Morphologgical Changes and Antioxidant Activity of *Stevia rebaudiana* under Salt Stress

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ABSTRACT

***Stevia rebaudiana*, a herbaceous perennial shrub contains steviol glycosides, as an alternative source of sugar for diabetic patients. Salinity is one of the limiting factors determining plant distribution and survival in natural ecosystem. The objective of this study was to investigate morphological changes and antioxidant activity of *Stevia rebaudiana* under salt stress and the ability of tolerance of *Stevia* plants to salt stress. Plants of *Stevia* were subjected to different levels of salt (1.58, 2.58, 5.2, 6.60, 10.17 and 13.7 EC mmhos/cm), whereas control plants were watered with tap water. Plant height (cm), plant spread (NS--EW), leaves number per plant, stem numbers/plant, stem fresh weight (g), leaves fresh weight (g), stem dry weight (g), leaves dry weight (g) and antioxidant enzyme activity (peroxidase) were assayed during the different cutting time. All these parameters were found to be severely affected under salt condition. Salt treatment caused an increase in electrolyte leakage compared to control. There is an increase in antioxidant enzyme activity under the high level of salt compared to untreated control plants. The experiment emphasizes that variety of stevia which used under this study can planting in the salt soil until 5.20 EC salt as an agricultural crop to meet the challenges for sugar and energy crisis.**

Keywords: *Stevia*, salt stress, morphology, antioxidant enzymes

INTRODUCTION

Stevia rebaudiana (Bertoni), a non-caloric sweetener (family-Asteraceae) is cultivated for its sweetening compounds (the steviol glycosides). The two main glycosides of *Stevia* are stevioside (5% - 10% of dry leaves) and rebaudioside-A (2% - 4%). Due to the non-caloric and sweetening properties, stevioside has gained attention with the rise in demand for low-carbohydrate, and low-sugar food alternatives (Kalpana *et al*., 2009). The stevioside contents present in the leaves of *Stevia*, enhances the leaf of the plant to contain a massive caloric sweetener. Moreover, it is essential to understand that, Stevioside is a glycoside and the main characteristic is the huge increase in the sweetening power, ranging from 100-400 times higher than sucrose (Gujral, 2004). *Stevia rebaudiana* is an herbaceous perennial (2n = 22) (Midmore and Rank 2006). Consequently, *Stevia* has been cited for its ability to aid against several conditions which includes the following: Candida, high blood pressure, weight loss, tooth decay and gingivitis, digestive ailments, nicotine and alcohol cravings, acne and other skin ailments, sweetener for the growing diabetic population as well as for medicinal and household purposes (Gujral, 2004). *Stevia rebaudiana* is a sweet, green leaves that contain high amount of sweetener but has no calories. Inside *stevia rebaudiana* leaves, there are many sweet components includes stevioside, rebaudioside A, B, D, E and dulcoside. Stevioside is the most abundant component and is a natural sweetener and 300 times sweeter than sucrose. It is an alternative to sugar, that suitable for people who suffer from diabetes and hypertension because it has a negligible effect on blood glucose. *Stevia* leaves contain a lot of other nutrition such as protein, fibers, carbohydrates, phosphorus, iron, calcium, potassium, sodium, magnesium, iron, zinc, vitamin C and vitamin A (Abou-Arab *et al*., 2010). Dry leaves of stevia are sweeter approximately 10 to 15 times than sucrose (Raymond, 2010) while glycemic index is zero, so it is sweetener with no caloric value (Seema, 2010; Puri *et al*., 2011) and with proven non-toxic effect on human health (Barriocanal *et al*., 2008). *Stevia rebaudiana* Bertoni is native from northeastern Paraguay, and today it is cultivated around the world. Nowadays, attention is concentrated upon using Stevia in food industries, in order to close the gap between the production and consumption. The *Stevia* plant was recently introduced to Egyptian agriculture in order to produce a natural sweetener than can cover some of the lack of sugar production in Egypt (Alaam, 2007). Stevia cultivation in the Egyptian agricultural environment; one feddan of Stevia may produce up to 400 kg of Stevia sugar, annually. Taking the sweetening powder of the *Stevia* sugar into consideration; these 400 Kg of Stevia sugar are equivalent to about 80,000 sweetening units. Note that one feddan of "Sugar cane" produces about 5,000 sweetening units and one feddan of "Sugar beet" produces about 3,500 sweetening units. A sweetening unit is equivalent to the sweetness of one kilogram of sucrose (Alaam, 2007). *Stevia* plants are a good source of carbohydrates (61.93% d.w.), protein (11.41% d.w.), crude fiber (15.52% d.w.), minerals (K,

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21.15; Ca, 17.7; Na, 14.93 and Mg, 3.26 mg/100 g d.w. and Cu, 0.73; Mn, 2.89; Fe, 5.89 and Zn, 1.26 mg/100 g d.w.) also essential amino acids were found in amounts higher than those recommended by FAO and WHO for adults as well as non- essential amino acids (Esmat Abou-Arab *et al*., 2010).

Isozymes are defined as multiple molecular forms of a single enzyme. These forms usually have similar, if not identical, enzymatic properties, but slightly different amino acid compositions due to differences in the nucleotide sequence of the DNA that codes for the protein. Often the only difference among isozymes is the substitution of one to several amino acids. Only those isozymes that have large variations in size or shape or that differ in net charge can be separated by electrophoresis (Kumari, *et al*., 2009). Salt stress, like other abiotic stresses can lead to oxidative stress through the increase in reactive oxygen species (ROS), such as superoxide (O^{2-}), hydrogen peroxide (H2O2) and hydroxyl radicals (OH), which are highly reactive and may cause cellular damage through oxidation of lipids, protein and nucleic acids (Ying, *et al.,* 2007). To minimize the effect of oxidative stress, plant cells have evolved a complex antioxidant system, which is composed of low molecular mass antioxidants (glutathione, Malate and carotenoids) as well as ROS scavenging enzymes, such as superoxide dismutase (SOD), Esterase (EST), Malate dehydrogenase (MDH) and Glutamate dehydrogenase (GDH). Plant peroxidases have been used as biochemical markers for various types of biotic and abiotic stresses due to their role in very important physiological processes, like control of growth by lignification, cross linking of pectins and structural proteins in cell wall, catabolism of auxins. This investigation was carried out to study the effect of different salt concentrations on morphological characteristics of *Stevia* plants such as plant height (cm), Plant spread (NS-EW), number of branches per plant, Leaves number per plant, fresh weight of leaves (g) , dry weight of leaves (g), fresh weight of stems (g) and dry weight of stems (g), estimate the content of Stevioside and Rebaudioside A *via* HPLC, assay peroxidase activity and calculate proline content on *Stevia* plants under salt levels.

