MORPHO-PHYSIOLOGICAL RESPONSE OF STEVIA (*STEVIA REBAUDIANA* BERTONI) TO SALINITY UNDER HYDROPONIC CULTURE CONDITION (A CASE STUDY IN IRAN)

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Abstract. Stevia (*Stevia rebaudiana* Bertoni) is a relatively unknown crop in Iran and many countries of world, with great potential as a natural sweeteners source. Stevia has a high content of sweeteners, which are up to 150 times sweeter than sugar, but virtually with no calories. An experiment was carried out to investigate the effect of salinity stress on root characteristics and physiological traits of stevia using six different concentrations of NaCl (0, 30, 60, 90, 120, and 150 mM) in Hoagland solution with four replications in open shading structures at Medicinal Plant Research Center, Shahed University of Tehran, Iran. The results showed that root characteristics (such as root fresh weight (RFW), root dry weight (RDW), root volume (RV), root length (RL), root area (RA), root diameter, root mass density (RMD), and dry root mass density (DRMD)) reduced with the intensification of NaCl. The effect of NaCl was significant on the protein content, activity of catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), and DPPH. The increasing of NaCl caused significantly enhancement of protein content, CAT activity, and total antioxidant activity. The POD activity showed a significant decrease by the increasing of sodium chloride rate. The PPO and POD activities by the increasing sodium chloride rate showed a significant decrease. Steviol glycosides (SVglys) compositions: stevioside (Stev), rebaudioside A (Reb A), rebaudioside B (Reb B), rebaudioside C (Reb C) and dulcoside A (Dulc A) and SVglys content showed changes under the influence of salinity. In 30 mM NaCl treatment was obtained the highest value of total SVglys yield and SVglys content. The findings from this study lead to the conclusion that, salinity stress caused reduces root characteristics and changes in physiological traits (protein content, activity of CAT, POD, PPO and total antioxidant activity DPPH). On the other hand, at the lowest salinity level (30 mM), the highest amount SVglys was obtained. It seems that the high level of SVglys at lower salinity levels is one of the reasons for salinity tolerance in Stevia, which requires further investigation.
Keywords: *enzyme activity, Hoagland, NaCl, peroxidase, root, steviol glycosides*

Introduction

The herb of stevia (*Stevia rebaudiana* Bertoni) also known as a honey leaf is a perennial plant and from the Asteraceae (Compositae) family native to Brazil and Paraguay (often referred to as the herb of Paraguay) (Karimi et al., 2015). The plant has a sweet taste due to the presence of diterpene SV glys. Stev and Reb-A are dominant glycoside compositions of the plant which makes that plant to be even 300 times sweeter than sucrose (Hajihashemi and Ehsanpour, 2014). This plant is of worldwide importance and high demand in many countries (such as Japan, Korea, China and South America) which are high potency natural sweeteners and low-calorie (Barbet-Massin et al., 2016).

The plant can, apparently, be successfully grown under different conditions regarding climate and soils (Hajar et al., 2014). The plant is adapted to poor soils, with low nutrient requirements. However, stevia shows some variability in what concerns the sensitivity or tolerance to salinity stress (Reis et al., 2015). There is often a tendency for

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a relation between growth and yield of crops, and salinity that is well established in the scientific literature: usually, the higher salinity levels the less growth and yield of the crop (Ityel et al., 2012; Jamil et al., 2012; Reis et al., 2015).

Salinity is one of the most hazardous and limiting environmental factors of crop product and plant growth particularly in arid and semi-arid regions (Gharsallah et al., 2016). FAO (2015) reported that 800 million ha of land and 32 million ha of agricultural land are estimated salt affected. The soil salinity of decreased water uptake by the root plants and causes osmotic stress, ion toxicity and mineral deficiencies (Munns and Tester, 2008).

Responses of plants to salinity stress (including physiological and morphological modifications) depending on the stage of growth and the genotype (Montana et al., 2014). Different types of salts exist in agricultural lands (such as Na_2SO_4, Na_2CO_3, KCl, $CaSO_4$, $MgSO_4$, $MgCl_2$ and etc) each of which can lead to the salinity stress (Bazrafshan and Ehsanzadeh, 2016). However, in salinity stress are dominant mostly Na^+ and Cl^- concentrations (Montana et al., 2014), thus NaCl is commonly the most widespread and disadvantageous salt in agricultural lands of Iran and other countries (Azizpour et al., 2010).

Salinity stress has been reported (Wu et al., 2010) to cause reactive oxygen species (ROS) formation and accumulation in the plant. Oxidative stress defenses happen through an enzymatic antioxidant mechanism including CAT, SOD, POD and non-enzymatic antioxidants as phenolics, flavonoids (Gharsallah et al., 2016).

