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Effect of Zn, B and Mo as treatments on sweetener compounds accumulation and gene expression level of UGT76G1 *Stevia rebaudiana* Bertoni.

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ABSTRACT

Stevia produces a low-calorie sweetener, which is a calorie-free sweetener, considered a helpful food additive for people with hypertension, diabetes, and obesity. The highly potent diterpene glycosides (stevioside and rebaudioside A), which are sweeteners, are 300 times sweeter than sucrose. The aim of the present study is to investigate the effect of micronutrient applications on diterpene glycosides (stevioside and rebaudioside A) in three stevia varieties using the HPLC analysis technique and to investigate the expression levels of UGT76G1 gene using gene-specific primers. In this context stevia sweeteners (Stevioside and Rebaudioside A) leaves contents were extracted and determined. The results indicated that different treatments resulted in variable steviol glycoside contents. In addition, the gene expression level of UGT76G1 was affected with the micronutrient applications, as an increase in the transcription abundance of the gene was observed. In conclusion, micronutrients were found to enhance stevia glycosides accumulation in the leaf tissues, especially in Egy1 and China1 varieties. In addition, the application of the three micronutrients (Zn, B and Mo) depicted the most favourable effects on stevia leaves.

Keywords: *Stevia; sweetener; glycosides; micronutrients; HPLC; UGT76G1 gene.*

INTRODUCTION

Stevia rebaudiana is a small perennial shrub of the Asteraceae family. The leaves contain different diterpenc glycosides. The herb produces high-potency, zero-calorie sweeteners in its leaf tissue, specifically stevioside and rebaudioside A. Steviol glycosides are 200-300 times sweeter than sugarcane [1-7]. Thus, stevia can be used as a replacement for high-calorie sugar sources in food products. It is recommended to be used in the management of diabetes and has been used by humans with no detected side effects [1,8-10]. It is used for medical purposes such as, the treatment of hypertension, obesity, dental care and used in cosmetics to cure acne. The two main glycosides of the stevia plant are Stevioside (traditionally 5-10% of the dry weight of the leaves) and Rebaudioside A (2-4%).

These glycosides are the sweetest compounds; however, other related compounds, including Rebaudioside C (1-2%), Duclosid A, Duclosid C, as well as other glycosides of flavonoids, coumarins, cinnamic acids, phenylpropanoids and some essential oils, exist in plant leaves [11-14]. The biosynthesis of these glycosides is linked to the biosynthesis of gibberellins, plant growth regulators. Thus, stevia is considered a potential source of glycosides and also gibberellins [15,16]. The biosynthesis of the sweet compounds takes place in green tissues (chloroplasts) and therefore, they are accumulated in the leaves and green tips of plant leaf tissues. As plant stems mature and lose their color, any steviosides present dissipate. Steviol glycosides are diterpenoids derived from steviol as the final step of glycosylation by the marker enzyme Uridine diphosphate glycosyltransferase (UGT). In the eight different steviol glycosides, the two main glycosides are stevioside and rebaudioside A; Stevioside traditionally makes up the majority of the sweetener (60~70% of the total glycosides content). It is also responsible for the bitter aftertaste. On the other hand, Rebaudioside A is usually present as 30-40% of total sweeteners. The UGT76G1 gene is the primary gene responsible for the conversion of stevioside to rebaudioside A. Rebaudioside A was found to be the sweetest glycoside with a reduced bitter aftertaste. Boron (B), molybdenum (Mo), and zinc (Zn) are three essential micronutrients required for the normal growth and development of plants. Several analytical techniques have been employed to assess the distribution and level of sweet diterpenoid glycosides in *S. rebaudiana*. The most common analytical method for their determination is high-performance liquid chromatography (HPLC). The present study investigated the effect of some micronutrients on steviol glycosides accumulation in stevia leaves using HPLC. Moreover, gene expression levels of UGT76G1 in the three stevia varieties were estimated.

Materials and Methods:

Plant materials

Three stevia varieties, namely China 1, Egy 1 and Sponti, were obtained from the Sugar Crops Research Institute (SCRI), Agricultural Research Centre (ARC), Ministry of Agriculture, Egypt.

