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Original Research

Effect of salinity on gene expression, morphological and biochemical characteristics of *stevia rebaudiana* Bertoni under *in vitro* conditions

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Abstract: *Stevia rebaudiana* Bertoni is a famous medicinal plant for its low calorific value compounds which are named steviol glycosides (SGs) and they are 150-300 times sweeter than sugar. Among various SGs, stevioside and rebaudioside A considered to be the main sweetening compounds. Soil salinity is one of the most essential stress in the world. Salinity affects the survival and yield of crops. In current study the effects of salinity and osmotic stress caused by different concentration of NaCl (0, 20, 40, 60 and 80 mM) on morphological traits, genes expressionand amount of both stevioside and rebaudioside Aunder *in vitro* conditions has been investigated. The morphological traits such as bud numbers, root numbers, shoot length (after 15 and 30 days) were evaluated. With increasing salinity, the values of all studied morphological traits decreased. To investigation of *UGT74G1* and *UGT76G1* genes expression that are involved in the synthesis of SGs, RT-PCR was done and there were significant differences between all media. The highest expression of both genes was observed in plantlets grown on MS media (with NaCl-free). Also, the lowest amounts of gene expression of the both genes were seen in MS+ 60 mM NaCl. Based on HPLC results, the highest amount of both stevioside and rebaudioside A were observed in plantlets grown in MS media (with NaCl-free). Finally, it can be concluded that stevia can survive under salt stress, but it has the best performance in the lower salinity.

Key words: *Stevia rebaudiana*; Tissue Culture; NaCl; RT- PCR; HPLC.

Introduction

The *Stevia rebaudiana* Bertoni (2n = 22) is one of 154 members of genus *Stevia* (family Asteraceae) of the South American that commonly known as sugar leaf, candy leaf, sweet weed, sweet grass or honey leaf. Stevia is famous for its low calorific value compounds. The active compounds of stevia are steviol glycosides (SGs) including: Rebaudioside A, B, C, D, E, F, M, Stevioside, Steviol bioside, Dulcoside A and Dulcoside C which are 150-300 times sweeter than sugar and differ to genotype and production environment.Stevioside and rebaudioside A considered to be the main sweetening compounds. These compounds are applied for medicinal, food and cosmetic industries (1-8).

The steviol glycosides biosynthesis pathway divided in two steps. These two steps consist of multiple genes such as *DXS, DXR, CMS, CMK, MCS, HDS, HDR, GGDPS, CDPS, KS, KO, KAH, UGT85C2, UGT, UGT74G1* and *UGT76G1*. The first step starts with 2-C-methyl-D-erythritol-4 phosphate (MEP) pathway that happens in plastids. The second step starts with steviol molecules to the latest synthesis of rebaudioside A is governed by three UGTs (UDP-glycosyltransferases) via *UGT85C2*, *UGT74G1*, and *UGT76G1*. An enzyme encoded by *UGT74G1* and *UGT76G1* has been recognized for giving rise to glucosylate steviolbioside and stevioside to stevioside and rebaudioside A, respectively (9-13).

Soil salinity is one the most essential abiotic stress characteristics which limits plant development and productivity. Plants grown in fields with high salinity are totally affected by osmotic stress and therefore they encounter with lot of different morphological, physiological and biochemical (14). Furthermore, salt effects on the survival and yield of *S. rebaudiana* so it has become important for us to know the changes in these characteristics (14-18).

Also, examining these responses is problematic under field circumstances, however plant tissue culture techniques are done under sterile and controlled environmental conditions. The benefits of this technique make several chances for researchers to study the reactions of plants against abiotic and non-abiotic stresses (19-20).

According to our literature review, there are some reports on the effect of salt stress on morphological, physiological and biochemical characteristics of stevia (15-16,21-22) but there have not investigated morphological traits, genes expression and amount of both stevioside and rebaudioside A under *in vitro* conditions simultaneously.

In this study, the *S. rebaudiana*response to salt and osmotic stress caused by different concentration of NaCl has been investigated. Therefore, the present study was carried out with an objective of studying the effect of salinity (NaCl) on morphological characteristics (bud number, root number and shoot length), expression level of genes (including *UGT74G1* and *UGT76G1*) in steviol glycosides biosynthesis pathway and the most important steviol glycosides (stevioside and rebaudioside A) contents of *S. rebaudiana* under *in vitro* condition.

