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STUDYING cDNA SCoT IN RESPONSE TO SALINITY STRESS IN *STEVIA REBAUDIANA* BERTONI

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SUMMARY

The present study was carried out with two cultivars of *Stevia rebaudiana* Bertoni i.e., 'Sugar High-A3' and 'Spanti' during 2018-2019 at Sugar Crops Research Institute, Giza, Egypt, and College of Agriculture Engineering Sciences, Baghdad University, Iraq. The objectives of said research were to determine the performance of Stevia cultivars for growth and yield related traits, and gene expression for salinity tolerance under varied NaCl stress levels. Calluses of these two Stevia cultivars (Sugar high-A3 and Spanti) were cultured on MS medium supplemented with NaCl at five concentration levels (0.00, 500, 1000, 2000, 3000 mg L^{-1}). Generally, the morphological characteristics of Stevia cultivars negatively responded to increase in NaCl concentrations *in vitro* such as callus survival and regeneration percentages, plantlets length, multiple shoots, leaves per plant, roots per plantlet and root length. The higher salinity concentration of NaCl (3000 mg L^{-1}) induced the lowest significant percentage of callus survival, especially with Spanti (32.65%) as compared with control (62.0%). The mean of regeneration percentage reached the maximum value with control (38.52%), and decreased gradually with increasing NaCl concentrations until it was 23.95% with 3000 mg L^{-1}. However, it was feasible that salinity did not reduce the average length of shoots, the highest significant value of multiple shoots was obtained with control with 0.00 NaCl (14.05), while the lowest significant value (8.46) was observed with 3000 mg L^{-1} NaCl. Moreover, leaves per plant were reduced under salinity stress, 0.00 and 500 mg L^{-1} NaCl treatments achieved the highest significant number of leaves (9.19 and 8.17), respectively, followed by 1000 mg L^{-1} (7.29). Cultivar sugar high-A3 was more tolerant than cultivar Spanti to salinity stress, and the average numbers of roots were 6.24 and 3.96, respectively and could be used as part of a better strategy to reclaim salt affected soils. The RNA extracted from the treated plants used to synthesis cDNA, five markers of cDNA- SCoT were used. Primer SCoT5

produced a fragment with a molecular size of 585 bp, also Primer SCoT6 produced fragments with a molecular size of 623, 450 and 313 bp, while primer SCoT7 produced fragments with a molecular size of 290 bp. The fragments produced by primers SCoT5, SCoT6 and SCoT7 that mentioned above were observed in salt tolerance plantlet and not found in the sensitive ones. The cDNA- SCoT markers technology may work well and identify and develop the salt stress tolerance lines in Stevia crop.

Key words: Stevia (*Stevia Rebaudiana* Bertoni), salt stress, NaCl, growth and yield traits, *in vitro*, SCoT, cDNA, callus, regeneration

Key findings: In inbreeding project, two cultivars of Stevia i.e., Sugar high-A3 and Spanti were screened to identify the salt tolerant lines by using the cDNA-SCoT markers. Results revealed that Stevia cultivar Sugar high-A3 was found more tolerant to salinity than Spanti.

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INTRODUCTION

Stevia rebaudiana Bertoni is a small perennial herb (2n = 22) belong to family Asteraceae. Stevia has an extensive root and brittle stems producing small leaves. The stevia plant is known to get the yield of steviol glycosides (SGs) that is about 300 times sweeter than sugar. The Stevoside and rebaudioside-A are considered being the main sweetening compounds in Stevia. These compounds applied in medicine, food industries (Akbari *et al*., 2017; Esmaeili *et al*., 2016).

Salt stress causes more than 50% yield losses in major crops around the world depending on the crop (Shahverdi *et al*., 2015; Al-Taweel *et al*., 2018). Salinity stress affects the many metabolic and physiological processes of the plant which can damage the cells. High salinity stress causes ionic and osmotic stresses in the plant tissue, which lead to several morphological, anatomical and physiological modifications (Hasan *et al*., 2018). In many crop species, the genetic diversity can be determined using morphological and physiological characteristics as well as bio-chemical and DNA marker analysis and to use in the development of the genotypes with genetic potential of salt tolerance (Liu, 1997).

Salinity is one of the most important and harmful environmental factors limiting, and 19.5% of the irrigated agricultural land is considered saline, so most of the crops are sensitive to salinity caused by high concentrations of NaCl in the irrigated water and soil. Salinity is one of important abiotic factors limited plants germinations, vegetative growth and productive in many crops (Al-Taweel *et al*., 2018). Furthermore, salt also effect the growth, yield and survival of *Stevia rebaudiana,* therefore, the said study has prime importance to study the effects of various salinity levels on the

agronomic traits and the changes caused in its physiological characteristics (Jamil *et al*., 2012; Islam *et al*., 2014).