Plant roots are the first organ to experience the effects of salt stress and essential to plant maintenance, as well as, have an important function in determining the yield of crops (Ober and Sharp, 2007). The widespread root system is positively correlated with salinity tolerance. Variations in the number of roots, root length, and its growth rate have been observed in different varieties and plants that these traits may create differences in plant characteristics such as tolerance to drought and salinity stresses (De-Oliveira and Varshney, 2011). The root of the plant's longer and more lateral roots than fewer plants that this attribute is more tolerant to salinity stress. An important factor in the tolerance to salinity how to develop their root system (Singh et al., 2000). Increased root surface is important since caused increased levels of absorption and increasing the efficiency of water and nutrients. Therefore, longer roots and more root surface can be provided possibility salinity tolerance (Aghaei et al., 2009).

The cultivation of stevia multipurpose plant caused increasing its production for medicinal and other purposes. There is much research on potential uses, micropropagation, and cell culture, secondary metabolite production of this plant. However, there are only a few reports available on biochemical modifications and without reported on root characteristics of stevia in NaCl salinity condition (Gupta et al., 2016). On the other hand, root characteristics and biochemical traits modifications are important for understanding salinity tolerance mechanism, but strategies differ among plant species (Liu et al., 2016). Therefore, there is a need to research on modifications of root parameters and biochemical traits of the plant. So, in the present research was planned with objectives the assess of six different concentrations of NaCl (0, 30, 60, 90, 120, and 150 mM) on root characteristics and physiological traits of stevia.

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Material and methods

Plant material and growth conditions

The present study was carried out based on a completely randomized design (CRD) with four replications in open shading structures at Medicinal Plant Research Center, Shahed University of Tehran, Iran. The seedling of stevia (*Stevia rebaudiana* Bertoni) propagation was carried out by tissue culture. The uniform two seedlings were transplanted into the pot (30 × 30 cm) containing a mixture of pumice and perlite (50:50 ratio). Two weeks after establishment of seedlings were subjected to salinity stress with different NaCl (Sodium chloride; Merck, Darmstadt, Germany) concentration (0, 30, 60, 90, 120, and 150 mM). Salinity treatment was applied according to the treatments 15 days after planting and continued until the end of the experiment. The pots were watered one time a day by mineral nutrients with 500 mL per pot of modified Hoagland solution (*Table 1*). The EC and pH of drainage water from pots were checked every week, and an additional 500 mL of distilled water was applied to minimize pH and EC changes in the root zone (Rahimi and Biglarifard, 2011). The whole plants were harvested at 62 days after transplanting and leaves and stems were separated and placed in the freezer (-30 °C) used for further assays.

Table 1. *Nutritional composition of used in the preparation of Hoagland solution*

Macronutrients	Chemical components	Stock solution	ml of stock solution/1 ml
KNO_3	2M KNO_3	202 g/L	2.5
H_2PO_4	1M KH_2PO_4 (pH to 6.0)	136 g/L	0.5
$Ca(NO_3)_2$	1M $Ca(NO_3)_2$•$4H_2O$	236 g/0.5L	2.5
NH_4NO_3	1M NH_4NO_3	80 g/L	1
Micronutrients			
Iron	Iron (Sprint 138 iron chelate)	15 g/L	1.5
$MgSO_4$	2M $MgSO_4$•$7H_2O$	493 g/L	1
H_3BO_3	H_3BO_3	2.86 g/L	1
$MnCl_2$	$MnCl_2$•$4H_2O$	1.81 g/L	1
$ZnSO_4$	$ZnSO_4$•$7H_2O$	0.22 g/L	1
$CuSO_4$	$CuSO_4$•$5H_2O$	0.051 g/L	1
Na_2MoO_4	Na_2MoO_4•$2H_2O$	0.12 g/L	1

Root parameters calculation

Underground organ, total roots were washed thoroughly and minimized damages with running water. Root fresh weight was measured immediately with 0.001g accuracy. RV was obtained by subtraction of root volume after inserting it in the certain volume of water to first RV with 0.1 mm accuracy. RA was evaluated by Atkinson method (Hajabbasi, 2001). Root diameter and volume was obtained by *Table 2* relations and other traits such as root fresh weight to soil volume (RMD), root dry weight to soil volume (DRMD), root dry weight to root volume, and root length to root fresh weight (RF) was calculated (Ganjali et al., 2003).