Materials and Methods

Plant materials and culture conditions

In present study, *stevia rebaudiana* Bertoni plantswere provided from Zagros Bioidea Co. Razi University Incubator, Kermanshah, Iran. Axillary buds of about 2 cm in length with two leaves were separated from the shoots. The culture medium which used to stevia propagation was MS (23), with 30 g/L sucrose supplemented and different concentrations of NaCl (0, 20, 40, 60 and 80 mM). The pH of the medium was adjusted to 5.8 and after adding 8 g/L agar and then autoclaved. Each medium contained five explants and cultures were incubated at 25 ± 1°C under 16 h light and 8 h dark photoperiod provided by cool white fluorescent lamps with 3000 Lux intensity and relative humidity 72 to 75%.

Morphological traits

Morphological characteristics including "number of buds and roots after 30 days" and "shoot length after 15 and 30 days" were recorded.

RNA extraction

Total RNA of fresh leaves was extracted using RNX plus™ kit (Cinnaclon) according to the manufacturer's recommendations. RNA quantification was done using NanoDrop Spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, and Wilmington, USA). All RNA isolates had an OD260:OD280 between 1.8 and 2.0, The RNA quality was furthermore tested by 1.0% agarose gel electrophoresis.

Expression analysis of *UGT74G1* and *UGT76G1* genes

The two-step semi-quantitative RT-PCR method was used to determine gene expression of UGT74G1 and UGT76G1 genes in stevia. For cDNA synthesis, 10 µg of total RNA was reversely transcribed with 100 U M-Mulv reverse transcriptase in a total volume of 20 µL of Master Mix containing 1 µL oligo (dT)18 primer, 2 µL of 10X M-MuLV buffer, 1 µL of each dNTP and Nuclease-free Water, according to the manufacturer's recommendations (Viva 2-steps RT-PCR Kit, vivantis, Malaysia). The *β-Actin* house-keeping gene was used as the internal control. Primers for target and *β-Actin* genes were designed using the Oligo 7 Primer Analysis Software and to achieve specific characters required for semi quantitative polymerase chain reaction (RT-PCR) (24; Table 1). RT-PCR reactions were performed for the targets and house-keeping gene. The PCR reaction mixture (25 µL) contained 2µL of cDNA, 0.5 µL of dNTPs (10 mM), 1 µL of each primer (Forward and Revers primer), 0.32 µL of MgCl2, 2.5 µL of 10x PCR buffer and 0.5 µL of *Taq* DNA polymerase (5U/µL). The PCR reaction was performed as initial denaturation at 94°C for 7 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C for 7 min.

The PCR products were separated by electrophoresis on a 1% agarose gel in TBE buffer. Four independent experiments were conducted. The amplicons were quantified by the Total Lab TL120 v2009 software (Nonlinear Dynamics Ltd). Which delivers quantitative estimates of the amplicon band intensities by changing them into corresponding numerical values. The expression levels of *UGT74G1* and *UGT76G1* were normalized relative to the amount of β-actin expression.

HPLC analysis

The contents of stevioside and rebaudioside A were estimated in the leaves of stevia treated by different concentration of NaCl by the method described earlier (25). Powder of dried leaves of stevia which treated by different concentrations of NaCl (0, 20, 40, 60 and 80 mM) was applied to extracting by 80% methanol for 3–4 times and after that dried in vacuo and defatted with hexane and residual extract was vacuum dried. Then extract was dissolved in acetonitrile and filtered. The chromatographic separation was achieved using a symmetry Xbridge amide column (4.6 × 150 mm, 3.5 lm, Waters, USA) at 50 °C, mobile phase involved acetonitrile:water (80:20) in isocratic elution mode with detector wavelength 210 nm. The injection volume was 10 µl with a flow rate of 0.8 ml/min. The stevioside and rebaudioside A estimations were achieved using three independent replicates.

Statistical analysis

Data analysis was performed by Excel and SPSS Ver. 16 softwares. Collected data had a normal distribution, so it was used directly for statistical analysis. Also mean comparison was performed by Duncan's multiple range test with critical value of $P < 0.05$.

Results and discussion

Effect of different concentrations of NaCl on Stevia morphological traits

Stevia plantlets, under different concentration of NaCl, showed significant differences (0.01 %) in the all measured traits, including bud number, root number and

Table 1. List of primers used in RT-PCR and house-keeping genes.

Gene	Primer sequence 5´→ 3´ (forward/reverse)	Amplicon length (bp)	Accession number
UGT74G1	AATCGGGCCAACACTTCCAT/ TCGGGTCCATGTTTCACCAG	174	AY345982
UGT76G1	GACCAACAACCGCCAAGTTC/ CCCAAGAACCCATCTGGCAA	185	AY345974
β-Actin	TTGCCCTGAGGTTCTGTTCC/ATCCGGTCAGCAATACCAGG	171	AP548026

Table 2. Mean squares for the effect of different concentration of NaCl on *stevia rebaudiana* Bertoni after 15 and 30 days of the experiment (where SL15= shoot length after 15 days, SL30= shoot length after 30 days, BN15= number of buds after 15 days, RN30= number of roots after 30 days).