Stevia plant cultivation and foliar spray of micro-nutrient solution treatments:

In December, the seeds of stevia plants were cultivated in pots filled with potting mixture (petmose: sand: clay) (1:1:1) at the greenhouse of Agricultural Research Station-El-Sabahia. All pots were kept under controlled temperature ranging from 16 to 23°C and photoperiod of 13-16 hours. The experimental farm within latitude of 31° 19' N and longitude of 29° 92' E. The soil of experimental pH was 7.8. Available Zinc (Zn), molybdnum (Mo), and Boron (B) in the soil were 0.95 mg/kg, 0.002 mg/kg, and 0.014 mg/kg, respectively.

After Twenty five days of cultivation, thirty two pots having 15-kg soil capacity were taken and 10-kg powdered soil collected from the Agricultural Research Station-El-Sabahia farm was filled up and the following treatments: T1: absolute control, T2: foliar (Zn as $ZnSO_4$@ 0.2% solution), T3: foliar (B as H_2BO_3 @10ppm), T4: foliar (Mo as $(NH4)_6Mo_7O_{24}$@ 1.0 g L^{-1}), T5: both (Zn as $ZnSO_4$@ 0.2% solution) and (B as H_2BO_3 @10ppm), T6: both Zn as $ZnSO_4$ @ 0.2% solution and Mo as $(NH4)_6Mo_7O_{24}$@1.0 g L^{-1}, T7: foliar application of B as H_2BO_3 @10ppm and Mo as $(NH4)_6Mo_7O_{24}$@ 1.0 g L^{-1}, T8: both (Zn as $ZnSO_4$@ 0.2% solution), (B as H_2BO_3@10ppm) and Mo as $(NH4)_6Mo_7O_{24}$@1.0 g L^{-1}. Each treatment was replicated four times in a completely randomized design (CRD). There were 32 pots altogether (8 ×4). The second spray was applied on day 20, following the first application, and then the plants were allowed to grow for 90 days after sowing. All data were recorded after

15, 30 and 45 days' post-foliar treatment. Additionally, leaves of some plants were cut from the plants for further analysis.

Preparation of leaves for extraction

The stevia plants with a maximum growth stage (mature stage before flowering) were harvested by cutting the plant at 5 – 10 cm from the ground surface. The brown and yellow leaves were removed from the plants. Then, the plant cuttings were washed in clean water and spread on trays covered with cheesecloth to remove excess water. Stevia leaves were dried in an electric oven (E. Schulz & Co. Inh. Franz. Skorezewsh KG) at 50°C.

Extraction of sweet diterpene glycosides from *S. rebaudiana*

The sample was prepared by accurately weighing 1.0 gm of stevia leaf powder and extracting it with methanol (100 ml). Then, the extracted tissues were soaked in 1.0 liter of water at 70°C for 30 minutes. The extracts were then filtered through whatman filter paper no 11. The filtrate solution was clarified with active charcoal (B.D.H. Laboratory Chemicals Division Poole, England) and finally left to recrystallize. The procedure was repeated three times until the formation of colorless crystals were observed [17,18].

Stevioside standard preparation

Stevioside standard preparation was carried out according to [19,20] as follows: Dried leaves of *Stevia rebaudiana* Bertoni (10.0g) that were collected from Sugar Crops Research Institute (SCRI), Agricultural Research Center (ARC); Ministry of Agriculture, Egypt) were extracted by soaking leaves in 1.0 liter of hot distilled water (85°C) for 30 minutes. The resulting liquid fractions were filtered using a Buchner filtration system, and the leaves were then washed with an additional volume of hot water (50 ml). The aqueous solution was concentrated to 50 ml in an (Edwards Model EF03, England). The extract was defatted using ethyl acetate, and then extraction with isobutyl alcohol (150 ml). The aqueous phase was discarded, and the organic phase was evaporated by rotary evaporator (Type 349, James Jobling and Co. Ltd., England) at 70°C until dryness. The dry pellets were dissolved in hot methanol (100 ml) and were kept overnight to crystallize. The crystals were separated by filtration and then re-dissolved in boiling methanol (50 mL) to obtain a concentrated solution. The solution was clarified with active charcoal (B.D.H. Laboratory Chemicals Division Poole, England) and recrystallised. The procedure was repeated three times until the formation of colorless crystals. The pure stevioside standard solution was subjected to HPLC analysis.