MATERIALS AND METHODS

The present investigation was carried out at Faculty of Agriculture Saba Basha, Alexandria University and Sabahia Agricultural Research Station during two harvest seasons 2014-2016. Samples of *Stevia rebaudiana* Bertoni were collected from healthy plants. *Stevia rebaudiana* (Bertoni) seeds were cultivated in pots (Bitmos: sand: clay) (1:1:1) at green house at Sabahia Agricultural Research Station. The plant material used in the present study consisted of one variety, named *Stevia rebaudiana* (Bertoni) C.V. Sponti (2n = 22). This genotype was obtained from Sugar Crops Research Institute (SCRI); Agricultural Research Center (ARC); Ministry of Agriculture, Egypt. After three weeks young plants were transferred to a large polythene bags and treated with different salt stress. Plants of *Stevia* were subjected to different levels of salt (1.58, 2.58, 5.2, 6.60, 10.17 and 13.7 EC mmhos/cm), whereas control plants were watered with tap water. When the plants became three months old they were evaluated and subjected to various analyses.

Morphological characteristics:

Plant height (cm), was expressed in cm, as the ratio between the measurements from the soil surface to the highest point of the plant; plant spread (NS--EW), was calculated from north to south and from west to east, leaves number per plant, calculated as the whole leaves number for five (each) plants; stem numbers/plant, recorded as mean number of branches per plant was obtained from total counting of branches from five plants; stem fresh weight (g), as yield of fresh stem matter was expressed in g/plant weighing five plants, using semi analytical balance; leaves fresh weight (g) calculated as leaves of fresh matter were expressed in g/plant weighing five plants; stem dry weight (g) recorded as stem yield of dry matter was expressed in g/plant, obtained weight of five plants with humidity between 10 and 15% and Leaves dry weight (g) calculated as leaves yield of dry matter was expressed in g/plant, obtained weight of five plants with humidity between 10 and 15%.

Extraction and estimation of Stevia sweeteners:

Stevia leaves were dried in an electric oven (E. Schulz & Co. Inh. Franz. Skorezewsh KG) at 50°C. *Stevia* sweeteners obtained from Stevia International Company for Agra industrial Projects (SKAP) then Stevioside standard preparation was carried out according to Nishiyama *et al*., (1992). Extraction of Stevia sweeteners from leaves were carried out by 0.5gm of dry stevia leaves. It was ground and dissolved in 0.5 ml methanol and put in shaking and heating for 30 minutes at 70°C then kept in room temperature for cooling then abukhner funnel was used for filtiration using Afilter paper one time after that we used Activated charcoal for filtration another a time finally we kept the filtrate frozen until analysis. Stevia leaves extract was separated and identified on HPLC by 210 nm (Agilent 1200PDA detector); Eclipse plus C18 column (3.5 mm 4.6x250 mm); linear gradient over 20min (84:55% CH3CN in H2O/ 0.1% TFA); flow rate 2.0 mL/min. Injection volume: 70 ml at ambient temperature (25°C). All the conditions used were

according to Makapugay *et al.,* (1984). For samples identification quantification and the retention time were as described by Makapugay *et al.,* (1984). Area under each peak was used to calculate the percent of each compound.

Isoenzymes electrophoresis:

Agar-starch-Polyvinylpyrrolidone (PVP) gel electrophoresis was carried out according to the procedures described by (Shaw & Kaen (1967) and Andrews (l981). The extracts were made by grinding from young leaves using of tissue in a mortar with 10 µl of electrode buffer and centrifuged for 15 sec, a sample of 10 µl of the homogenate was then absorbed onto a small rectangle (about 4mm X 2mm) of filter paper that was placed on the original line of gel plates, and after storage at 4 °C for 30 minutes, it was removed. This buffer was prepared by dissolving 92.75 gm of 0.3M Boric acid and 12 gm sodium hydroxide in 5 Liters of distil- led water then the solution adjusted to pH 8.3. The gel buffer used was 0.07 M Tris 0.007 M citric acid, pH 8.3. One liter of the gel buffer was prepared by dissolving 9.21 gm Tris, l.05 gm citric acid in distilled water, and kept in a refrigerator until experimental use. Agar-starch-Polyvinylpyrrolidone (PVP) gel was prepared by dissolving 1.0 gm, 0.5 gm PVP and 0.5 gm of hydrolyzed starch with 10 ml electrode buffer and 90 ml distilled water. The mixture was shaking vigorously and cooked in a boiling water bath until the solution become transparent. The hot liquid gel was poured on glass plates (20 X 30 cm) to produce a smooth surface layer with thickness of 0.8-0.9 mm, and kept at 4 °C until used (El-Metainy *et al*., 1977). Electrophoresis experiment were conducted in an incubator refrigerator adjusted at 4°C using a 250 volts AC electrical current, with constant voltage throughout the 90 minutes of the running period. Phosphate buffer with 0.l M, pH 7.0, was used as a staining buffer by adding 39 ml of 0.l M solution of monobasic sodium phosphate to 61 ml of 0.1 M solution of sodium dibasic phosphate and completed the final volume to 200 ml with distilled water. Each gel was incubated in l00 ml phosphate buffer, pH 7.0 containing 20 mg α-naphthyl acetate (α-NA), 20 mg β-naphthyl acetate (NA) dissolved in l ml acetone and completed to 5 ml by distilled water. The 50 mg fast blue RR salt, dissolved in 5 ml distilled water, was added after three minutes of the addition of α- and β-NA. Incubation was extended for thirty minutes at room temperature and complete darkness. Plates were than distained in distilled water until a clear background of gel plate (Youssef *et al*., 1989).

Proline Determination:

Proline was determined according to the method of Bates *et al*. (1973). 3% Aqueous Sulfosalicylic Acid, Acid Ninhydrin: 1.25 gm Ninhydrin, 30 ml glacial acetic acid, 20 ml 6M phosphoric acid. The mixture was warmed with agitation until dissolved then kept cool at 4 ^{o}C until use. 0.5 gm sample of leaf material was homogenized in 10 ml extraction buffer. The homogenate was filtered through Whitman No. 2 filter paper. 2 ml of filtrate were reacted with 2 ml acid ninhydrin and 2 ml glacial acetic acid in a test tube for 1 h at 100 ^{o} C, the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene mixed vigorously in a test tube with a stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was determined with spectrophotometer at 520 nm using toluene as a blank. Proline standard solution was prepared by dissolving 100 mg proline in 100 ml. of 3% aqueous sulfosalicylic acid. Aliquots of 10 µl to 50 µl of the Proline solution were put into test tubes. Then, the total volume was adjusted to 1 ml using 3% aqueous sulfosalicylic acid. Each tube was treated as previously described.