		Mean Squares			
Source	df	BN15	RN30	SL15 (mm)	SL30 (mm)
Salinity	4	2.300**	7.633**	2463. 710**	41488.080**
Error	25	0.246	0.906	213.860**	919.21*
CV (%)		30	35	8	22

ns= non-significant; ** = Significant differences in the levels of 0.01; * = Significant differences in the levels

shoot length (both after 15 days and 30 days) (Table 2).

The mean comparison results were shown in Table 3 and Figure 2. According to the table, the lowest bud numbers (0.66 buds) were observed in MS medium+ 80 mM NaCl that it had significant differences with other media. Also the highest amount of this trait (2.16 buds) was seen in MS medium without any additional NaCl. There are no significant differences between other NaCl concentrations in bud numbers. Additionally, the highest root numbers were seen in NaCl-free MS medium (3.83) and there were significant differences between NaCl-free and other medium with different concentrations of NaCl except MS+ 20 mM NaCl. Also, the lowest root numbers (1.16) were measured in MS+ 80 mM NaCl medium, but there were no significant differences between this medium and MS+ 40 and MS+ 60 mM NaCl. On the other hand, the highest amount of shoot length both in 15th day (64.67 mm) and 30th day (232.17 mm) was seen in MS media with NaCl-free, and the lowest shoot length was observed in MS+ 80 mM media for both on the 15th day (8.83 mm) and 30th day (18.67 mm) of measurement. Obviously, the best media for stevia in this research was MS media NaCl-free (control). Shoot proliferation of nodal explants in various mediums (MS+ different concentration of NaCl) after 30 days is shown in Figure 1.

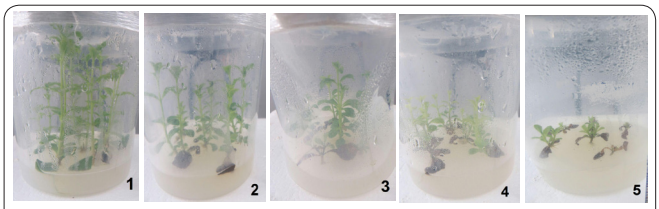

Figure 1. Shoot proliferation from nodal explants on medium (MS+ different concentration of NaCl) after 30 days. (Where, 1=0 mM NaCl (MS), 2 = MS + 20 mM NaCl; 3 = MS + 40 mM NaCl; 4 = MS + 60 mM NaCl; 5= MS + 80 mM NaCl).

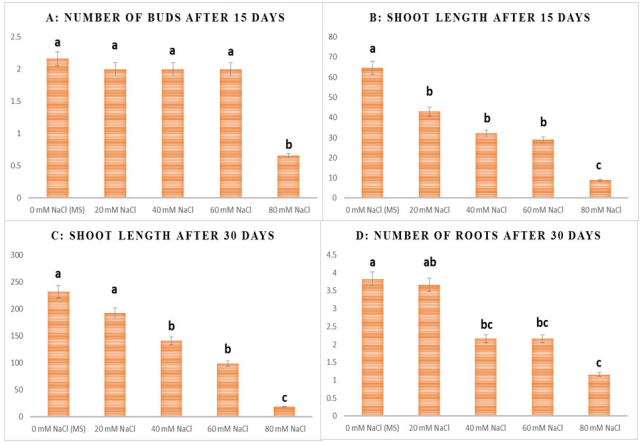

Figure 2. Effect of different concentrations of NaCl on *Stevia rebaudiana* after 15 and 30 days of culture. In each of the graphs, horizontal curves indicating the trait numbers (Where, 1=0 mM NaCl (MS), 2 = MS + 20 mM NaCl; 3 = MS + 40 mM NaCl; 4 = MS + 60 mM NaCl; 5= MS + 80 mM NaCl), (A: number of buds after 15 days, B: shoot length after 15 days, C: shoot length after 30 days, D: number of roots after 30 days).