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Table 2. *Method of calculation parameters associated with root*

Parameters	Formula	Reference
Root length (RL)	RL = (roots weight) × 0.890	Ganjali et al., 2003
Root area (RA)	$RA = 2(\text{Root volume} \times \pi \times RL)^{0.5}$	Hajabbasi, 2001
Root diameter (RD)	$RD = (4 \times \text{Root fresh weight}/(RL \times \pi))^{0.5}$	Akhavan et al., 2012
Root Surface Area Density RSD)	RSD = (RL × RD × π)	Akhavan et al., 2012

π = constant number 3.14

Protein and antioxidant enzymes assay

Samples were frozen in liquid nitrogen and stored at -30 °C. One g of frozen leaf was homogenized in a mortar with 5 mL of 50 mM potassium phosphate buffer (pH = 7.5) containing 1 mM ethylenediaminete-traacetic acid (EDTA), 1 mM dithiotreitol and 2% polyvinyl pyrrolidon (PVP). The homogenate was centrifuged at 15,000 g for 25 min and the supernatant was used for protein and antioxidant enzyme assay.

Protein content

For determining the amount (concentration) of protein, spectrophotometer with Bradford method (1976) was used. The basis of this method is binding the Coomassie Brilliant blue G-250 to protein in an acidic environment and determine maximum absorption from 595 to 465 nm. The absorbance at 595 nm had a direct comparison to protein concentration.

Catalase assay

The CAT activity assay was performed using Chance and Maehly (1995) method. Three mL reaction mixture containing 2.5 mL 0.05 mM sodium phosphate buffer (pH = 7), 30 µg protein solution was added to quettes and at the time of measurement, 30µL H_2O_2 (30%) was added to reaction mixture and the absorbance at 240 nm, at 60 s, and at 25 °C was recorded.

Peroxidase assay

POD activity was assayed adopting the method of Polle et al. (1994). According to this method, POD activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol ($\epsilon = 26.6\ mM^{-1}cm^{-1}$). The reaction mixture contained 100 mM potassium phosphate buffer (pH = 7.0), 20.1 mM guaiacol, 10 mM H_2O_2 and enzyme extract. The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 3 min.

Polyphenol oxidase assay

The polyphenol oxidase (PPO; E.C. 1.10.3.1) activity was measured according to the method of Raymond et al. (1993) in absorbance at 430 nm. The reaction mixture contained 1900 µl 50 mM sodium phosphate buffer (pH = 6.8), 500 µl pyrogallol 0.02 mM and 100 µl enzyme extract.

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DPPH activity

The measurement of the DPPH radical scavenging activity was performed according to the methodology described by Brand-Williams et al. (1995). The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution 0.5 mM in ethanol. The changes in color (from deep violet to light yellow) were read at 517 nm after 100 min of reaction. The mixture of ethanol (3.3 mL) and sample (0.5 mL) serve as a blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL).

Determination of SVglys content and compositions

The SVglys content and compositions of stevia leaves were determined according to the procedures used by other researchers (Ceunen and Geuns, 2013; Karimi et al., 2015). 0.1 g of powdered leaves (dried at 65 °C for 48 h in hot air avon) was transferred to 15 mL tubes, 3 mL distilled water were added and kept in a water bath for 30 min at 80 °C. The resultant solution was firstly centrifuged at 12,000 × g for 5 min and the supernatant recovered. Then, 3 mL distilled water was added to the pellet and then centrifuged. This process was repeated three times and the supernatant from each process was pooled. The pooled supernatant was centrifuged again (12,000 × g for 5 min) and the new supernatant was transferred to new tubes. The volume of the final supernatant was exactly diluted to 10 mL using distilled water and filtered using 0.45 m nylon filter attached to a syringe. Then, a C18 cartridge was used for SVglys purification. The C18 cartridge was firstly washed with 3 mL methanol and then conditioned with 3 mL of distilled water. Thereafter, 0.5 mL of the filtered supernatant was loaded into the cartridge and then the cartridge was washed with acetonitrile/water mixture (20:80, v/v). Finally, SVglys were eluted from the C18 cartridge with 1 mL of acetonitrile/water (80:20, v/v) and kept in 1.5 mL tubes at -20 °C until further analysis.

For the chromatographic SVglys analysis, two reverse-phase C18 columns were connected in series and a UV-vis detector set at 202 nm was used. A solvent gradient of acetonitrile and water, as mobile phases, were created with a flow rate of 0.5 mL min^{-1}. The acetonitrile ratio was increased into the solvent gradient in 50, 65, 80, 80 and 50% during 0-10, 10-18, 18-22, 22-24 and 24-30 min, respectively. In order to carry out SVglys assay, 40 µL of the purified extract was injected into the HPLC pump. Among SVglys compositions, Reb A, Stev, Reb F, Reb C and Dulc A were detected. For quantification purposes, pure Stev and Reb A (purity > 99%) were used as external standards. Then, Reb F, Reb C, and Dulc A were quantified by their molecular weight ratio to Reb A, because it has been shown that all SVglys have similar molar extinction coefficients (Geuns, 2010). The HPLC peak area was calculated by Chromstar 7.0 software and the results of SVglys content were expressed as a percentage of leaf dry weight (W/W), using the calibration curves obtained from the relationship between external standards (ppm) and their relative HPLC peak area.