Morphological and physiological traits are subjective and quantitative in practice, and can be affected by many environmental factors of various stress conditions and the experience of evaluators (Bolaric *et al*., 2005). Regarding to the literatures review, there are some reports on the effect of salt stress on physiological, morphological and biochemical characteristics of Stevia (Rathore *et al*., 2014; Zeng *et al*., 2013; Gupta *et al*., 2014; El-Housini *et al*., 2015). Shahverdi *et al*. (2017) findings indicated that the Stevia plants which irrigated with Hoagland nutrient solution and low level of NaCl (30 mM) during 62 days in the greenhouse caused the highest percentage of Stevoside, Rebaudioside-A and Stevioside + Reb-A, Chlorophyll a, carotenoids, total sugar, and the Stevia was considered as a moderate salt tolerant plant. However, at the same time their study has not shown data on investigation of morphological traits, genes expression and amount of both Stevoside and rebaudioside-A under *in vitro* conditions simultaneously.

Molecular techniques have been proposed to be appropriate powerful tools for identification of some clonal variation, stress tolerance and establish genetic stability (Rahman and Rajora, 2001; Bennic *et al*., 2004; Javanmardi *et al*., 2011; Khaled *et al*., 2018). A method for gene differential expression in plants based on the start codon targeted polymorphism (SCoT) DNA marker technique, called cDNA-SCoT (Wu *et al*., 2013). The cDNA-SCoT is advantageous method compared to all other existing ones because it is relatively more efficient, cheaper, faster, simpler to operate, and the results can be easily reproduced (Luo *et al*., 2014). A cDNA-SCoT (cDNA starts codon-targeted) markers used for determining the gene expression in *Phoenix dactylifera, Saccharum officinarum, Mangiferaindica*, sugarcane, Olive tree and *Dendrobium officinales* (Munns and Tester, 2008; Chen, *et al*., 2013; AL-Janabi and Al-Rawi, 2018). However, the cDNA-SCoT markers are used for genetic diversity assessment in various plant species (Luo *et al*., 2010; Al-Qurainy *et al*., 2015). The cold resistance related genes have been studied in sugarcane under cold and drought stress using the cDNA-SCoT technique (Chen *et al*., 2010; Li *et al*., 2013; Abd El-maksoud *et al*., 2018).

Knowledge of molecular mechanisms in *Stevia rebaudiana* Bertoni under salinity stress conditions is limited. Therefore, in present study, the experiments were carried out on two cultivars of *Stevia rebaudiana* (Sugar high-A3 and Spanti) to determine the performance for growth and yield related traits and gene expression profiling under different NaCl salinity stress levels.

MATERIALS AND METHODS

Plant material

The study was carried out on two cultivars of *Stevia rebaudiana* Bertoni, the first one was 'Sugar High-A3' which has many branches, larger leaves, bushy plant and contain high glycosides, and its seeds imported from Ever Stevia company, Canada. The second cultivar was 'Spanti' which has small leaves and contain less

glycoside than 'Sugar High-A3', having Spanish origin, and its seeds imported from Fito Seed Company, Spain for seeds production and improved by tissue culture propagation with single plant selection programs at Sugar Crops Research Institute, Giza, Egypt. The plant's leaves were used as the source of explants for callus initiation, regeneration, multiplication and rooting. The leaves were washed with tap water and sterilized by 70% ethanol for 5 min, followed by immersion in chemical disinfectant Clorox at 20% sodium hypochlorite (NaOCl 5.25%) supplemented with 150 mg L^{-1} of ascorbic acid for twenty min., then rinsed four times each for ten min in distill water.

Mode of excision

Margins of expanded leaves (10-12 mm long) removed, the remaining part cut transversely to the midrib into two portions. Then the leaf portions dissected into small pieces (0.3 cm) and cultured in MS media (Murashige and Skoog, 1962).

Callus induction shoots regeneration and rooting

Leaf segments cultured in MS medium supplemented with 3% sucrose, 1.10 mg L^{-1} BA, 0.55 mg L^{-1} IBA, 0.7% agar and the pH adjusted to 5.8. Explants were grown at 25°C and 16 h light photoperiod with a light intensity of 2000 lux provided by white cool fluorescent tubes. Callus cultured in MS media supplemented with 0.00, 500, 1000, 2000 and 3000 mg L^{-1} NaCl as a source of salinity for *in vitro* salt stresses. The subculture performed every four weeks using the same media, and then the maintained calluses then transferred to the same previous MS media which supplemented with 0.5 mg L^{-1} IBA and 2.10 mg L^{-1} BA for the regeneration of shoots. The number of shots per callus pieces recorded after two months from transferring the callus to the regeneration media. multiple shoots separated and transferred vertically on ½ MS with 2% sucrose, 0.7% agar, 0.5 mg L^{-1} IBA and PH adjusted to 5.5 as a rooting media for four weeks. Data recorded as percentages of survival and regeneration of callus, plantlet length (cm), multiple shoots, leaves per plant, roots per plant, and root length (cm).