Investigation of *UGT74G1* and *UGT76G1* genes expression

The results of RT- qPCR were normalized to the level of the housekeeping gene of β-actin in plants subjected to different concentrations of NaCl. There were significant differences between all media with different concentrations of NaCl. The highest expression of both *UGT74G1* and *UGT76G1* genes that are involved in the synthesis of steviol glycoside was observed in plantlets grow on in MS media without any additional NaCl (control) accounting for exactly 1.0 (Figure 3). Also, the lowest amounts of gene expression of the both genes were seen in MS+ 60 mM NaCl. For both genes, there was a decreasing trend from NaCl-free media to medium with 60 mM NaCl but it was seen that expression raised when NaCl was increased from 60mM to 80 mM in tissue culture medium of stevia. These increasing was from approximately 0.55 to nearly 0.6 for *UGT74G1* and it was from just over 0.6 to over 0.7 (Figure 3). On the other hand, in media containing NaCl except MS+20 NaCl, expression of *UGT76G1* was more

Table 3. Effect of different concentrations of NaCl on *Stevia rebaudiana* after 30 days of culture. MS culture without NaCl as a control. (Where SL15= shoot length after 15 days, SL30= shoot length after 30 days, BN15= number of buds after 15 days, RN30= number of roots after 30 days) Mean values within a column with the same letter are not significantly different based on least significant difference (LSD) at p = 0.05.

	Salinity treatments	BN15	RN30	SL15 (mm)	SL30 (mm)
1	MS (control)	2.16 a	3.83 a	64.67 a	232.17 a
2	MS + 20 mM NaCl	2.00 a	3.66 ab	43.00 b	192.68 a
3	MS + 40 mM NaCl	2.00 a	2.16 bc	32.16 b	141.33 b
4	MS + 60 mM NaCl	2.00 a	2.16 bc	29.00 b	98.50 b
5	MS + 80 mM NaCl	0.66 b	1.16 c	8.83 c	18.67 c

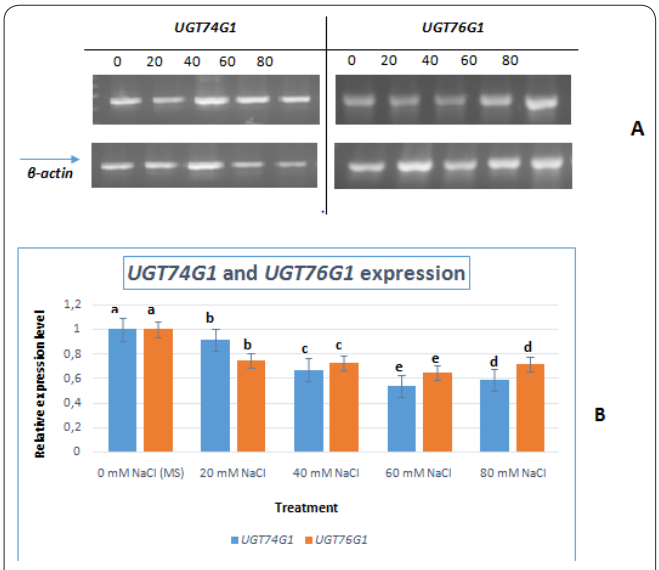

Figure 3. Expression of *UGT74G1* and *UGT76G1* in the leaves of *stevia rebaudiana* Bertoni under different concentrations of NaCl. (a) Semi-quantitative RT-PCR analysis of *UGT74G1* in plants treated with 0, 20, 40, 60 and 80 mM NaCl for 30 days. *β-actin* was used as an internal control. The final value was the average of at least four independent experiments. Only the best pictures are shown. (b) The relative expression level of *UGT74G1* and *UGT76G1* (related to *β-actin*) under different concentrations of NaCl. Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncan's test).

than the amount of that for *UGT74G1*. It suggests expression of *UGT76G1* is NaCl-affected less than it is for *UGT74G*.

HPLC analysis of steviol glycosides

Firstly, HPLC fingerprinting was performed on the pure marker compounds, including standard of Stevioside (St) and Rebaudioside A (Re). Fingerprint patterns procured from the studied samples under different concentrations of NaCl showed significant differences between the amounts of two intended steviol glycosides. The highest amount of both stevioside and rebaudioside A were observed in plantlets grown in MS media without additional NaCl (NaCl free). Interestingly, there was a decreasing trend for St and Re from NaCl freemedia to Ms+80 mM media from approximately 18 to near 10 for St and from around 4 to thereabout 1 for Re. So the least amount of these two steviol glycosides was seen in plantlets grown in MS+80 mM media (Figure 4). HPLC chromatograms obtained from all the samples showed the peaks corresponding to standard stevioside and rebaudioside-A marker compounds (Figure 5B-F)