Statistical analysis

All data was analysis with SAS 9.2 software and means comparisons was performed by Duncan multiple ranges in 5% of probability.

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Results

Root characteristics

The effect of salinity was significant on RFW, RDW, RV, RL, RA, root diameter, RSD, RMD and DRMD (*Table 3*). With the increasing amount of sodium chloride showed a significant decrease in all root traits measured (*Table 4*). The highest mean traits of RFW, RDW, RV, RL, RA, root diameter, RSD, RMD, and DRMD were in the NaCl 0 mM, however, the traits of RDW, RL and DRMD in the NaCl 30 mM did not show significant decrease compared to the control (non-stress) (*Table 4*). The salinity of NaCl 60 mM compared to the control (NaCl 0 mM) causes decreased approximately 50% in the mean of root characteristics (mentioned above). Salinity level of NaCl 150 mM compared to the control decreased 80.16, 73.24, 72.10, 73.23 and 72.73% at RFW, RDW, RV, RL and RA, respectively (*Table 3*). In *Figure 1*, the structure of root morphology shown in different levels of sodium chloride.

Table 3. *Analysis of variance for effect of salinity (NaCl) on root characteristics of Stevia*

Sources of variance	Mean square (MS)										
	df	RFW	RDW	RV	RL	RA	Root diameter	RSD	RMD	DRMD	RF
Salinity (NaCl)	5	737.2**	10.4**	703.0**	8.29**	894.1**	0.21*	960.1**	0.038**	0.00055**	0.001 ns
Experimental error	18	20.69	0.30	20.50	0.24	12.01	0.08	18.61	0.001	0.00001	0.001
CV (%)	-	21.15	17.26	20.12	17.26	12.34	9.33	15.64	21.15	17.26	22.16

ns, * and ** non-significant, significant at 5% and 1% respectively
(RFW: Root Fresh Weight, RDW: Root Dry Weight, RV: Root Volume, RL: Root Length, RA: Root Area, RSD: Root Surface Area Density, RMD: Root Mass Density, DRMD: Dry Root Mass Density, RF: Root Fineness (Root Length/ Root Fresh Mass)

Table 4. *Mean comparison of salinity levels (NaCl) on root parameters of stevia*

Salinity (mM)	RFW (g/plant)	RDW (g/plant)	RV (cm^3)	RL (m)	RA (cm^2)	Root diameter (cm)	RSD ($m^2\ m^{-3}$)	RMD ($g\ m^{-3}$)	DRMD ($g\ m^{-3}$)
$NaCl_0$	44.57 a	5.42 a	47.50 a	4.82 a	53.47 a	3.43 a	51.98 a	0.323 a	0.039 a
$NaCl_{30}$	28.74 b	4.81 a	26.50 b	4.28 a	37.61 b	2.92 b	38.87 b	0.208 b	0.034 a
$NaCl_{60}$	20.94 c	2.98 b	19.75 c	2.65 b	25.54 c	3.17 ab	26.38 c	0.151 c	0.021 b
$NaCl_{90}$	16.83 c	2.90 b	14.25 c	2.58 b	21.29 c	2.89 b	23.27 c	0.122 c	0.021 b
$NaCl_{120}$	9.07 d	1.66 c	13.75 c	1.48 c	15.95 d	2.78 b	12.97 d	0.065 d	0.012 c
$NaCl_{150}$	8.84 d	1.45 c	13.25 c	1.29 c	14.58 d	2.96 b	11.96 d	0.064 d	0.010 c

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test
(RFW: Root Fresh Weight, RDW: Root Dry Weight, RV: Root Volume, RL: Root Length, RA: Root Area, RSD: Root Surface Area Density, RMD: Root Mass Density, DRMD: Dry Root Mass Density, RF: Root Length/ Root Fresh Mass)

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Figure 1. *The effect of NaCl concentration on the root morphology*

Physiological traits

The effect of NaCl was significant on the protein content, activity of CAT, POD, PPO and DPPH ($P \leq 0.01$) (*Table 5*). The increase of NaCl concentration caused significantly enhuncment protein content so that the highest protein content was in the highest levels of sodium chloride (120 and 150 mM) and the lowest protein percentage was control level of salinity (*Table 6*).

