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Original Research

Metal oxides as a biostimulator for upregulation of genes involved in the biosynthesis of Rebaudioside A

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Abstract: *Stevia rebaudiana Bertoni* is a kind of perennial medicinal plant with sweetening properties which belongs to *Asteraceae* family. Its leaves with fundamental glycoside compounds consist of both a sugar part and a non-sugar sector. One of the glycoside compounds is Rebaudioside A which has a greater importance in business. This experiment was conducted to evaluate the effects of Ag_2O, CrO_3, PbO, Fe_2O_3, BaO and TiO_2 on the expression pattern of these genes in the *Stevia rebaudiana*. Rebaudioside A biosynthesis was repeated 3 times with concentrations of 50, 100 and 200µM. Also, the results of the study pertaining to the expression pattern of these genes showed that metal oxides have led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A. According to the expression profile, it was found that its effect on DXR, HDS, HDR, IDI and CPPS genes is more than other genes. The peak HPLC indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of all treatments. In general, the expression profile of these genes and the results of HPLC show that whatever going to the end of the pathway of production of Rebaudioside A, the activity of the enzymes increases under the influence of these treatments, and eventually a greater amount of Rebaudioside A will be produced. This process shows that metal oxides will have a significant effect on the biosynthesis of Rebaudioside A.

Key words: Stevia; Rebaudioside A; Genes; Expression pattern.

Introduction

In recent years, the development of natural sweeteners is increasing, especially those containing sucrose which is unabsorbed in the digestive system, and is not too caloric, so it is suitable for some diseases such as diabetes and obesity. One of the promising alternatives is glycoside family, especially steviol glycoside (1). Steviol glycosides are mostly found in stevia leaves. In 1931, two French chemists, Bridel and Lavieille, managed to extract stevia glycoside sweeteners. They obtained natural stevia glycosides thorough a series of hydrolysis experiments which lack the enzyme (2). In fact, steviol glycoside is a diterpene glycoside extract of stevia which has been identified in various parts of *Stevia rebaudiana* and is the most important sweetener group in stevia (3). Steviol glycosides include compounds such as dalcoside A, Ribaudioside E-A, Steviol biocides, stevioside and steviol glycoside composition; therefore, 34 compounds are now known in steviol glycosides and eight of them are in the form of isomers. Among these compounds, Ribaudioside A and stevioside, are more important than other compounds and have been identified as a combination of other compounds in the leaves of *Stevia rebaudiana* dominant glycosides which has great commercial importance (4). Stevia also has protein, fiber, carbohydrates, phosphorus, iron, calcium, sodium, potassium, magnesium, zinc, and vitamins A and C. These sweet compounds pass through the digestive processes without chemical decomposition, so it is helpful for those who need to control blood sugar (5). In stevia, pyruvate and glyceraldehyde 3-phosphate during the MEP pathway are converted to isomers dimetilalildiphosphate (DMADP) and isopentyl diphosphate by genes DXS, DXR, MCT, CMK, MDS, HDS and HDR and ultimately, these isomers lead to the production of the steviol glycoside and Rebaudioside A and the regulation of sugar in the leaf tissue (6). The results of the current studies show different responses of different species of plants to the prepared food in the form of metal oxides. For example, Veau and partners in 2000 examined the amount expression of genes DXS (1-deoxy-D-xylulose 5-phospate synthase), DXR (1-deoxy-D-xylulose 5-phospate reductoisomerase) and MDS (2-C-methyl-D-erytrithol 2,4-cyclodiphosphate synthase) under the treatment of dichlorophenoxy acid in the catharanthus roseus plant. Their results showed that the treatment of dichlorophenoxy acid leads to the reduction of the expression in these genes. The results of biochemical analyzes showed that treatment dichlorophenoxy acid leads to reduced pigments photosynthesis (chlorophyll a and b, carotenoids) (7). Kumar *et al.* (2012) have reported 15 genes which were effective in biosynthesis pathway of Rebaudioside A. In their study, the gibberellin, kinetin and methyl jasmonate treatments were performed. It was shown that the gib-