cDNA SCoT reaction

The RNA extracted from treated and control plants according to the Trizol method as reported by Luo *et al*. (2014). The RNA used to synthesis cDNA by adding 1 µl of oligo dT to RNA and incubated at 66°c for 5 min. After thawing on ice for two minutes, reverse transcriptase 1 µl 5 x buffers, two µl of dNTPase and one µl of reverse transcriptase enzyme were added. The samples incubated for one cycle in PCR at 42°c for one hour and another termination cycle at 70°c for 5 min carried out. cDNA concentration was measured using Fluorometer, and 100 ng of cDNA was used to conduct the reaction for all the samples.

cDNA SCoT PCR reaction

The cDNA-SCoT technique was used for the comparison the response of *Stevia rebaudiana* cultivars changed at various concentrations of NaCl

Table 1. SCoT primers sequences used in PCR reaction.

No.	Primer code	Primers sequence (5`-3`)
1	SCoT4	CAA CAA TGG CTA CCA CCT
2	SCoT5	CAA CAA TGG CTA CCA CGA
3	SCoT6	CAA CAA TGG CTA CCA CGC
4	SCoT7	CAA CAA TGG CTA CCA CGG
5	SCoT8	CAA CAA TGG CTA CCA CGT

under salinity stressconditions, which could be possible due to the expression of different genes. Five primers (cDNA- SCoT Oligo primer, macro gene Company) were used in present investigations (Table 1).

Amplification conditions

All the components of reaction mixture (25 µL) were taken for the amplification and further development of the SCoT markers. PCR reaction performed on MJ 200CT 96-well thermal cycler. Initial denaturation set out at 95°C for 4 min, followed by 40 cycles at 95°C for 51°C for 1_min, 72°C for 1_min, and a final extension at 75°C for 5 min. The amplification products separated on 1.3% agarose gel containing ethidium bromide against 100 bp DNA ladder. The agarose gel visualized under an Uvidoc HD6 (UVITEC Cambridge-UK).

Statistical analysis

This experiment was carried out in a completely randomized design. Each treatment was performed in ten jars containing five explants and each experiment replicated three times. Data were analyzed with two-way analysis of variance using SPSS statistical program (Version 25 64x edition). The treatment means was compared using Duncan's Multiple Range Test at $P < 0.0528$. The banding patterns generated by SCoT primers used to determine the difference between the two cultivars of *Stevia rebaudiana* using 1D software (Total Lab software v2009, Nonlinear Dynamics, UK).

RESULTS AND DISCUSSION

Plantlets of *Stevia rebaudiana* showed significant differences in response against different salt stresses in all measured traits, including percentages of survival and regeneration of callus, multiple shoots, leaves per plant, plant length, roots per plant, and root length (cm).

The comparison of mean data revealed that salt stress reduced the values for all traits as compared to control, and the reduction ranged between 14-40% (Figures 1-5). Cultivar Sugar high-A3 recorded with low reduction values for all studied traits than cultivar Spanti. The most affected trait was the number of multiple shoots which reduced by 40% and the lowest reduction was recorded in root length (14%), followed by plantlet length (28%) as compared to control. The lowest callus survival value was observed in cultivar Spanti (32.65±2.89%) grown on medium supplemented by 300 mg L^{-1} NaCl and has significant reduction (47.3%)