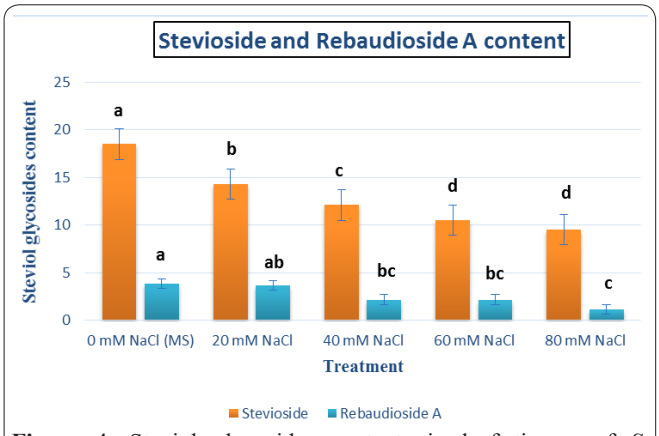

Figure 4. Steviol glycosides contents in leaf tissues of *S. rebaudiana* subjected to different concentration of NaCl.

Conclusion

Since the importance of steviol glycosides are the main components of Stevia, the most important aspect of the study on Stevia is improvement the growth and steviol glycoside content. So, the aim of the studies about Stevia can be raising the production of this compounds.

According to the results, stevia growth is impacted by salt stress. Actually, salt has a negative effect on Stevia. As it is seen, the best final production of stevia was in MS media without any additional NaCl. Zeng et al (2013) studied the effects of different NaCl concentrations on the growth, physiological responses, and steviol glycoside composition of *Stevia rebaudiana.* As they reported, some traits decreased and some of them increased when NaCl added to the media. Finally, it indicates that *S. rebaudiana* is moderately tolerant to salt stress. "Hypohaline soil can be utilized in the plantation of *S. rebaudiana* and may be profitable for optimizing the steviol glycoside composition" (16). Nevertheless, the best media for Stevia growth in the present study was NaCl freemedium. Notwithstanding that we observed decrease in steviol glycoside content under salt stress conditions, Gupta et al. (2016) concluded that "in spite of causing some growth reduction, application of chemical stress can enhance the production of SGs up to three fold compared to control plants" (26). Also, based on some reports on the effect of salt stress on morphological, physiological and biochemical characteristics

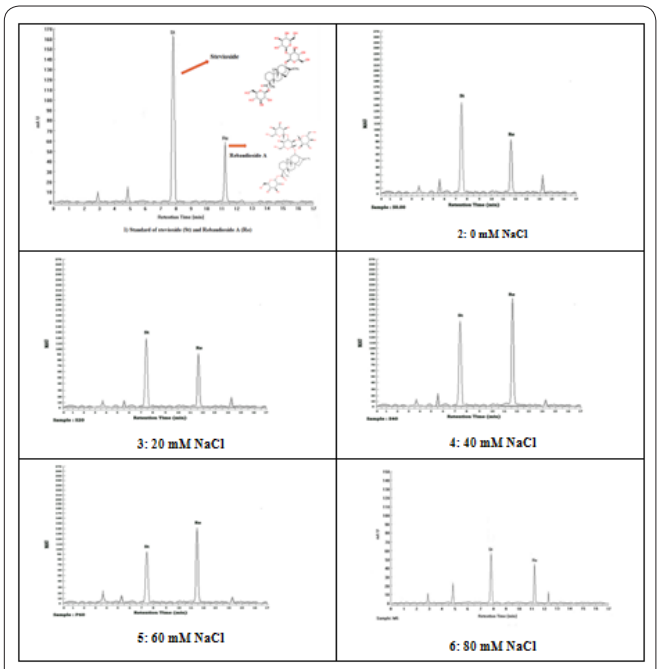

Figure 5. Representative HPLC chromatograms for quantification of stevioside and Rebaudioside A in methanolic extract of *S. rebaudiana* leaf tissues. 1) Standard of stevioside (St) and rebaudioside A (Re); 2) St. and Re. contents subjected to 0 mM NaCl; 3) St. and Re. contents subjected to 20 mM NaCl; 4) St. and Re. contents subjected to 40 mM NaCl; 5) St. and Re. contents subjected to 60 mM NaCl; 6) St. and Re. contents subjected to 80 mM NaCl.

of stevia (15-16, 21-22) it can be concluded that Stevia is moderately tolerant to abiotic stress.

On the other hand, our results suggest that both the best growth and the highest expression of *UGT74G1* and *UGT76G1* genes were occurred in MS media (NaCl free) and thereafter, the highest content of Stevioside and Rebaudioside A were obtained in the same media. Finally, it can be summed up that Stevia can survive under salt stress, but it has the best performance in the absence of salt. Application of medicinal plants have been suggested in different holy books (27).

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