Table 5. *Analysis of variance for effect of salinity (NaCl) on biochemistry traits of stevia*

Sources of variance	Mean square (MS)					
	df	Protein	CAT	POD	PPO	DPPH
Salinity (NaCl)	5	31.45**	38.33**	2.85**	29.64**	7.94**
Experimental error	18	1.05	0.17	0.01	0.79	0.064
CV (%)	-	10.11	11.95	9.84	25.15	10.74

ns, * and ** non-significant, significant at 5% and 1% respectively

Table 6*. Mean comparison of salinity levels (NaCl) on biochemistry traits of stevia*

Salinity (mM)	Protein (%)	CAT (U/gFW)	POD (U/gFW)	PPO (U/gFW)	DPPH (U/gFW)
$NaCl_0$	5.84 e	0.021 e	2.64 a	8.02 a	0.61 e
$NaCl_{30}$	8.27 d	0.123 e	2.03 b	5.24 b	1.30 d
$NaCl_{60}$	9.88 c	2.34 d	1.71 c	3.53 c	2.03 c
$NaCl_{90}$	11.17 bc	5.18 c	0.89 d	2.36 cd	2.36 c
$NaCl_{120}$	12.46 ab	6.26 b	0.73 d	1.42 de	3.67 b
$NaCl_{150}$	13.43 a	7.04 a	0.50 e	0.67 e	4.19 a

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test

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The highest and lowest CAT activity were in NaCl 150 mM and control levels respectively, in other words, increasing sodium chloride increased CAT activity (*Table 6*).

The POD activity showed a significant decrease in the increasing amount of sodium chloride so that the highest activity of this enzyme was in control level and lowest activity was at the highest level of salinity (NaCl 150 mM) (*Table 6*).

The activity of PPO and POD showed a significant decrease by the increasing amount of sodium chloride (on the contrary CAT activity), so that in the NaCl 0 mM and NaCl 150 mM observed the highest and lowest activity of this enzymes respectively (*Table 6*).

The total antioxidant activity DPPH significantly increased by the increasing sodium chloride. In other words, salinity stress was significantly increased the antioxidant capacity of the plant, so that the lowest of activity was in control level and the highest activity was NaCl 150 mM (*Table 6*).

SVglys production

The salinity caused a significant variation in SVglys compositions and SVglys content of stevia (*Table 7*). In the NaCl 30 mM obtained the highest value of total SVglys yield and SVglys content (0.51 g/plant and 12.47% of the leaf dry weight, respectively) (*Table 8*). However, regarding SVglys yield of stevia, there was no significant difference between control, NaCl 30, and NaCl 60 mM treatment.

The effect of NaCl treatment was significant on SVglys compositions (Such as Stev, Reb A, Reb B, Reb C and Dulc A). The highest value of Stev, Reb A, Reb B, Reb C and Dulc A were achieved in NaCl 30 mM level. The Svglys compositions increased by NaCl 30 mM treatment and thereafter decreased when the stress became more severe. Reb A/Stev ratio (sweetness quality) was significantly affected by NaCl treatment with the highest value in NaCl 90 and NaCl 120 mM treatments (*Table 8*).

Discussion

Salinity is one of the factors reducing growth and yield of many crops around the world and also, affect on growth and composition of secondary metabolites in medicinal plants (Aghaei Joubani, 2015). The NaCl stress caused a decrease in all root traits measured (Such as RFW, RDW, RV, RL, RA, root diameter, RSD, RMD, and DRMD). By increasing salinity levels, root morphology were bulky, shorter and slower growth (*Figure 1*). Reported that salinity stress decrease RL, RF, and RDW in quinoa plant (Panuccio et al., 2014). Root is an organ which could affect resource conversion, use efficiency and transduction of production sources. So structural changes in root morphology caused by salinity stress could alter root structure and affected physiological process occurred in the root (water absorbance and nutrient availability) (Malamy, 2005; Panuccio et al., 2014).

Safwat et al. (2016) reported that salinity decreased root number and length in stevia. The RFW and RDW in *Periploca sepium* Bunge were reduced strongly with increasing salinity levels (Sun et al., 2011). Researchers mentioned the reduction of photosynthesis, degradation of cell membranes, reduction water availability to roots and accumulation of sodium ions as the main factors reduce plant growth under salt stress (Sharifi et al., 2007).

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Table 7. *Analysis of variance for SVglys compositions of stevia under NaCl treatments (0, 30, 60, 90, 120 and 150 mM)*

Sources of variance	Mean square (MS)								
	df	Stev	Reb A	Reb B	Reb C	Dulc A	Total SVglys	Reb A/Stev ratio	SVglys yield
Salinity (NaCl)	5	18.06**	1.33**	0.041**	0.0061**	0.036**	34.25**	0.0151**	0.104**
Error	18	0.026	0.023	0.0004	0.00009	0.0038	0.043	0.0018	0.009
CV (%)	-	3.16	7.53	7.92	6.20	10.23	2.53	10.58	27.14

ns, * and ** non-significant, significant at 5% and 1% respectively
(SVglys: Steviol glycosides; Stev: Stevioside; Reb A: RebaudiosideA; Reb C: Rebaudioside C; Dulc A: Dulcoside A)