RESULTS AND DISCUSSIONS

Morphological studies of *Stevia rebaudiana*:

The data in Table 1 for plant height (cm) in *Stevia rebaudiana* under different salt concentrations indicated that with the increase in salt levels the value of this character was decreased from 71.34 to 50.43 cm in range of 20.91 cm, the highest value was recorded to the tap water with average of 71.34 and the lowest value was recorded to the maximum salt level 13.7 EC. Data showed that no significant variation between tap water and the second salt concentrations (1.58 EC), also the same results detected among the high salt concentrations (10.17 and 13.7 EC). On the other hand, the results clearly indicated that there were high significant variations between the other salt concentrations with $L.S.D._{.0.05}$=3.25. Although, the decrease in plant height with the increase in salt levels but the plant height stilling in good condition and the decrease was almost 29.31% comparing with the control.

Calculating the plant spread (NS--EW) in *Stevia rebaudiana* as morphological parameter under different salt concentrations recorded in Table 1. The data for the plant spread from north to south (N--S) indicated that with the increase in salt levels the value of this character was decrease from 25.09 to 12.72 cm in range of 12.37 cm. The highest value was recorded to the tap water with average 25.09 and the lowest value was recorded to the maximum salt level 13.7 EC by 12.72 cm. The data for the plant spread from East to West (E--W) indicated that with the increase in salt levels the value of this character was decrease from 23.11 to 9.16 cm in

range 13.95 cm, the highest value was recorded to the tap water with average 23.11 and the lowest value was recorded to the maximum salt level 13.7 EC by 9.16 cm. Data in Table 1 showed that no significant variation between tap water and the second salt concentrations (1.58 EC), also between third & fourth salt concentrations and the same results detected among the high salt concentrations (6.60 and 10.17 EC) for the plant spread from north to south (N--S) while it was between concentrations (10.17 and 13.70 EC) for the plant spread from East to West (E--W). On the other hand the results clearly indicated that there were high significant variations between the other salt concentrations with $L.S.D._{0.05}$=2.01 and 1.87, in respect.

Data in Table (1) indicated that with the increasing in salt levels the value of this character was decreased from 144.20 to 95.28 cm in range of 48.92 cm, the highest value was recorded to the tap water with average 144.20 and the lowest value was recorded to the maximum salt level (13.7 EC) was 95.28. Data showed that no significant variation between tap water and the second and third salt concentrations (1.58 and 2.58 EC) also the same results detected among the high salt concentrations (10.17 and 13.7 EC). The results clearly indicated that there were high significant variations between the salt concentrations with $L.S.D._{0.05}$=6.43.

The data in Table 1 for number of branches/plant in *Stevia* under different salt concentrations indicated that with the increase in salt levels the value of this character was decreased from 3.80 to 2.50 cm in range of 1.3 branch/plant, the highest value was recorded to the tap water with average 3.80 branch and the lowest value was recorded to the maximum salt level 13.7 EC was 2.50 branch/plant. There is relationship between the stem number and leaves number per plant and both are the most parameters which help the breeders to show the highest level of salt can add to the *Stevia* plants and the data proved that although the decrease in plant height with the increase in salt levels but the number of branches per plant stilling high and comparing with the control.

Data in Table 2 for fresh weight of leaves and stems (g) in *Stevia rebaudiana* under different salt concentrations 1.58, 2.58, 5.20, 6.60, 10.17, 13.70 EC indicated that with the increase in salt levels the value of both characters were decrease from 5.34 and 9.23 to 2.30 and 4.92 g, in respect with range 3.04 and 4.31 g, respectively. The highest value was recorded to the tap water with average 5.34 and 9.23 g and the lowest value was recorded to the maximum salt level 13.7 EC with 2.30 and 4.92 g. Data showed that no significant variation between tap water, second, third and forth salt concentrations, also data detected that the same results among the high salt concentrations 6.60, 10.17 and 13.7 EC. On the other hand the results clearly indicated that there were high significant variations between the other salt concentrations with $L.S.D._{0.05}$=1.67 and 2.04, in respect. Data in Table 2 for dry weight of leaves and stems (g) in *Stevia* indicated that with the increase in salt levels the value of both characters were decrease from 3.21 and 7.01 to 1.01 and 4.91 g, in respect with range 2.20 and 2.10 g, respectively. The highest value was recorded to the tap water with average 3.21 and 7.01 g and the lowest value was recorded to the maximum salt level (13.7) with 1.01 and 4.91 g. Data showed that no significant variation between tap water, second, third and forth salt concentrations, also data detected that the same results among the high salt concentrations (10.17 and 13.70 EC). On the other hand, the results clearly indicated that there were high significant variations between the other salt concentrations with $L.S.D._{0.05}$=0.85 and 1.98, in respect.

These results are in agreement with that mentioned by Abdullateef and Osman (2011) who evaluated 10 *stevia* accessions using morphological parameters and collected data were subjected to 't' test analysis. Also, the present results are agreed with Kassahun *et al.* (2012) who worked on some morphological character on *Stevia rebaudiana* Bertoni.

Table 1. Morphological variations of *Stevia rebaudiana* under different salt concentrations

Characters & Salt (EC)	Plant height (cm)	Plant spread NS	Plant spread EW	Leaves number/plant	Number of branches/plant
Control	71.34[a]	25.09[a]	23.11[a]	144.20[a]	3.80[a]
1.58	69.21[a]	24.19[ab]	23.00[a]	140.27[a]	3.40[ab]
2.58	65.30[b]	22.87[b]	21.10[b]	138.11[a]	3.40[ab]
5.20	61.34[c]	22.05[b]	20.09[b]	130.22[b]	3.10[abc]
6.60	55.91[d]	17.13[c]	15.10[c]	122.19[c]	3.00[bc]
10.17	51.39[e]	15.17[c]	10.43[d]	100.29[d]	2.88[bc]
13.70	50.43[e]	12.72[d]	09.16[d]	95.28[d]	2.50[c]
$L.S.D._{0.05}$	3.25	2.01	1.87	6.43	0.75

* 1.58: (0.585 g/L), 2.58: (0.585 g/L), 5.20: (0.585 g/L), 6.60: (0.585 g/L), 10.17: (0.585 g/L), 13.70: (0.585 g/L)