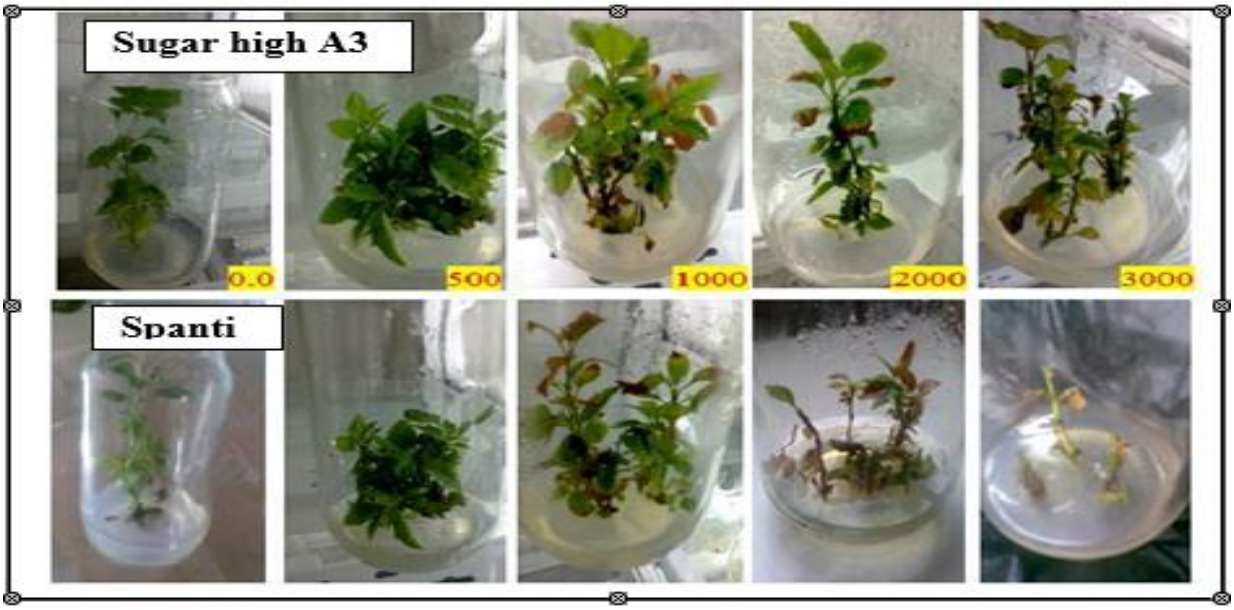

Figure 1. Effect of NaCl with different concentrations on the growth and development of two Stevia cultivars (Sugar high-A3 and Spanti).

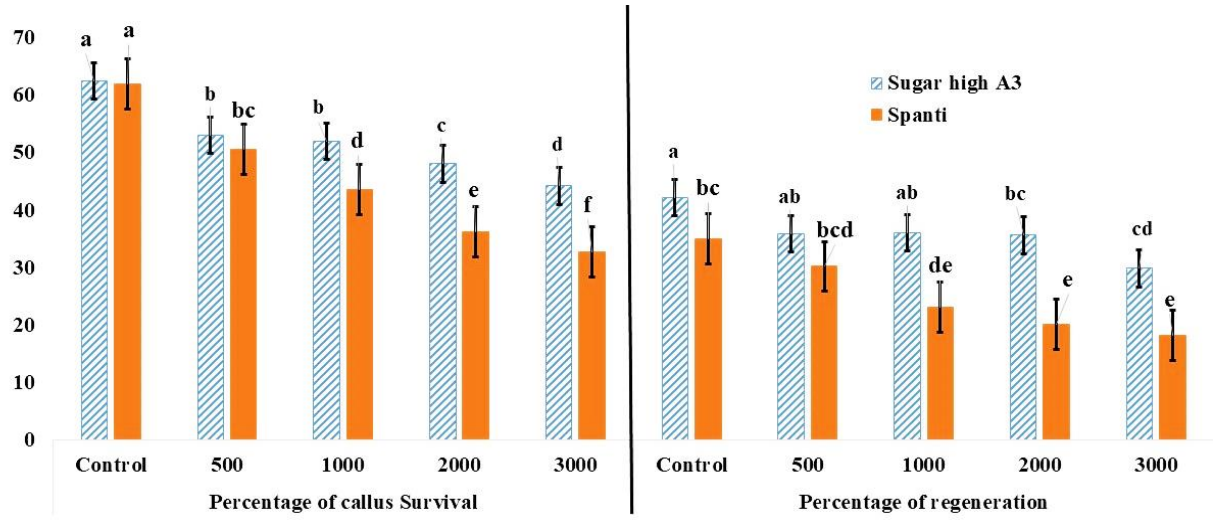

Figure 2. Effect of NaCl with different concentrations on the percentage of callus survival and percentage of regeneration in two Stevia cultivars.