Table 8*. Mean comparison of salinity levels (NaCl) on SVglys compositions of stevia*

Salinity (mM)	Stev (%)	Reb A (%)	Reb B (%)	Reb C (%)	Dulc A (%)	Total Svglys (percent of leaf dry matter)	Reb A/Stev ratio	SVglys yield (g/plant)
$NaCl_0$	5.74 c	2.03 c	0.26 c	0.16 c	0.61 bc	8.83 c	0.35 c	0.46 ab
$NaCl_{30}$	8.25 a	2.82 a	0.43 a	0.21 a	0.75 a	12.48 a	0.34 c	0.51a
$NaCl_{60}$	6.83 b	2.54 b	0.34 b	0.18 b	0.66 b	10.57 b	0.37 bc	0.47 ab
$NaCl_{90}$	3.88 d	1.84 c	0.20 d	0.13 d	0.57 bcd	6.65 d	0.47 a	0.33 b
$NaCl_{120}$	3.28 e	1.56 d	0.18 d	0.12 de	0.53 cd	5.70 e	0.47 a	0.16 c
$NaCl_{150}$	2.99 f	1.30 e	0.17 d	0.10 e	0.48 d	5.07 f	0.43 ab	0.15 c

Means in each column followed by similar letter (s), are not significantly different at 5% probability level, using Duncan's Multiple Range Test
(SVglys: Steviol glycosides; Stev: Stevioside; Reb A: RebaudiosideA; Reb C: Rebaudioside C; Dulc A: Dulcoside A)

The salinity stress an important subject is the generation of excessive ROS which caused membrane destruction, cell toxicity, and cell death (Chookhampaeng et al., 2008). According to researchers opinion, salinity stress converts superoxide radical (O_2) to hydrogen peroxide (H_2O_2) within the cell, this conversion prevents Calvin cycle activity and the process carbohydrates in plants. Therefore increase in activity of antioxidant enzymes such as CAT and SOD was from adverse effects of hydrogen peroxide formed which impact on the carbohydrates production in the chloroplasts. High levels of antioxidant activity in plants under stress are not the only mechanism of salinity tolerance, but also this mechanism can be compatible with components suppliers such as proline and carbohydrates to increase crop tolerance (Abo-Kassem, 2007).

Also, in this experiment, salinity increased CAT activity and total antioxidant activity DPPH in stevia (*Table 6*). CAT is an important antioxidant enzyme that converts H_2O_2 to water in the peroxisomes. In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration. Higher activity of CAT and Ascorbate peroxidase decreased H_2O_2 rate in cell and increased the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2directly inhibits CO_2 fixation (Bhutta, 2011). The activity

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of the enzyme of CAT is higher than that of POD, PPO, and DPPH at NaCl 150 mM, which suggests that CAT provides a better defense mechanism against NaCl stress-inducted oxidative damage in stevia. The results are similar to Sun et al. (2011); Yeonghoo et al. (2004).

Stevia plants have gained importance as sweeteners because of their Stev and RebA contents. The results indicated that NaCl 30 mM (low salinity level) compared to the non-salinity (control) caused increases of SVglys compositions and SVglys yield. Similar results were also obtained by Zheng et al. (2013), who found that lower salinity concentration in soil could alter the composition of SVglys by distinctly improving Reb A content. However, higher NaCl levels (90, 120 and 150 mM) causes prominent decrease of SVglys composition (Such as Stev, Reb A, Reb B, Reb C and Dulc A) and SVglys yield. Zheng et al. (2013) also reported similar results. It seems that when plants lived under high salinity stress conditions, energy was first allocated to the process of maintaining metabolic homeostasis, such as synthesis of simple osmolytes and enhancing activities of antioxidant enzymes (Abrol et al., 2012) rather than the synthesis of complex secondary metabolites. The concentrations of various secondary plant products are strongly dependent on the growing conditions and have an impact on the metabolic pathways responsible for the accumulation of the related natural products (Ramakrishna and Ravishamkar, 2011).

Conclusion

The NaCl stress caused changes in physiological traits and reduces of root characteristics (caused decrease approximately 50% in the mean traits of root characteristics). The increaseing antioxidant defense system (DPPH, CAT, POD and PPO) also responded to this stress. The level of NaCl 30 mM (low salinity) compared to the non-salinity caused increase of SVglys compositions and SVglys yield. It seems that increasing the SVglys rate in low levels of salinity stress can be one of the tolerance mechanisms in the plant, which requires further research.

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REFERENCES

[1] Abo-Kassem, E. E. (2007): Effects of salinity: Calcium interaction on growth and nucleic acid metabolism in five species of Chenopodiaceae. – Turkish Journal of Botany 31(2): 125-134.