Table 2. Fresh and dry weight of *Stevia rebaudiana* under different salt concentrations

Characters	Fresh weight of leaves (g)	Fresh weight of stem (g)	Dry weight of leaves (g)	Dry weight of stem (g)
Control	5.34[a]	9.23[a]	3.21[a]	7.01[a]
1.58	5.10[a]	9.11[a]	3.01[a]	7.00[a]
2.58	4.54[ab]	8.29[ab]	2.91[a]	6.71[ab]
5.20	4.00[abc]	8.21[ab]	2.53[ab]	6.00[abc]
6.60	3.36[bcd]	6.27[bc]	2.01[bc]	5.21[abc]
10.17	2.64[cd]	5.03[c]	1.29[cd]	4.31[c]
13.70	2.30[d]	4.92[c]	1.01[d]	4.91[bc]
$L.S.D._{0.05}$	1.67	2.04	0.85	1.98

* 1.58: (0.585 g/L), 2.58: (0.585 g/L), 5.20: (0.585 g/L), 6.60: (0.585 g/L), 10.17: (0.585 g/L), 13.70: (0.585 g/L)

Data on survival count, survival percentage, number of branches/seedlings, number of leaves/branches and number of leaves/seedlings were recorded. The results showed that Mean squares from analysis of variance revealed the existence of a very highly significant influence ($P < 0.001$) of cutting position, node number and interaction effect of cutting position with node numbers on all the parameters considered in the study. The current work is agreed with Shizhen, and Wanzhong (1988) studied the variation of 14 important quantity character of *Stevia rebaudiana* Bertoni and the influence of different density on the variation. The results indicate the variation tended to appear similar in the same character. Some of the characters were more stable, namely the paired leaf numbers, number of nods before transplanting, transplanting and plant height, number of nods, length of leaf at harvest time. Some of the character was not stable, namely the number of branch and the yield of dry, fresh culm, leaf. The result of the analysis for character correlation was shown that some characters, closely related to yield, first of all were dry leaf weight, fresh leaf weight, fresh culm weight, dry culm weight per plant, then, culm width, number of branch, and the third, plant height, number of nod, paired leaf number before transplanting. The results agree with Oliveira *et al.* (2004) who examined some morphological features of strains of *Stevia rebaudiana.*

Amino acid composition of *Stevia rebaudiana* (g 100^{-1} dry matter)

Data in Table 3 for essential and non essential amino acid composition in *Stevia rebaudiana* under different salt concentrations 1.58, 2.58, 5.2, 6.60, 10.17 and 13.7 EC indicated that with the increase in salt levels the value of both Valine and Proline were increased from 0.71 and 0.020 to 1.70 and 1.78 g 100^{-1} dry matter, in respect with range 0.99 and 1.61 g 100^{-1} dry matter, respectively. Data in Table 3 showed that when the salt level increased the essential and non-essential amino acid composition was changed. The mostly two amino acid have high expression were Valine and Proline.

Biochemical markers (Isoenzymes)

The study of genetic polymorphism in the peroxidase enzyme system was conducted in *Stevia* plants under study. Data were presented for control and different cutting time to illustrate differences in the patterns. Zymograms showing electrophoretic profiles of the peroxidase enzyme system in *Stevia* plants.

In contrast, as shown in Figure (1, Peroxidase isozyme exhibited a wide range of variability among the different salt concentration. Two cathodal (Pex.2c and Pex.3c) were found as common band for all the samples and one band was different among all samples and control (Pex. 1c). While the results detected four anodal (Pex.1a; Pex.2a; Pex.3a and Pex.4a) bands were recorded in one common band for all samples (Pex.3a) and other one (Pex.1a) for cutting time one, two and cutting three. Pex.2a was recorded for control, cutting time one, two and three. Twelve loci were detected for the cutting time three comparing with control (7), cutting time one (8.67) and two (10) (see Figure 1 at top) The fourth band appeared in control and cutting time one. That mean when *Stevia* plants subjected to salt stress plants try to increase the antioxidant by increase the enzyme activity. With increase of NaCl the plants show high activity and density of peroxidase enzyme as show in Figure (1). Peroxidase isozyme exhibited a wide range of variability among the different salt concentration in the fourth and fifth cutting data comparing with control plants (Figure 1). Two cathodal (Pex.1c and Pex.2c) were found as common band for all the samples with the different between samples. The data showed that (Pex.1c) was common band for all plants and (Pex.2c) was different.

While the results detected two anodal (Pex.1a and Pex.2a) bands were recorded in common band for all samples (Pex.2a) and other one (Pex.1a) for cutting time four and five.

Table 3. Amino acid composition (E.A.A. and N.E.A.A.) of *Stevia rebaudiana* leaves (g 100 $^{-1}$ dry matter) under different salt concentrations

Amino acids	Control	1.58	2.58	5.20	6.60	10.17	13.70	Refrances	
								Abou-Arab *et al.*, (2010)	Li *et al.*, (2011)
E.A.A. g (100 $^{-1}$ dry matter)									
Arginine	0.61	0.56	0.56	0.46	0.44	0.35	0.31	0.45	0.81
Lysine	0.40	0.41	0.45	0.40	0.39	0.30	0.30	0.70	0.15
Histidine	1.67	1.66	1.13	1.13	1.00	0.29	0.30	1.13	0.34
Ph-alanine	0.79	0.77	0.70	0.70	0.70	0.66	0.70	0.70	0.88
Leucine	1.98	1.58	1.48	1.30	0.94	0.78	0.68	0.98	1.30
Methionine	1.33	1.30	1.30	1.22	1.21	1.00	1.00	1.45	ND
Valine	0.71	0.70	0.84	0.84	1.55	1.64	1.70	0.64	0.94
Threonine	1.10	1.00	1.01	0.93	0.91	0.81	0.77	1.13	0.75
Isoleucine	0.60	0.60	0.58	0.56	0.55	0.43	0.40	0.42	0.72
N.E.A.A. (g 100 $^{-1}$ dry matter)									
Aspartate	1.07	1.11	1.00	1.00	0.96	0.91	0.88	0.37	1.72
Serine	0.46	0.46	0.44	0.40	0.40	0.33	0.30	0.46	1.02
Glutamic	0.43	0.43	0.40	0.40	0.38	0.32	0.30	0.43	1.90
Proline	0.020	0.48	0.33	0.85	1.26	1.62	1.78	0.17	1.72
Glycine	0.25	0.25	0.25	0.20	0.20	0.19	0.15	0.25	0.85
Alanine	0.56	0.56	0.50	0.50	0.44	0.40	0.39	0.56	0.95
Cystenine	0.50	0.50	0.45	0.44	0.41	0.39	0.33	0.50	ND
Tyrosine	1.08	1.08	1.00	0.99	0.98	0.78	0.78	1.08	0.49

* 1.58: (0.585 g/L), 2.58: (0.585 g/L), 5.20: (0.585 g/L), 6.60: (0.585 g/L), 10.17: (0.585 g/L), 13.70: (0.585 g/L)

The enzyme activity was different from plant to other plant under the two different cutting data. In general there are high variations between the two different cutting time data after treatments with salt levels and the data showed that the control plants in cutting time five were separate of cutting time four as shown in Figure 1.