Analysis of SGs by HPLC

The High Performance Liquid Chromatography (HPLC) technique can be used to directly measure the levels of steviol glycosides (rebaudioside A and stevioside) in *Stevia rebaudiana* Bertoni. The levels of stevia sweetener compounds were estimated at the Central Laboratory, Faculty of Science, Alexandria University, Alexandria, Egypt. Leaf extracts were separated and identified by HPLC according to [17,21,22] as follows: the stevioside solution was filtered through a Millipore membrane (13.0 mm diameter, 0.5 µm pore size) and analyzed using HPLC with a stevioside standard as an internal standard (10.0 mg/ml). Different extracts of stevia leaves were injected into an HPLC instrument (Shimadzu, Tokyo, Japan; model SPD-6AV) equipped with an LC-GA UV-V is detector and an Alex C-R 4 A recorder. The separation was carried out on a Zorbax NH2 column (25 cm × 0.4 mm I.D.; Dupont, Wilmington, DE, USA) using acetonitrile (HPLC grade, Fisons Co., England) as the mobile phase (acetonitrile: water, 80:20 v/v, adjusted to pH 5.0 with H_3PO_4). The flow rate was 2.0 mL/min; the UV detection wavelength was 210 nm; the recorder chart speed was 20.0 mm/min; and the analysis was performed at an ambient temperature of 25 °C. Two samples per variety were analyzed, and the quantities of stevioside and rebaudioside A were calculated from the area under each peak.

Isolation of total RNA

RNA extraction BS82314-50 Preps EZ-10 Spin Column Plant RNA Mini-Preps Kit (BIO BASIC, Canada) was used.

Synthesis of cDNA

The first strand of cDNA was synthesized using M-MuLV Reverse Transcriptase (BioLabs Inc. New England). The samples were incubated at 42°C for 1.0 hour then 72°C for 10 minutes. The obtained cDNA samples were then stored at -20ºC. Each 20.0µl of reverse-transcription mixture containing 1.0 µl templete RNA, 2.0 µl Oligo (dT)-primer, 2.0 µl 10x M-MuLV Buffer, 1.0 µl M-MuLV RT (200U/µl), 1.0 µl 10 mM dNTP Mixture and nuclease - free water up to a total volume of 20.0 µl.

Quantitative real-time PCR analysis (qRT-PCR)

For the relative quantification of the gene expression. RT quantitative PCR was conducted in Eppendorf Master cycler using the following PCR cycles: initial denaturation cycle 2 min at 95°C, 40 cycles of 5s at 95°C, 10 s 60°C and 5s at 72°C and followed by melting curve analysis. Quantitative RT-PCR determined expression of (UGT76G1) genes; Thermo Scientific PikoReal 96 Real-Time PCR System (www.thermoscientific.com/pikoreal) with SYPER Green SensiFAST™ SYPER® No-ROX Kit (BIOLINE). The quantitative real-time PCR was performed in 10.0µl volume containing 1.0µl cDNA, 5.0µl 2x SensiFAST SYPER® No-ROX Mix, 0.5µl, Forward primer, 0.5µl Reverse primer and 3.0µl H_2O. All expression data analyses were performed after comparative quantification of amplified product using the $2^{-\Delta\Delta Cq}$ method as previously described.

Table 1. Primer set designed for qRT-PCR used in the current study

Gene	Primer sequence	Reference
Stevia actin FP	5' CCCGCCATGTATGTCGCCATTCAA 3'	[23]
Stevia actin RP	5' TCAGTGAGGTCACGACCAGCAAGA 3'	
UGT76G1 FP	5' AACGTCAGTCAAACCCAATG 3'	[24]
UGT76G1 RP	5' CTCACATAACCAACAACCATCC 3'	

Data analysis

The expression level of target genes was normalised using the stevia actin gene as an internal control, and Relative transcript levels were calculated according to [25,26].