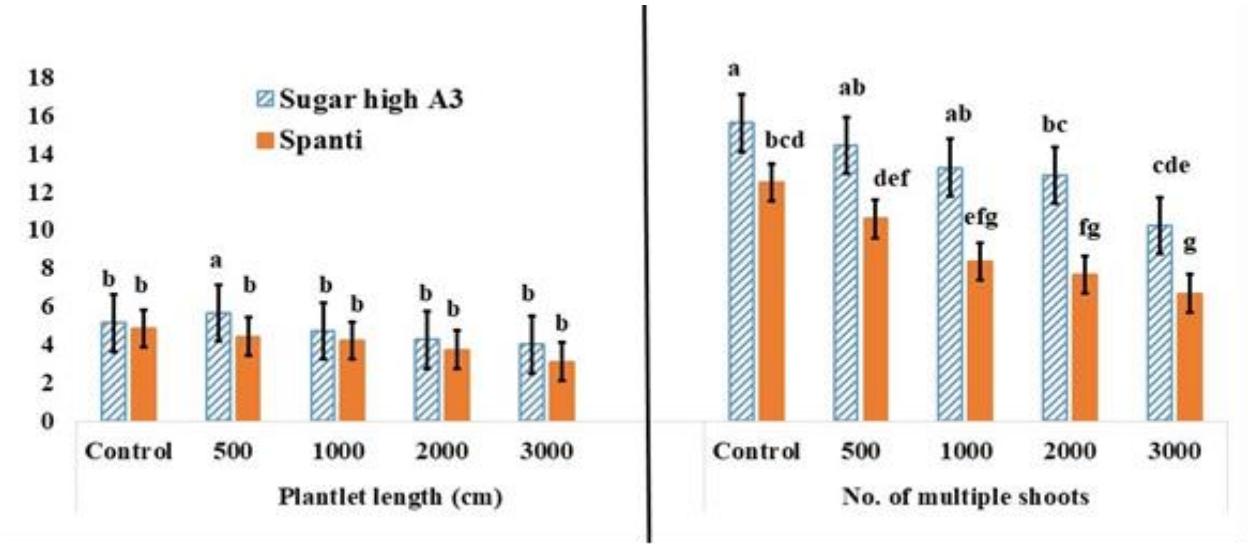

Figure 3. Effect of NaCl with different concentrations on plantlet length and number of multiple shoots in two Stevia cultivars.

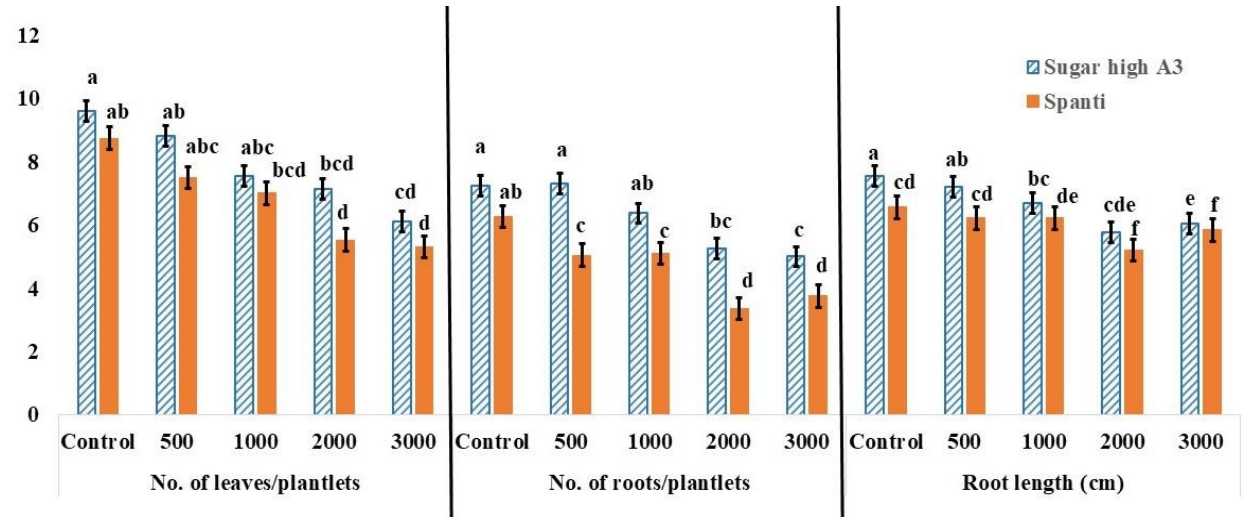

Figure 4. Effect of NaCl with different concentrations on leaves per plantlets, roots per plantlets and root length in two Stevia cultivars.