In contrast, as shown in Figure 1, Peroxidase isozyme exhibited a wide range of variability among the different salt concentration in the sixth and seventh cutting time data comparing with control plants. Three cathodal (Pex.1c, Pex.2c and Pex.3c) were detected and one of them Pex.2c found as common band for all the samples. The data showed that almost Pex.1c was also as common band for all plants except samples one in time six and control in time seven. While the results detected two anodal (Pex.1a and Pex.2a) bands were recorded for the last cutting date as common band for all samples (Pex.3a) was increase in the time six and the data showed three common band for this time. The enzyme activity were different from plant to other plant under the two different cutting data, the results indicated that when increase the salt or time of subject under salt the enzyme activity was increase and more locus were appear. *Stevia* is susceptible to water stress and that results in severe cell damages and growth reduction. The anti-oxidative capacity is also suppressed under severe stress. The necessity of carrying out this work was concerned as there is little knowledge of *Stevia* as an agricultural crop and our further research will highlight the strategy adopted for development of drought resistant *Stevia* plants which will alleviate the future threats for proper growth of Stevia plants in water deficit areas.

There was a high variability in enzyme activities observed in the leaves of well watered and stress plants of *Stevia*. The Payable On Death (POD) activity gradually declined in control plants where an ascending pattern was observed in stress plants with the greatest increase in moderately and mildly stressed plants at 1.58. 2.58 and 5.20 EC salt, accompanied by a gradual decrease in all conditions at 6.60, 10.17 and 13.70 EC.

This result was similar to that obtained by Bai *et al*., (2006). The level of antioxidants and the activities of antioxidant enzymes such as POD generally increased in plants under stressed conditions and in several cases their activity correlated well with enhanced tolerance, Prasad *et al*. (1994), Foyer *et al*. (1997). Ibrahim and Akladious (2014) also observed that drought stress resulted in considerable increase in the activity of GR, SOD and APX in shoots of soybean cv. Giza 22 and

111 as compared with control plants. The antioxidant potential of Stevia plants was high under mild and moderate stress but in severe stress it was low due to the fact that the plants were not able to defend against the extreme conditions of water stress.

Proline Determination

Proline content was determined in the present study as an indicator for salt tolerancet in of *Stevia rebaudiana* Bertoni. Results showed that the proline content was increased by increasing concentration of salt. The regression coefficient was done to determine the relationship between the two variables. Proline was considered as the dependent one (Y) while the salt concentration was determined as the independent variable (X) for *Stevia rebaudiana* Bertoni as shown in Figure 2 and Table 4. Results showed that increasing or decreasing in proline was due to the change in salt concentration. The results in Table 4 showed if salt increased from 1.58EC to 5.20EC, proline increased by almost 50% in mean from 0.488 to 0.846. Also with the high levels of salt the results were doubled that reached from 1.623 to 1.78 under 10.17 and 13.70 EC.

The decrease or increase of proline induction due to iso-osmotic stress (salt) indicated the suitability or tolerance of the species to afford abiotic stress, so that, when the proline production is increased, this indicates that the genotype can afford salt stress and it is tolerant to iso-osmotic stress. Again this indicates that *Stevia rebaudiana* Bertoni may be tolerant to salt stress. The disturbances in plant metabolism induced by abiotic treatments affect generally the various metabolic pools of iso-osmotic stressed plants.