Results and Discussion

Chemical analysis of stevia sweeteners by HPLC analysis of SGs

Plant nutrition is a significant factor that influences the growth and development of plants, and they have been classified as macro and micronutrients**.** Micronutrients are essential elements which are used by plants in small quantities [27,28]. The yield and quality of agricultural products increase with the utilization of these essential elements. The relationship between micronutrients and plant growth involves a complex balance of minerals, which are essential and beneficial for optimum plant growth and development. Boron (B), molybdenum (Mo), and zinc (Zn) are three essential micronutrients required for the growth and development of higher plants [29-31].

In the current work, stevia sweeteners (Stevioside and Rebaudioside A) were analyzed and determined by employing "HPLC" technique. The data obtained are given in Table (2). The data indicated that different treatments resulted in variable steviol glycoside contents. The concentration of stevia sweeteners was the highest in variety Egy1 when T5 and T6 treatments were applied. The results also showed that the variety China1 glycosides increased in treatments T6, T7 and T8. Finally, in variety Sponti, the highest value of stevia glycosides was obtained with T5 and T8 treatments.

Table (2): Concentration of stevia sweeteners

Treatments	Egy1		China1		Sponti	
	Stevioside %	Rebaudioside A %	Stevioside %	Rebaudioside A %	Stevioside %	Rebaudioside A %
T1 (co)	12.276	14.48	14.186	15.541	21.46	13.02
T2 (Zn)	20.166	20.631	13.48	18.67	8.547	6.606
T3 (B)	22.5	26.942	12.56	27.6	3.814	0.498
T4 (Mo)	3.524	4.299	19.121	18.98	11.847	8.547
T5 (Zn+B)	27.06	40.49	9.931	11.82	25.93	32.66
T6 (Zn+Mo)	24.2	40.97	42.44	42.3	6.78	6.516
T7 (B+Mo)	18.39	14.93	9.369	46.116	20.43	8.37
T8(Zn+B+Mo)	17.07	6.775	53.96	25.73	30.95	21.46

Stevioside accumulations in the leaves were marginally increased (up to 30%) by the foliar application of Zn+B+Mo to varieties China1 and Sponti. These results indicated that these treatments effectively increase stevia glycosides in the plant tissue. Additionally, gene expression data indicate that differential gene expression was observed. These results might be attributed to the increasing levels of photosynthetic pigments. Stevioside content obtained from this study was higher than those reported by [32,33], whose concentration ranged from 2.8-5.49%. [32,34] found that glycosides concentration ranged from 6.98-12.16%. For rebaudioside A content, China1 had the highest with 46.11% while Sponti gave the least with 32.66%.

Lower recorded rebaudioside content was observed than that reported by [32,35]. The variation in steviol glycosides content in stevia mother plant collection was linked to the open-pollination nature of stevia [36,37]. It is noteworthy that Rebaudioside A is more preferred than stevioside due to its desirable flavor profile as it reduces the after-test effect [38]. Stevioside, rebaudioside A and steviol contents were carefully determined through different methods, as indicated in the scientific literature, including enzymatic hydrolysis, chemical detection using Gas Chromatography (GC), overpressure Thin Layer Chromatography (TLC), densitometry, High Pressure Liquid Chromatography (HPLC), and capillary electrophoresis [39]. To address the issues of high cost and complex operation associated with current chemical laboratory methods, HPLC technology and near-infrared (NIR) spectroscopy techniques were developed to directly measure the content of steviol glycosides (rebaudioside A and stevioside) in the leaves of *S. rebaudiana* Bertoni [40].

Quantitative Real-Time PCR (qRT-PCR)

There are several genetic approaches available to investigate the action of glycosyltransferases in plants and how their catalytic activities may be related to physiological functions, whereby glycosylation can serve as a regulatory mechanism altering the levels of metabolites [41, 42]. In the present study, Real-Time quantitative PCR was used to detect the relative expression of UGT76G1 gene in three stevia varieties.