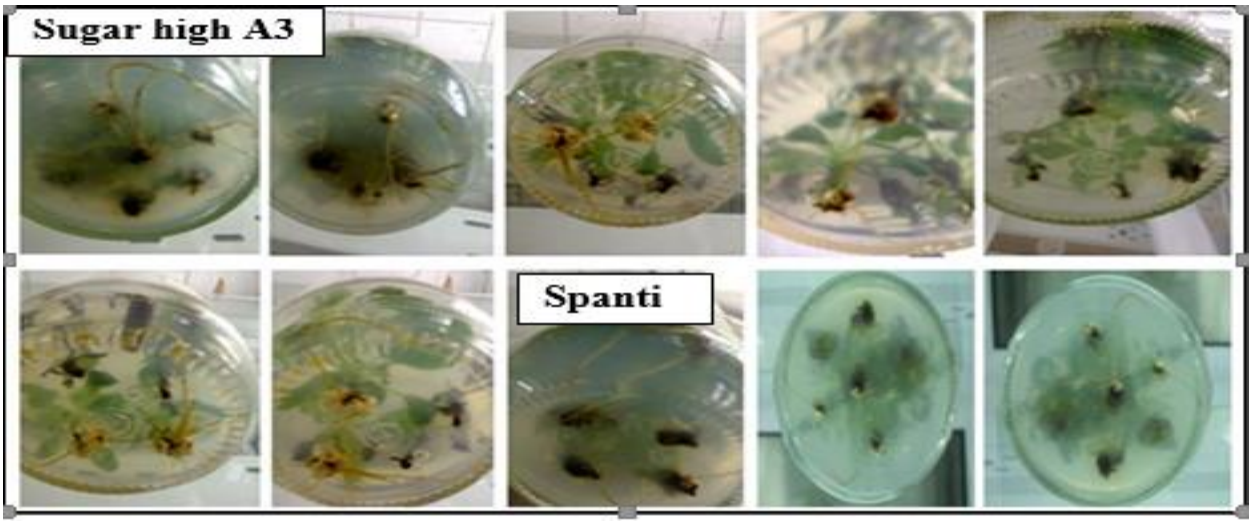

Figure 5. Development of rooting in two stevia cultivars (Sugar high-A3 and Spanti) plants *in vitro*.

(Figure 2). However, the highest value of callus survival with salt stress was seen in cultivar Sugar high-A3 (53.09±0.625%) grown on medium supplemented by 500 mg L^{-1} NaCl. On the other hands, the control without any additional NaCl recorded with callus survival values in Spanti (62±0.125%) and Sugar high-3 (62.5±0.125%). The ability of the Stevia cultivars i.e., Spanti and Sugar high-A3 to renew growth has significantly differed under the stress conditions caused by different NaCl salinity levels (Shahverdi *et al*., 2017).

The mean regeneration percentage gain the maximum value with control (38.52%), which gradually decreased with enhancement in NaCl concentrations i.e., 3000 mg L^{-1} (23.95%) (Figure 2). Cultivar Sugar high-A3 (35.90%) was affected less by salinity treatments as compared to cultivar Spanti (25.23%). Concerning interactions between salinity and Stevia cultivars, it prevailed that the regeneration percentage was significantly highest in cultivar Sugar high-A3 cultured on 0.00 NaCl (42.11%), while the lowest significant mean was obtained with cultivar Spanti cultured at 3000 mg L^{-1} NaCl (18.00%). For average length of shoots, the results were feasible and salinity did not effect and reduce the shoot length in both cultivars (Figure 3). The metabolic pathways are altered as a result of salinity/NaCl stress in the case of the formation of callus, shoot, and root of *S. rebaudiana* (Javed and Gurel, 2019).

The concentration of 500 mg L^{-1} NaCl exhibited the highest value of shoot length (5.05 cm). However, a significant difference detected between both cultivars, Cultivar Sugar high-A3 revealed significantly greater value (4.76 cm) as compared to cultivar Spanti (4.08 cm) (Figure 3). Regarding the interactions between cultivars and salinity levels, cultivar Sugar high-A3 achieved significantly highest mean value (5.66 cm) at 500 mg L^{-1} as compared to other treatments resulted nonsignificant differences. Salinity stress caused

reduction in leaf fresh and dry weight, chlorophyll a, b, and the total chlorophyll and enunciated negative relationship between NaCl concentrations and glycoside levels in *stevia rebaudiana* plants (Shahverdi *et al*., 2017).

The multiple shoots were significantly affected by rising of salinity levels in the medium (Figures 1 and 3). The highest significant mean of multiple shoots was obtained with control at 0.00 NaCl (14.05) while the lowest significant mean was obtained with 3000 mg L^{-1} (8.46). Cultivar Spanti was more affected by salinity as compared to cultivar Sugar high-A3. Concerning the interaction effect of cultivars and salinity on the said trait, the control gave the highest significant mean value for multiple shoots (15.6) as compared to other treatments. However, the lowest significant mean value was achieved in cultivar Spanti at 3000 mg L^{-1} NaCl (6.68). Salinity also affected the leaves per plant in both cultivars of *stevia rebaudiana* (Figure 3). The treatments having 0.00 and 500 mg L^{-1} NaCl achieved the highest significant numbers of leaves per plant (9.19 and 8.17), respectively, followed by 1000 mg L^{-1} NaCl (7.29). However, significantly lowest and same leaves per plant were recorded at 2000 and 3000 mg L^{-1} NaCl (6.34 and 5.71). Cultivar Sugar high-A3 revealed the significantly highest number of leaves per plant (7.85) as compared to Spanti (6.82). Hence, NaCl plays the role of abiotic stress elicitor, causing an accumulation of reactive oxygen species and thus altering metabolic processes and physiology of Stevia cultivar under in vitro culture conditions (Javed and Gurel, 2019). Genetic approaches are available to identified the action of the related enzymes in the plants and how their catalytic enzymec activities may be related to physiological functions.

Plant response to salinity involves the perception of signal stress by receptors at the membrane level followed by signaling transduction in the cell, inducting a multiplicity of biochemical mechanisms involved in the protective role of secondary metabolites. The present findings are in analogy with Pandey and Chikara (2015) as reported that NaCl significantly affect the number of shoots, shoot length, root number, and root length of Stevia. However, with increase in NaCl concentration the leaf dry weight (DW), stem DW, root DW, shoot DW and leaf FW were markedly decreased by 30-70 % and 50-55 %, respectively at 100mM, and suggested that NaCl is an enhancer acting as a repressor of transcription to genes of steviol glycoside biosynthesis pathway that could alter the production of steviol glycosides.