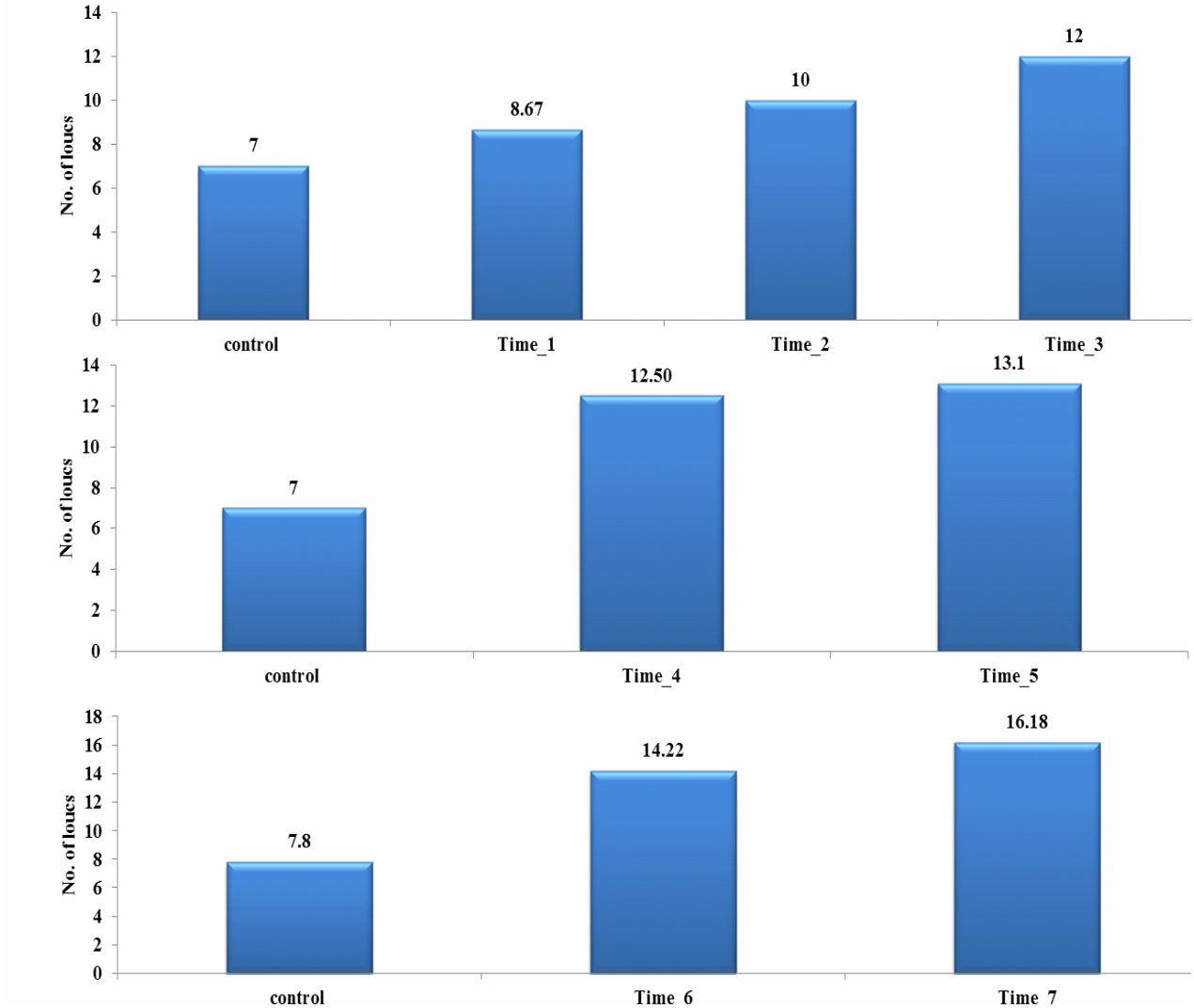

Figure 1. Peroxidase activity of *Stevia rebaudiana* Bertoni cultivar at different cutting times comparing with control under different salt levels

These changes in the contents of the various metabolites under salt treatments may indicate an enhancement or retardation in the synthesis, accumulation or consumption of these cellular metabolites. Proline, which frequently accumulates in stressed cells than any other free amino acid, was always correlated with the stress to which the plant cell is subjected. However, the values of these contents varied according to the degree of stress, the plant species was tested and organ analyzed.

These results are in accordance with those obtained by other authors, i.e. the accumulation of proline is frequently reported for most of the plant cells and tissues exposed to stress (Nayyar and Walia, 2003 and Errabii *et al.* 2007) change in proline content has been correlated with its capacity to tolerate and adapt to salinity conditions.

Chemical analysis for Stevia sweeteners (HPLC)

Stevia sweeteners were analyzed and determined employing the High Performance Liquid Chromatography HPLC. The data obtained are given in Table 5. From these data there were differences between the different salt treatments. With increase of salt levels from control to 13.7 EC data showed high variations in the total stevia sweeteners. The Concentration of total Stevia sweeteners (mg. sweeteners/g. dried leaves) were in control (65.44) compared with other treatments to be (30.1) in treatment (13.7 EC). There is no significant variation from the 0 to 5.20 EC in total Stevia sweeteners, while with the increase of salt levels the amount of total Stevia sweeteners were decreased. These Results gave evidence that these treatments are different and differential gene function was observed.

Table 4. proline content in *Stevia rebaudiana* Bertoni under different salt concenteration

Cultivar	Salt (EC)	Mean ± standard Error
Stevia rebaudiana Bertoni	Control	0.020 ± 0.002
	1.58	0.4885 ± 0.011
	2.58	0.3245 ± 0.010
	5.20	0.846 ± 0. 101
	6.60	1.2605 ± 0.161
	10.17	1.6235 ± 0.173
	13.70	1.7805 ± 0.189

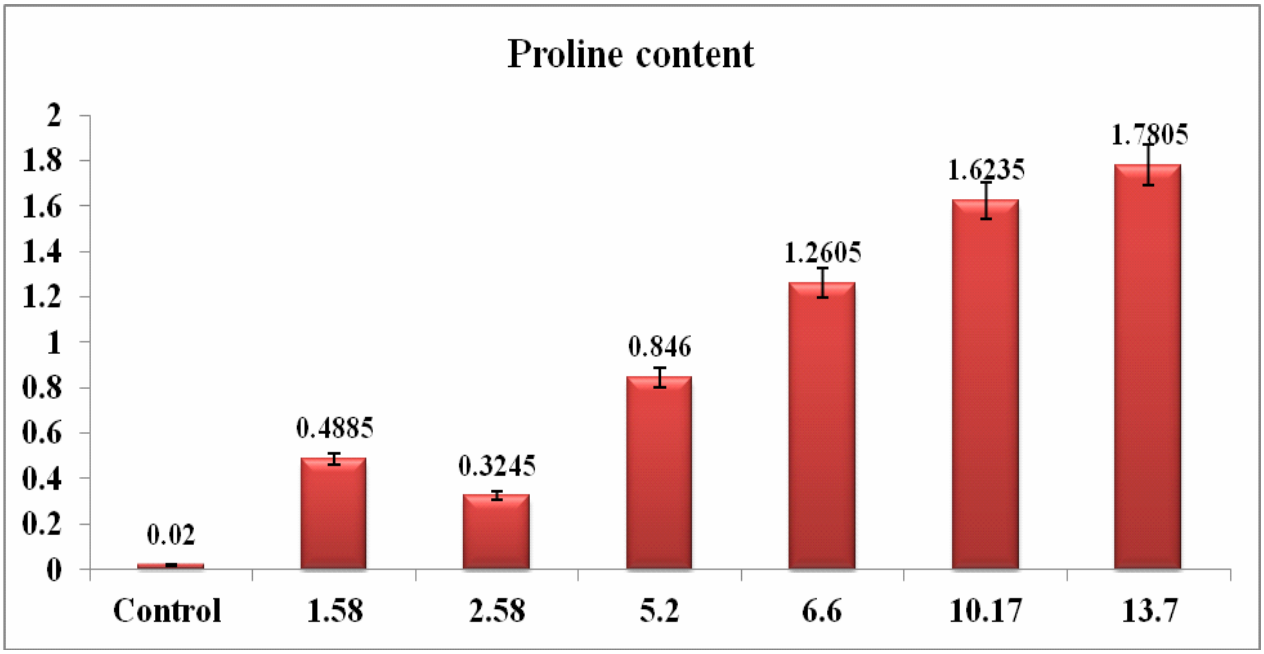

Figure 2. proline content in *Stevia rebaudiana* Bertoni under different salt concentrations