berellin (GA3) treatment up-regulated the expression of SrMCT, SrCMK, SrMDS and SrUGT74G1, whereas methyl jasmonate and kinetin treatments down-regulated the expression of all the fifteen genes of the pathway. In 2012, Kumar and colleagues investigated the gene expression MDS Stevia in response to light, indoleacetic acid (IAA) and abscisic acid (ABA). Their results showed that MDS gene expression is strongly influenced by light, so that the highest levels of gene expression in the early morning and high light intensity part is observed. Also, IAA hormone leads to the reduction of MDS gene expression and ABA hormone does not have much impact on gene expression (8). However, no study has yet been conducted to measure the effect of metal oxides on the expression of these genes. Therefore, this study is aimed to evaluate the effects of five different metal oxides containing CrO_3, PbO, Fe_2O_3, BaO and TiO_2 on expression of genes involved in the biosynthesis Rebaudioside A named DXS: 1-deoxy-D-xylulose 5-phospate synthase; DXR: 1-deoxy-D-xylulose 5-phospate reductoisomerase; MCT: 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol 4-phosphate synthase; CMK: 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol kinase; MDS: 2-C-methyl-D-erytrithol 2,4-cyclodiphosphate synthase; HDS: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HDR: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IDI: isopentenyl diphosphate isomerase; GGDPS: geranylgeranyl diphosphate synthase; CPPS: copalyl diphosphate synthase; KS: kaurene synthase; KO: kaurene oxidase; KAH: kaurenoic acid hydroxylase; UGT85C2: UDP glucosyltranserase – 85C2; UGT74G1: UDP glucosyltranserase – 74G1; and UGT76G1: UDP glucosyltranserase – 74G1.

Materials and Methods

Planting and the growing conditions

Very small and thin (approximately 3 mm) light brown seeds of stevia (*Stevia rebaudiana* bertoni) were purchased from Techno Kesht shiraz Company (Shiraz, Iran). These seeds were surface-sterilized with sodium hypochlorite (5%). Then, a piece of paper filter was put into each petri dish and 5 ml of distilled water was added. The seeds were transferred onto the paper filter, with 10 seeds in each of the 32 petri dishes. A distance of 1cm or longer was kept between the seeds. The dishes were covered with their lids and sealed with parafilm. After preparing the media and sealing the dishes, all petri dishes were placed in an incubator. More than 80% of the seeds germinated in the dark within five days at room temperature. The seedlings developed roots with at least 25 mm length and were then transferred into the jardiniere containing cocopeat and perlite with proportion 70 to 30, respectively. During the experimental period, the jardiniers were irrigated twice a week. The seedlings received tap water at greenhouse conditions with about 60% relative humidity for two weeks until the size of leaves reached about 10 cm. Diurnal cycles at each temperature (27/24°C) were set at 16h light and 8h dark cycles. At this state, the seedlings were treated with 50 mL of different concentrations (50, 100, 200 µM) of metal oxides loaded onto the gelatin dispersion. The equal concentrations of gelatin dispersion were used as control treatments conditions which continued for three days. Young leaves were harvested and freezed in liquid nitrogen for twenty-four hours after each treatment, and then stored at -70 °C for the RNA extraction and real-time PCR analysis. For biochemical analysis, young leaves were harvested and stored at 4°C.

RNA preparation

Total RNA was extracted from 100 mg of leaf matter using RNX-Plus buffer (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The quantification of total RNA was performed with a Nano-drop ND 1000 spectrophotometer at 260 nm (Thermo Fisher scientific, wilmington, DE, USA). The RNA integrity was checked by visual observation of 28S rRNA and 18S rRNA bands on an agarose gel electrophoresis before Real-time PCR analysis Fig. 1.

DNase treatment and cDNA synthesis

DNase treatment was carried out using a Fermentas DNase Kit (Fermentas, Hanover, MD, Germany) using the manufacturer's protocol and again the integrity of total RNA was checked by electrophoresis in agarose gel. Five µg of DNase-treated RNA was converted to cDNA with a revert aid first strand cDNA Synthesis Kit (Thermo Scientific, Fermentas) using the manufacturer's protocol in a 20 µL final volume.

Primer design

Primers were designed using AlleleID7 software (Premier Bio soft Intl, Palo Alto, CA, USA) for target genes are prepared in Table 1. Elongation factor genes were used as the internal control (whose expression proved not to be influenced by metal oxides treatment) for data normalization (9,10). Two primer pairs were designed for each individual gene which are represented in Table 1. Primers for the PCR reactions were designed to have a melting temperature of about 55 °C to 65 °C and to give a PCR product between 100 and 200 bp.