Relative expression level of the UGTSr gene in different tissues of stevia viz tender leaf, mature leaf, stem, and flower was quantified by ΔΔCt quantification method [25]. The same method was adopted in the current study to quantify the relative gene expression levels of UGT76G1 in the leaves of stevia plants, and the obtained data are demonstrated in Table 3. The different gene expression values of UGT76G1 gene were observed in different treatments of Egy1 variety comparing to the control. The highest gene expression value (16.17) was recorded in treatments T5 (Zn+B) and the lowest gene expression value (0.183) was recorded in treatments T2. In contrast, in **Table (3)** the highest value (9.85) of expression of UGT76G1 gene was reported for China1 variety in treatments T8 (Zn+B+Mo) and the lowest gene expression value (0.882) was recorded in treatments T2. The results in **Table (3)** revealed that the highest value of expression of UGT76G1 gene was obtained for Sponti variety (18.64) in treatments T8 (Zn+B+Mo) comparing to the control.

Table (3): Relative gene expression of stevia UGT76G1 gene in Egy1 variety with different micronutrients treatments using RT-quantitative PCR

Treatments	ΔΔcq expression of UGT76G1 gene		
	Egy1	China1	Sponti
T1(co)	1	1	1
T2 (Zn)	0.183	0.882	2.531
T3 (B)	0.489	2.345	1.879
T4 (Mo)	1.993	2.675	4.890
T5 (Zn+B)	16.167	1.164	15.242
T6 (Zn+Mo)	11.471	4.0843	1.892
T7 (B+Mo)	2.620	5.982	5.098
T8 (Zn+B+Mo)	0.254	9.849	18.635

The increased level of UGTSr gene expression in the mature leaf supports the conclusion reported by [23] that the active phase of diterpene glycoside synthesis in stevia is associated with an increase in stevia glucosyltransferases gene expression. [41,43] studied the gene expression of the genes responsible for the biosynthetic pathway of steviosides UGT74G1, 76G1, and 85C2 using RT qPCR technique. They found that UGT76G1gene was the main gene responsible for the conversion of stevioside to rebaudioside A.

Conclusion

The results illustrated in the current study indicated that different treatments in variable steviol glycoside contents. The concentration of stevia sweeteners was the highest in variety Egy1 when plants were treated with Zn, B and Zn, Mo (T5 and T6 treatments), respectively. The results also showed that variety China1 glycosides were increased in treatments T6, T7 and T8. Finally, in variety Sponti, the highest value of stevia glycosides was obtained with T5 and T8 treatments. In addition, different gene expression values for the UGT76G1 gene were obtained for each treatment of the Egy1 variety compared to the control. The highest gene expression value was recorded in treatments T5 and the lowest gene expression value was recorded in treatments T2. Meanwhile, the highest value of UGT76G1 gene expression was reported for China1 variety in treatments T8 and the lowest gene expression value was recorded in treatments T2. Finally, the highest value of UGT76G1 gene expression was obtained for Sponti variety that was treated with Zn, B and Mo (T8 treatment) comparing to the control.

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تاثير الزنك، البورون والمولوبيديم كمعاملة رش ورقى على تراكم المواد المحلاة والتعبير الجينى لجين 1UGT76G فى نبات الاستيفيا.

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الخلاصة
تعد نباتات الاستيفيا من النباتات الواعدة التى تتميز بوجود مادة محلاة تفوق 300 مرة المواد العادية. وتعتبر مفيدة بالاخص لمرضى السكر وتحتوى على مواد هامة مثل ***stevioside and rebaudioside A***. ويعد الغرض الرئيسى من الدراسة تقييم تاثير الزنك، البورون والمولوبيديم كمعاملة رش ورقى على تراكم المواد المحلاة والتعبير الجينى لجين ***UGT76G***1 فى نبات الاستيفيا باستخدام طرق مختلفة. حيث اوضحت النتائج اختلافات معنوية عالية فى محتوى ***stevioside and rebaudioside A*** بالاخص فى صنفى ***Egy1 and China***1.

الكلمات المفتاحية: الاستيفيا – المغذيات الصغرى – التعبير الجينى.