Table 5. Percentages of Stevia sweeteners component

Sweeteners	Salt concentrations						
	Control	**1.58**	**2.58**	**5.20**	**6.60**	**10.17**	**13.70**
Steviolbioside %	65.44	62.92	55.51	58.58	42.04	34.73	30.1
Stevioside %	16.86	15.99	14.42	12.59	8.70	6.26	6.0
Rebaudioside C %	1.23	1.0	0.09	--	--	--	--
Rebaudioside A %	3.79	3.07	3.06	2.34	2.77	2.77	2.11
Unknown %	12.68	17.02	26.92	26.49	46.49	56.24	61.79

These results are in agreement with Shibata *et al*. (1995) who observed a wide range of variation in the four main glycosides and found that dulcoside A and stevioside, and rebaudioside A and C, and it were positively correlated with each other. Stevioside and rebaudioside A, and dulcoside and rebaudioside C, were negatively correlated with each other. These correlations can be partially explained by the biosynthetic relationships between the individual glycosides because stevioside is the substrate for the synthesis of rebaudioside A, plants high in rebaudioside A will probably be low in stevioside. The current results is agree with Huang *et al*. (1995) who investigated the seedling population and clone variation from the difference of sweet component and content in *Stevia rebaudiana* Bertoni. The results showed: (1) there are 7.3% high yielding plants in seedling population, in which the proportion of R-A type with content of R-A over St content is 10.96%, and their R-A content varies from 3.3 to 12.0%. (2) Among the selected improved clone J-2, there are high significant difference in leaf size and steviosides content. The R-A content varies from 4.5 to 12.2%. (3) The individuals with R-A content of 7.04 apprx 12.03% from seedling population (original content 3.86%) and individuals with R-A content of 10.15 apprx 12.15% from improved individual clone (original content 9.10%) were selected out. Reis *et al.* (2015) concluded that stevia is suitable to be grown in semiarid and saline regions, if there is only one harvest; to obtain two or more harvests only fresh water with low electrical conductivity should be used. Moreover, it was shown that stevia crop tolerance to salinity was greater than the one of the sugar cane, and crop sensitivity to salinity was lower in stevia than in the conventional sugar crops. As final remarks, more research efforts are needed to generalize the use of stevia as a sugar substitute crop, such as the studies on the sweetener content of their leaves and its response to the application of fertilizers and composts.

REFERENCES

Abdullateef, R. A., and M. Osman. 2011. Influence of genetic variation on morphological diversity in accessions of Stevia rebaudiana Bertoni. Int. J. Biol. 3(3): 66-72.

Abou-Arab, A., MF Abu-Salem. 2010. Physico-chemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana Bertoni plant. Afr. J. Food Sci. 4:269–281.

Alaam, A.I. 2007. Sugar crops council: Future view. The Proceeding of Thirty-eight Annual Conference, Egyptian Sugar Expertese Society Hawamdia, Egypt

Amzad-Hossain,M., A.Siddique., S.Mizanur-Rahman., M.Amzad- Hossain. 2010. Chemical composition of the essential oils of Stevia rebaudiana Bertoni leaves. Asian Journal of Traditional Medicines 5 (2): 56–61

Andrews, A.T. l981. Electrophoresis: Theory, Techniques and Biochemical and Clinical Application. Clarendon Press, Oxford, pp. 336.

Ashraf, M., J.C, Harris. 2004. Potential biochemical indicators of salinity tolerance in plants. Plant Science, 166 :3–16.

Bai, L.-P., F.-G.Sui, T.-D.Ge, Z.-H. Sun, Y.-Y. Lu, and G.-S. Zhou. 2006 Effect of Soil Drought Stress on Leaf Water Status, Membrane Permeability and Enzymatic Antioxidant System of Maize. Pedosphere, 16, 326-332. http://dx.doi.org/10.1016/S1002-0160(06)60059-3 [

Barriocanal L., M.Palacios, G.Benitez, S.Benitez, J.T.Jimenez, N.Jimenez. 2008. Apparent lack of pharmacological eff ect of steviol glycosides used as sweeteners in humans, a pilot study of repeated exposures in some normatensive and hypotensive individuals and in type 1 and type 2 diabetics. Regulatory Toxicology and Pharmacology 51 (1): 37-41

Bates. L, RP.Waldren. ID.Teare. 1973. Rapid determination of free proline for water stress studies. Plant and Soil, 39, 205-207

EL-Metainy, A.Y., A.Y. Abou-Youssef & M. El-Hadad. 1977. Starch degrading isozymes in Triticumn aestivum, Triticumn pyramidal, and their interspecific hybrid. Egypt.J.Genet.Cytol., 6: 375-379.

Errabii T., C.B.Gandonou., H.Essalmani, J.Abrini, M.Idaomar, N.S. Senhaji. 2007. Effects of NaCl and mannitol induced stress on sugarcane (Saccharum sp.) callus cultures. Acta Physiologiae Plantarum, 29: 95–102.

Esmat, A., A.Abou-Arab, Azza Abou-Arab and M. Ferial Abu-Salem. 2010 Physico-chemical assessment of natural sweeteners steviosides produced from Stevia rebaudiana bertoni plant African Journal of Food Science Vol. 4(5) pp. 269- 281,

Foyer, C.H., H.Lopez-Delgado, J.F. Dat, and I.M.Scott.1997 Hydrogen Peroxide- and Glutathione-Associated Mechanisms of Acclimatory Stress Tolerance and Signalling. Physiologia Plantarum, 100, 241-254.

Gujral SR. 2004. Stevia 0% Calorie, 100% Sweet, 100% Nature. Science Tech. Entrepreneur, 12(10).

Huang, S. Ying, G,Guo. Ai , Y. Qian, Cheng, Y. Lan, Gu and F. Hong. 1995. Studies on the variation of steviosides content and selection of type R-A in Stevia rebaudiana. Journal of Plant Resources & Environment. 4(3): 28-32.

Ibrahim, M.H., and S.A. Akladious. 2014. Influence of Garlic Extract on Enzymatic and Non Enzymatic Antioxidants in Soybean Plants (Glycine max) Grown under Drought Stress. Life Science Journal, 11, 46-58.

Kalpana,M., M.Anbazhagan, and V. Natarajan. 2009. Utilization of Liquid Medium for Rapid Multiplication of Stevia rebaudiana Bertoni. Journal of Ecobiotechnology, 1, 016-020

Kassahun, B.M, Z.D.Zigene, W. Kebede, and H.Gebremeskel. 2012. Performance of Stevia (Stevia Rebaudiana Bertoni) for morphological and economic traits under different ecologies of Ethiopia, Journal of Agriculture and Development (JAD), vol. 2, no. 2, pp. 54-67.