[2] Abrol, E., Vyas, D., Koul, S. (2012): Metabolic shift from secondary metabolite production to induction of antioxidative enzymes during NaCl stress in Swertia chirata Buch. – Ham Acta Physiol Plant 34: 541−546.

[3] Aghaei, K., Ehsanpour, A., Shah, A., Komatsu, S. (2009): Proteome analysis of soybean hypocotyl and root under salt stress. – Amino Acids 36(1): 91-98.

[4] Aghaei-Joubani, K. A. T., Najmeh, A. K., Mohammad, R. A., Yazdani, M. (2015): Effect of salt stress on some physiological and biochemical parameters of two Salvia species. – Journal of Plant Process and Function 3(9): 85-96.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1601_017028

[5] Azizpour, K., Shakiba, M., Sima, N. K. K., Alyari, H., Mogaddam, M., Esfandiari, E., Pessarakli, M. (2010): Physiological response of spring durum wheat genotypes to salinity. – Journal of Plant Nutrition 33(6): 859-873.
[6] Barbet-Massin, C., Giuliano, S., Alletto, L., Daydé, J., Berger, M. (2016): Towards a semi-perennial culture of *Stevia rebaudiana* (Bertoni) under temperate climate: effects of genotype, environment and plant age on steviol glycoside content and composition. – Genetic Resources and Crop Evolution 63(4): 685-694.
[7] Bazrafshan, A., Ehsanzadeh, P. (2016): Evidence for differential lipid peroxidation and antioxidant enzyme activities in *Sesamum indicum* L. genotypes under NaCl salinity. – Journal of Agricultural Science and Technology 18(1): 202-222.
[8] Bhutta, W. (2011): Antioxidant activity of enzymatic system of two different wheat (*Triticum aestivum* L.) cultivars growing under salt stress. – Plant Cell Environ 57: 101-107.
[9] Bradford, M. M. (1976): A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. – Annual Review of Biochemistry 72: 248-254.
[10] Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995): Use of free radical method to evaluate antioxidant activity. – Lebensm Wiss Technology 28: 25-30.
[11] Ceunen, S., Geuns, J. (2013): Influence of photoperiodism on the spatiotemporal accumulation of steviol glycosides in *Stevia rebaudiana* (Bertoni). – Plant Science 198: 72-82.
[12] Chance, B., Maehly, A. C. (1995): Assay of Catalase and Peroxidase. – Academic Press, New York.
[13] Chookhampaeng, S., Pattanagul, W., Theerakulpisut, P. (2008): Effects of salinity on growth, activity of antioxidant enzymes and sucrose content in tomato (*Lycopersicon esculentum* Mill.) at the reproductive stage. – Science Asia 34: 69-75.
[14] De Oliveira, A. C., Varshney, R. K. (2011): Root Genomics, pp. 1-10. – Springer, Berlin.
[15] Ganjali, A., Kafi, M., Bagheri, A., Shahriari, F. (2003): Allometric relationship between root and shoot characteristics of chickpea seedlings (*Cicer arietinum*). – Journal of Agriculture Science and Technology 18: 67-80.
[16] Geuns, J. M. C. (ed.) (2010): Stevia and Steviol Glycosides. – Euprint, Heverlee.
[17] Gharsallah, C., Fakhfakh, H., Grubb, D., Gorsane, F. (2016): Effect of salt stress on ion concentration, proline content, antioxidant enzyme activities and gene expression in tomato cultivars. – AoB Plants 8: 1-22.
[18] Gupta, P., Sharma, S., Saxena, S. (2016): Effect of abiotic stress on growth parameters and steviol glycoside content in *Stevia rebaudiana* (Bertoni) raised in vitro. – Journal of Applied Research on Medicinal and Aromatic Plants 3(4): 160-167.
[19] Hajabbasi, M. A. (2001): Tillage effects on soil compactness and wheat root morphology. – Journal of Agriculture Science and Technology 3: 67-77.
[20] Hajar, E. W. I., Sulaiman, A. Z. B., Sakinah, A. M. M. (2014): Assessment of heavy metals tolerance in leaves, stems and flowers of *Stevia rebaudiana* plant. – Procedia Environment Science 20: 386-393.
[21] Hajihashemi, S., Ehsanpour, A. A. (2014): Antioxidant response of *Stevia rebaudiana* B. to polyethylene glycol and paclobutrazol treatments under in vitro culture. – Applied Biochemistry and Biotechnology 172(8): 4038-4052.
[22] Ityel, E., Lazarovitch, N., Silberbush, M., Ben-Gal, A. (2012): An artificial barrier to improving root-zone conditions for horticultural crops: response of pepper plants to matric head and irrigation water salinity. – Agriculture Water Management 105: 13-20.
[23] Jamil, M., Bashir, S., Anwar, S., Bibi, S., Bangash, A., Ullah, F., Shikrha, E. (2012): Effect of salinity on physiological and biochemical characteristics of different varieties of rice. – Pakistan Journal of Botany 44: 7-13.
[24] Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., Tavarini, S., Guglielminetti, L., Angelini, L. G. (2015): The effect of soil moisture depletion on stevia (*Stevia rebaudiana*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1601_017028