Results revealed that salinity had a significant effect on the average number of roots per plantlet (Figures 4 and 5). The highest significant value achieved with control at 0.00 NaCl (6.76), while the lowest significant value was recorded at 3000 mg L^{-1} NaCl (4.38). Cultivars showed the same trend, however, Sugar high-A3 was found more tolerant than Spanti to salinity with mean number of roots i.e., 6.24 and 3.96, respectively. The interactions between studied variables revealed significant differences for number of roots in both cultivars grown with 0.00 NaCl (7.26 and 6.27), respectively. The significantly least number of roots was recorded with cultivar Spanti (3.36 and 3.76) cultured at 2000 and 3000 mg L^{-1}, respectively. Also, the data shown in Figure 4, the effect of exposure to

Table 2. Primers and the molecular size of the amplified fragments of *Stevia rebaudiana* treated with different concentrations of NaCl (mg L^{-1}).

Primers Name	M.S. bp	500	1000	2000	3000
SCoT4	877	+	+	+	+
	864	+	+		
	735		+	+	+
	706	+			
	649	+	+	+	+
	587	+			
SCoT5	729	+	+	+	+
	585		+	+	
	531	+	+	+	+
	397		+	+	+
	298	+	+	+	+
	233	+	+	+	+
	217		+	+	+
SCoT6	668	+	+	+	+
	623		+	+	
	450		+	+	
	429	+			
	400			+	
	360				+
	313		+	+	
	269	+	+	+	+
	93	+	+	+	+
SCoT7	451	+	+	+	+
	292		+	+	
	236	+	+	+	+
	202	+	+	+	+
	193	+	+	+	+
	169	+	+	+	+

salinity in-vitro on the root length, the control (0.00 NaCl) and 500 mg L^{-1} treatments were recorded with significantly highest mean for root length (7.06 and 6.72 cm), respectively. However, significantly least mean values for said trait were recorded with 2000 mg L^{-1} (5.49 cm) followed by 3000 mg L^{-1} NaCl (5.95 cm). The cultivar means exhibited that cultivar Sugar high-A3 produced the significantly lengthy roots (6.66 cm) as compared to Spanti (6.02 cm) (Figure 4). Genetic approaches are available to identified the action of the related plant's enzymes and hormones and how their catalytic activities may be related to physiological functions (Hasan *et al*., 2018).

In interactions of cultivars and salinity levels, the highest significant average root length was achieved by cultivar Sugar high-A3 cultured with 0.00 NaCl. While the lowest significant means (5.21 and 5.85 cm) were recorded with cultivar Spanti cultured with 2000 mg L^{-1} followed by 3000 mg L^{-1} NaCl, respectively. Cultivar Spanti showed more sensitivity to salt stress than Sugar high-A3. Hossain (2008) and Pratibha *et al*. (2010) also found that plant response to salt stress varies at different developmental stages. Increased salinity during plant

