
<td>12.33 ± 0.33a</td>
<td>26.6 ± 0.76a</td>
<td>1.02 ± 0.18b</td>
<td>0.15 ± 0.02b</td>
<td>0.38 ± 0.11b</td>
<td>0.06 ± 0.02</td>
<td>0.22 ± 0.12</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>10.16 ± 0.44b</td>
<td>0.33 ± 0.33b</td>
<td>0.66 ± 0.68a</td>
<td>8.66 ± 0.88a</td>
<td>20 ± 1.15b</td>
<td>0.52 ± 0.10b</td>
<td>0.09 ± 0.02b</td>
<td>0.29 ± 0.07b</td>
<td>0.05 ± 0.01b</td>
<td>0.62 ± 0.08</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>200</td>
<td>6.83 ± 0.44c</td>
<td>0c</td>
<td>0</td>
<td>6 ± 0.57c</td>
<td>14.6 ± 1.33c</td>
<td>0.46 ± 0.12b</td>
<td>0.09 ± 0.01c</td>
<td>0.23 ± 0.01c</td>
<td>0.05 ± 0b</td>
<td>0.14 ± 0.04</td>
<td>0.03 ± 0</td>
</tr>
<tr>
<td>300</td>
<td>5.16 ± 0.72d</td>
<td>0c</td>
<td>0</td>
<td>4.66 ± 0.33d</td>
<td>11.3 ± 1.76d</td>
<td>0.42 ± 0.06b</td>
<td>0.08 ± 0c</td>
<td>0.33 ± 0.04b</td>
<td>0.05 ± 0b</td>
<td>0.07 ± 0.02</td>
<td>0.01 ± 0</td>
</tr>
</tbody>
</table>

±: Standard error; means with the same letters within columns are not significantly different according to Duncan's multiple range test at confidence level of 95%.

Table 3. Comparison of physiological parameters in 4-week-old regenerants produced from shoots on MS medium supplemented with different concentrations of NaCl.

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>Length of shoots (cm)</th>
<th>No. of nodes</th>
<th>No. of leaves</th>
<th>FW of shoots (g)</th>
<th>DW of shoots (g)</th>
<th>FW of leaves (g)</th>
<th>DW of leaves (g)</th>
<th>FW of stems (g)</th>
<th>DW of stems (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.83 ± 0.24b</td>
<td>2.66 ± 0.4b</td>
<td>10 ± 1.18b</td>
<td>0.16 ± 0.03b</td>
<td>0.22 ± 0.01b</td>
<td>0.01 ± 0b</td>
<td>0.03 ± 01b</td>
<td>0.03 ± 0b</td>
<td>0.01 ± 0b</td>
</tr>
<tr>
<td>100</td>
<td>4.1 ± 0.16c</td>
<td>4.6 ± 0.33c</td>
<td>13 ± 0c</td>
<td>0.43 ± 0.03c</td>
<td>0.30 ± 0.03c</td>
<td>0.02 ± 0c</td>
<td>0.09 ± 0.01c</td>
<td>0.02 ± 0.01c</td>
<td>0.01 ± 0</td>
</tr>
</tbody>
</table>

±: Standard error; means with the same letters within columns are not significantly different according to Duncan's multiple range test at confidence level of 95%.

Table 4. Comparison of antioxidant activities in 6-week-old calli produced from leaf explants on MS medium supplemented with different concentrations of NaCl.

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>TFC (µg/mg of DW)</th>
<th>TPC (µg/mg of DW)</th>
<th>TAC (µg/mg of DW)</th>
<th>TRP (µg/mg of DW)</th>
<th>DPPH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.88 ± 0.01b</td>
<td>4.4 ± 0.02b</td>
<td>10.46 ± 0b</td>
<td>9.9 ± 0.01b</td>
<td>50.38</td>
</tr>
<tr>
<td>100</td>
<td>2.65 ± 0.01a</td>
<td>5.83 ± 0.02a</td>
<td>11.5 ± 0.01a</td>
<td>10.5 ± 0.01a</td>
<td>64.04</td>
</tr>
<tr>
<td>200</td>
<td>1.72 ± 0.02b</td>
<td>4.1 ± 0.01c</td>
<td>8.52 ± 0c</td>
<td>9.68 ± 0.01c</td>
<td>30.58</td>
</tr>
</tbody>
</table>

±: Standard error; means with the same letters within columns are not significantly different according to Duncan's multiple range test at confidence level of 95%.
Figure 3. Roots grown from shoots raised on PGR-free media under 0, 100, 200, and 300 mM NaCl stress after 4 weeks.

Figure 4. Effect of NaCl concentrations (0 and 100 mM) on shoots after 4 weeks regarding Reb A content, represented with red bars, and ST content, blue bars. Error bars are shown as standard deviation for each bar.