Bertoni) grown in greenhouse conditions: Growth, steviol glycosides content, soluble sugars and total antioxidant capacity. – Scientia Horticulturae 183: 93-99.
[25] Liu, H., Tang, C., Li, C. (2016): The effects of nitrogen form on root morphological and physiological adaptations of maize, white lupin and faba bean under phosphorus deficiency. – AoB Plants 8: 1-14.
[26] Malamy, J. (2005): Intrinsic and environmental response pathways that regulate root system architecture. – Plant, Cell & Environment 28(1): 67-77.
[27] Montaña, L. A., Fischer, G., Magnitskiy, S., Zuluaga, G. (2014): Effect of NaCl salinity on seed germination and seedling emergence of purple passion fruit (*Passiflora edulis* Sims). – Agronomía Colombiana 32(2): 188-195.
[28] Munns, R., Tester, M. (2008): Mechanisms of salinity tolerance. – Annual Review Plant Biology 59: 651-681.
[29] Ober, E. S., Sharp, R. E. (2007): Regulation of root growth responses to water deficit advances in molecular breeding toward drought and salt tolerant crops, pp. 33-53. – Springer, Dordrecht.
[30] Panuccio, M., Jacobsen, S. E., Akhtar, S. S., Muscolo, A. (2014): Effect of saline water on seed germination and early seedling growth of the halophyte quinoa. – AoB Plants 6: 1-18.
[31] Polle, A., Otter, T., Seifert, F. (1994): Apoplastic peroxidase and lignifications in needles of Norway Spruce *Picea abies* L. – Plant Physiology 106: 53-60.
[32] Rahimi, A., Biglarifard, A. (2011): Influence of NaCl salinity and different substracts on plant growth, mineral nutrient assimilation and fruit yield of strawberry. – Notulae Botanicae Horti Agrobotanici Cluj-Napoca 39(2): 219.
[33] Ramakrishna, A., Ravishankar, G. A. (2011): Influence of abiotic stress signals on secondary metabolites in plants. – Plant Signaling and Behavior 6(11): 1720-1731.
[34] Raymond, J., Rakariyatham, N., Azanza, J. L. (1993): Purification and some properties of polyphenol oxidase from sunflower seeds. – Phytochemistry 34: 927-931.
[35] Reis, M., Coelho, L., Santos, G., Kienle, U., Beltro, J. (2015): Yield response of stevia (*Stevia rebaudiana* Bertoni) to the salinity of irrigation water. – Agriculture Water Management 152: 217-221.
[36] Safwat, A. A., Amany, K. I., Marwa, S. G., Walid, W. M., Salah, K. (2016): Diatomite alleviates the adverse effects of salinity stress on growth and yield of *Stevia rebaudiana*. – International Journal of Advanced Biotechnology and Research 7(1): 10-21.
[37] Sharifi, M., Ghorbanli, M., Ebrahimzadeh, H. (2007): Improved growth of salinity-stressed soybean after inoculation with salt pre-treated mycorrhizal fungi. – Journal of Plant Physiology 164(9): 1144-1151.
[38] Singh, D., Massod Ali, R., Basu, P. (2000): Genetic variation in dry matter partitioning in shoot and root influnces of chickpea to drought. – Paper presented at the 3rd International Crop Science Congress, Hamburg.
[39] Sun, J. K., Li, T., Xia, J. B., Tian, J. Y., Lu, Z. H. (2011): Influence of salt stress on ecophysiological parameters of *Periploca sepium* Bunge. – Plant Soil Environment 57: 139-144.
[40] Wu, Q., Zou, Y., Liu, W., Ye, X., Zai, H., Zhao, L. (2010): Alleviation of salt stress in citrus seedlings inoculated with mycorrhiza: changes in leaf antioxidant defense systems. – Plant Soil Environment 56(10): 470-475.
[41] Yeonghoo, K., Joji, A., Takuji, N., Norikazu Shinji, S., Kenji, U. (2004): Antioxidative responses and their relation to salt tolerance in Echi*nochloa oryzicola*Vasing and *Setaria virdis* (L.) Beauv. – Plant Growth Regulation 44: 87-92.
[42] Zheng, J., Chen, C., Li, D., Yi, B., Wy, W. (2013): Effects of salt stress on the growth, physiological responses, and Glycoside contents of *Stevia rebaudiana* Bertoni. – Journal of Agricultural and Food Chemistry 61: 5720-5726.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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