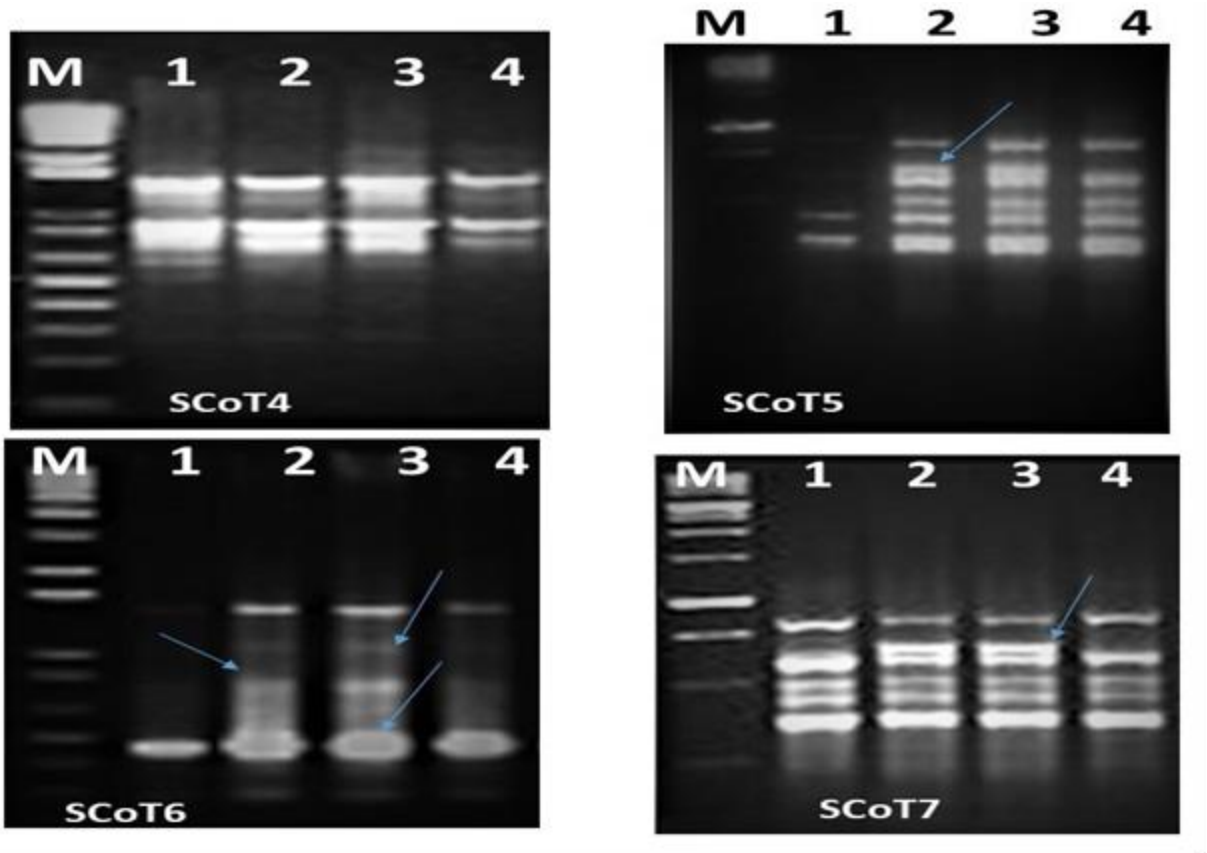

Figure 6. cDNA-SCoT markers profiling generated from individual plant leaf of Stevia at NaCl different concentrations (SCoT primer 4, 5, 6, 7). Lane M: 100 bp ladder; lanes 1 and 4 (mother plant); lanes 2 and 2 cultivars of Stevia.

development stage would delay germination, reduction in vegetate growth and formation of thinner roots. Stalinization can inhibit both cells division and cells expansion in growing tissues of Stevia plant. 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 (Javed and Gurel, 2019). In addition, transcript expression profiling of Stevia genes involved in steviol glycoside biosynthesis path way showed an increased in expression genes i.e., UGT85C2, KAH, UGT74G1 and UGT76G1 in 50, 75 and 100 mM of NaCl concentrations as compared to control (Pandey and Chikara, 2015).

In this investigation, five cDNA-SCoT primers (Oligo primer, Macro gene Company) were used with two Stevia cultivars. A cDNA-SCoT marker profile generated with primers SCoT4, SCoT5, SCoT6 and SCoT7 (Figure 6). The cDNA-SCoT primers produced 134 bands, 29 of these (21.6%) were polymorphic between the two cultivars as shown in Table 2 and Figure 6. Primers SCoT5 produced polymorphic fragment with a molecular size 585 bp, and Primer SCoT6 produced polymorphic fragments with a molecular size of 623, 450 and 313

bp, while primer SCoT7 produced polymorphic fragments with a molecular size of 290 bp. The polymorphic fragments produced by primers 5, 6 and 7 revealed that these such type of fragments were only observed in salt tolerance plantlets but not found in the sensitive ones. Therefore, these fragments could be declared as positive marker for salt tolerance in *Stevia rebaudiana*, and cDNA-SCoT markers may identify as a salt stress marker, so we device to repeat this study on another *stevia rebaudiana* cultivars for final conclusion to use cDNA-SCoT markers profiling to appear a variations in different gene expressions between treated and untreated Stevia plants under various salinity stress, as reported by Al-Qurainy *et al*. (2017) in their study on Phoenix plant.

CONCLUSION

High concentrations of NaCl caused negative influence on all studied variables of both cultivars of *Stevia Rebaudiana*. Increasing NaCl concentrations reflected varied behavior of different explants under *in vitro* conditions. Results suggested that varying NaCl concentrations imposes osmotic imbalance in *Stevia Rebaudiana* plants. In present investigation, the cDNA-SCoT marker technique was used to identify the salinity tolerance in cultivars of Stevia under salinity stress. A cDNA-SCoT marker showed variation in different gene expressions profiling between the treated plantlets with different NaCl concentrations and untreated planlets. The polymorphic fragments produced by primers SCoT5, SCoT6, and SCoT7 in the salt tolerant plantlets but not observed in the sensitive ones. Therefore, these fragments declared as positive markers for identification of Stevia populations by cDNA-SCoT markers. The regenerated and acclimatized plantlets will be transplanting in the field for further studies to be used in future breeding programs to develop a range of Stevia lines with genetic potential of salinity tolerance.

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