Khatab,A.2015. Peroxidase Isozyeme and Proline Content In Relation To Salinity Tolerance in Stevia Plant. Alex. Science Exchange J.3:206-212

Kumari, S.S., S.B. Adiver, Mallesh AND Malik Ahmed Pasha 2009 Isozyme Variabilty in Phaeoisariopsis personata (Berk. and Curt.) von Arx Causing Late Leaf Spot of Groundnut (Arachis hypogaea L.) International Journal of Plant Protection, Vol. 2 No. 2 : 219-23 (April to September, 2009)

Li G., Wang R, AJ.Quampah, Z .Rong, Shi C, Wu J. 2011. Calibration and Prediction of Amino Acids in Stevia Leaf Powder Using Near Infrared Reflectance Spectroscopy. J. Agric. Food Chem. 59:13065–13071.

Makapugay H., N.Nanayakkara, A.Kinghorn. 1984. Improved high performance liquid chromatographic separation of the Stevia rebaudiana sweet diterpene glycosides using linear gradient elution. Journal of Chromatography 283: 390–395

Midmore, J.D., A.H. Rank 2006. An intense natural sweetener-laying the ground work for a new rural industry; May 2006 RIRDC Publication No 06/020 RIRDC Project No UCQ-17A

Nayyar, H. and D.P. Walia. 2003. Water stress induced proline accumulation in contrasting wheat genotypes as affected by calcium and absisic acid. Biol. Plant., 46: 275-279.

Nishiyama, P., M.Alvarez, and L. G. E. Vieira. 1992. Quantitative analysis of stevioside in the leaves of Stevia rebaudiana by near infrared reflectance spectroscopy. J. Sci. Food Agric. 59: 277–281.

Oliveira, D. M. V., E. R. Forni-Martins, P. M. Magalhaes and M. N. Alves. 2004. Chromosomal and morphological studies of diploid and polyploidcytotypesofSteviarebaudiana (Bertoni) Bertoni (Eupatorieae, Asteraceae). Journal of Genetics and Molecular Biology. 27(2): 215-222.

Prasad, T.K., M.D.Anderson, B.A.Martin, and C.R. Stewart. (1994) Evidence for Chilling-Induced Oxidative Stress in Maize Seedlings and a Regulatory Role for Hydrogen Peroxide. The Plant Cell, 6, 65-74. http://dx.doi.org/10.1105/tpc.6.1.65

Puri, M., D.Sharma, A.K.Tiwari. 2011. Downstream processing of stevioside and its potential applications, Biotechnology Advances 29: 781-791.

Raymond K.W. (2010). General Organic and Biological Chemistry, John Wiley & Sons, USA, (516): 364-368.

Reis, M., L.Coelho, G.Santos, U.Kienlec, J.Beltrão. 2015. Yield response of stevia (Stevia rebaudiana Bertoni) to the salinity of irrigation. Agricultural Water Management (152): 217–221.

Seema T. 2010. Stevia rebaudiana: A medicinal and nutraceutical plant and sweet gold for diabetic patients, International Journal of Pharmacy & Life Sciences ISSN: 0976-7126

Shaw, M. D., and A. L. Kaen.1967. Starch gel electrophoresis of enzymes. Biological. Research laboratory publication. Howthron center.

Shibata, H., Y. Sawa, T. Oka, S. Sonoke, K. K. Kim, and M. Yoshioka .1995.Steviol and steviol-glycoside:Glucosyltransferase activities in Stevia rebaudianaBertoni-Purification and partial characterization. Journal of Archives of Biochemistry and Biophysics. 321(2): 390-396.

Shizhen, S., and W. Wanzhong.1988. The Variation of Stevia rebaudiana Bertoni Quantity Character and Relations to The Yield. Journal Acta Agronomia.14(2): 167-173.

Ying J., Li H., P. Murray., Z. Gao., Y.W.Chen., Y.Wang., K.Y. Lee., A.T.Chan., R.F. Ambinder., G.Srivastava, et al. 2007 Tumor-specific methylation of the 8p22 tumor suppressor gene DLC1 is an epigenetic biomarker for Hodgkin, nasal NK/T-cell and other types of lymphomas. Epigenetics, 2:15–21.

Youssef, M.K., E.A. Badr, A. Abou-Youssef. & S. El-Deeb.1989. Comparative electrophoretic pattern of isozymes in four species of tilapia, third Egyption British conference on Animal, Fish and Poultry production.Alexandria, 7-10 October, 849-858.

الملخص العربي

التغيرات المورفولوجية ونشاط مضادات الاكسدة للاستيفيا تحت الاجهاد الملحى

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محصول الاستيفيا من المحاصيل الواعدة الجديدة فى المجتمع المصرى كمحصول سكر هام يتميز بزيادة نسبة المادة المحلاة به مقارنتا بالمحاصيل السكرية الاخرى مثل قصب السكر وبنجر السكر ولة اهمية اقتصادية كمحلى طبيعى يستخدم فى الصناعات الغذائية وانتاج مواد التجميل ويتعرض هذا المحصول الى تاثير سلبى على المحصول بتغير الظروف البيئية خاصة الملوحة والجفاف. وتم دراسة تأثير الاجهاد البيئى مثل الملوحة على الصفات المورفولوجية والبيوكيمائية فى النبات وتقدير الحمض الامينى البرولين لتوظيف الاصناف الناتجة فى برامج الانتخاب والتربية وقياس مدى النشاط الانزيمى لنبات الاستيفا تحت الاجهاد الملحى والجفاف واخيرا قياس محتوى المادة المحلاة فى النباتات الناتجة. اظهرت النتائج وجود اثر معنوى فى جميع الصفات المدروسة. كما اظهرت النتائج ايضا انة مع زيادة تركيز الملح يحدث نقص فى بعض الاحماض الامينة وزيادة فى البعض وبالاخص الحامض الامينى البرولين حيث ذاد افراز هذا الحامض مع زيادة تركيز الملح وكما هو معروف علاقتة بتحمل النباتات للضغوط البيئة الغير حيوية ومنها الملوحة والجفاف حيث ذاد نسبة البرولين من ٠,٠٢٠ الى ١,٧٨ فى تركيز الملح الاعلى. وتم تقدير نشاط انزيم البروكسيديز فى نبات الاستيقا على فترات مختلفة تحت ظروف الاجهاد الملحى وكانت النتائج واضحة انة مع زيادة تركيز الملح وزيادة مدة بقاء المحصول فى الارض نجد انة يزداد افراز هذا الانزيم كأداة للدفاع عن النباتات اثر التعرض للاجهاد الملحى.