Figure 5. Effect of NaCl concentrations (0, 100, 200, and 300 mM) on regenerants after 4 weeks regarding Reb A content, represented with blue bars, and ST content, red bars. Error bars are shown as standard deviation for each bar.
Almost all of the parameters declined with increment in salinity from the control to 5000 ppm, 7500 ppm, and 10,000 ppm concentrations of NaCl. The highest NaCl concentration produced a lethal effect leading to death of in vitro-grown plantlets. These results agree with our data on root formation but disagree with our callus and shoot formation results. Zeng et al. (2013) investigated the reduction in physiological parameters upon increase of NaCl stress up to 90 mM, which is consistent with our root formation results, but not our callus and shoot formation findings. The study conducted by Pandey and Chikara (2014) illustrated an increase of all growth and development parameters with up to 100 mM of NaCl stress for Stevia shoots and roots. These results are in agreement with our findings regarding callus and shoot formation, while they disagree in the context of root formation. In addition, Pandey and Chikara (2015) examined the significant effect of 25 mM, 50 mM, 75 mM, and 100 mM NaCl concentrations on different growth parameters of S. rebaudiana and found their decrease with increasing NaCl concentrations up to 100 mM. Gupta et al. (2016) showed an increase of SG content upon increasing NaCl salt concentration in vitro-grown S. rebaudiana plants. S. rebaudiana was found to be a moderately salt-tolerant species by Hussin et al. (2017), which is consistent with our findings of shoot formation whereby after reaching a certain threshold the SG increment stopped. Moreover, a significant decrease of SG contents was observed from 100 mM to 200 mM and 300 mM NaCl concentration during root formation. Fallah et al. (2017) showed a significant decrease of SGs with increasing NaCl stress in S. rebaudiana. The highest amounts of SGs were formed in the case of the control treatment in this study. Moreover, in our study, SGs were not detected in calli obtained by callus formation. The reason for this might be that energy was utilized in maintaining metabolism of enzymatic and nonenzymatic antioxidants rather than the synthesis of complex SG molecules and secondary metabolites.

Metabolic pathways are altered as a result of salinity/NaCl stress in the case of callus, shoot, and root formation of S. rebaudiana. The disturbance of metabolic processes

<table>
<thead>
<tr>
<th>Conc.</th>
<th>TFC (µg/mg of DW)</th>
<th>TPC (µg/mg of DW)</th>
<th>TAC (µg/mg of DW)</th>
<th>TRP (µg/mg of DW)</th>
<th>DPPH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.44 ± 0.01b</td>
<td>6.97 ± 0.01b</td>
<td>13.77 ± 0b</td>
<td>10.22 ± 0.02b</td>
<td>48.03</td>
</tr>
<tr>
<td>100 mM</td>
<td>5.04 ± 0.01a</td>
<td>7.48 ± 0a</td>
<td>14.63 ± 0a</td>
<td>10.39 ± 0.01a</td>
<td>49.48</td>
</tr>
</tbody>
</table>

±: standard error; means with the same letters within columns are not significantly different according to Duncan's multiple range test at confidence level of 95%.

<table>
<thead>
<tr>
<th>Conc. of NaCl (mM)</th>
<th>TFC (µg/mg of DW)</th>
<th>TPC (µg/mg of DW)</th>
<th>TAC (µg/mg of DW)</th>
<th>TRP (µg/mg of DW)</th>
<th>DPPH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.65 ± 0.01b</td>
<td>5.83 ± 0b</td>
<td>11.5 ± 0.01a</td>
<td>10.5 ± 0.01b</td>
<td>64.04</td>
</tr>
<tr>
<td>100</td>
<td>3.89 ± 0.02a</td>
<td>5.91 ± 0a</td>
<td>10.86 ± 0.01b</td>
<td>10.6 ± 0.01a</td>
<td>63.91</td>
</tr>
<tr>
<td>200</td>
<td>1.94 ± 0.01c</td>
<td>5.31 ± 0c</td>
<td>7.22 ± 0d</td>
<td>10.14 ± 0.01d</td>
<td>67.45</td>
</tr>
<tr>
<td>300</td>
<td>1.7 ± 0.01d</td>
<td>5.13 ± 0.01d</td>
<td>9.52 ± 0.01c</td>
<td>10.49 ± 0.01c</td>
<td>69.82</td>
</tr>
</tbody>
</table>

±: standard error; means with the same letters within columns are not significantly different according to Duncan's multiple range test at confidence level of 95%.
leads to increased phenols, flavonoids, total antioxidant activity, total reducing power, and DPPH-free radical scavenging activity. All phytochemicals increase in the calli and shoot leaves formed in MS medium containing up to 100 mM NaCl concentration. However, a decline in phytochemical activity is observed in regenerant leaves during root formation taking place in MS medium having 100 mM, 200 mM, and 300 mM NaCl concentration. Stevia has previously shown such types of responses regarding the accumulation of phenols in shoots containing up to 75 mM NaCl concentration (Rathore et al., 2014). Moreover, an increment of enzymatic antioxidant activities in soil-grown shoots was estimated with increasing concentrations of NaCl salt (Zeng et al., 2013). The most probable mechanism involves formation of ROS causing oxidative stress by supplementing NaCl to the medium. This causes plant cells to form more secondary metabolites.

In conclusion, the in vitro growth of *S. rebaudiana* calli and shoots revealed significant salt tolerance by calli and shoots at up to 100 mM NaCl concentration augmented to MS growth medium. However, the obtained roots indicated salt susceptibility with the rise of NaCl concentration from the control to 100 mM and above. Hence, this study suggests that *S. rebaudiana* plants possess the capability to grow in saline soils because of the significant tolerance of this plant to the deleterious effects of salt stress, but only up to the threshold level of 100 mM NaCl. Therefore, we may consider this plant to be moderately halotolerant.

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**References**