Real-time quantitative PCR analysis

Reaction Real-Time PCR using Bioer devices made

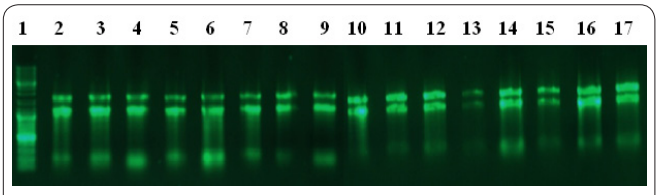

Figure 1. 1. Gene RulerTM DNA Ladder Mix (Fermentas); RNAs extracted from stevia leaves for genes; 2. DXS: 1-deoxy-D-xylulose 5-phospate synthase; 3. DXR: 1-deoxy-D-xylulose 5-phospate reductoisomerase; 4. MCT: 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol 4-phosphate synthase; 5. CMK: 4-(cytidine 5′ diphosphate)-2-C-methyl-D-erythritol kinase; 6. MDS: 2-C-methyl-D-erytrithol 2,4-cyclodiphosphate synthase; 7. HDS: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; 8. HDR: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; 9. IDI: isopentenyl diphosphate isomerase; 10. GGDPS: geranylgeranyl diphosphate synthase; 11. CPPS: copalyl diphosphate synthase; 12. KS: kaurene synthase; 13. KO: kaurene oxidase; 14. KAH: kaurenoic acid hydroxylase; 15. UGT85C2: UDP glucosyltransferase – 85C2; 16. UGT74G1: UDP glucosyltransferase – 74G1; and 17. UGT76G1: UDP glucosyltransferase – 74G1

Table 1. Sequences of primers used for real-time PCR amplification.

Number	Primer	Sequence	TM
1	DXS	F: GATCTACAAAAGTTACCGGTTC	53.3
		R: TCCTCTACGGTAAGTAAGACTTC	52.52
2	DXR	F: TTGAGCTATCTATCTCCAACAC	52.37
		R: TATCTGTTCAGCAAGAAGAGTC	52.51
3	MCT	F: AGACAAGATTCTGTTTTTAGTG	49.28
		R: GAGTTGTAACCTTGATGTTAGT	48.94
4	CMK	F: AATCTATATCGCAAGAAGACTG	51.79
		R: CTTCCAGACATAAAAACAGAAT	51.52
5	MDS	F: GAGCCTGGATACCCTCTCATC	58.56
		R: CCTCTTTATGCGGGCTTAACT	58.4
6	HDS	F: TTTCTTGGCTCCGTATCG	50.9
		R: TGAGGCTACATCTGAATAGG	50.6
7	HDR	F: AAACAATTTGATGTCATTGATAA	52.63
		R: GGTTCTTTCTACTAGTTTTCCAA	52.56
8	IDI	F: TATGAGTTACTCCTTCAGCAAC	52.47
		R: AGGTAGTCAAGTTCATGTTCTC	51.19
9	GGDPS	F: CGATTGGTTTGTTGTTTCAG	50.8
		R: GCTTCCTTGTTTAATTTCTCC	50.4
10	CPPS	F: CTACACGGCTTCGCTTTG	53.1
		R: GTCACATCTACTCCATCTTGC	53.4
11	KS	F: GAGAGAAGCTATATGGACAAGAG	52.9
		R: GATGTCCTTCACAGTATCAAGA	52.82
12	KO	F: GTTGAAGGAGAAGAAACCTTAC	52.61
		R: CAACATATAAGCTCTCCACATC	52.66
13	KAH	F: GGTTTGGTTCCTCGTGGGGT	57.2
		R: TGCGTGAAGCTATGGACGAG	53.8
14	UGT85C2	F: ATGATGTATTGGACACTTGCTG	54.2
		R: CCTTGAGACGGATGCCTTC	54.7
15	UGT74G1	F: CCTTGGTCTCAGATGTTGTTTG	55
		R: CGTCCACTCTATTACCTCTTCC	55
16	UGT76G1	F: CATCTTTCACACCAACTTCAAC	53.5
		R: GCGTCGTAATTCGTCAGC	53.5
17	Elongation Factor	F: GATGCTTCCGACTAAACCTATGG	56.6
		R: CACTCTTGATAACACCGACTGC	56.9

Primer design (in form of exon junction) was carried out using Allele ID 7 software for the internal control EF and test genes.

in china and SYBR Premix Ex Taq II kit Takara Company was performed. For this purpose, the cDNA made from leaf samples under controlled conditions (samples not treated) and leaf samples Treatment as a template and primers RT-F1 and RT-R1 genes studied as specific primers of the target gene and primers (EF1) Elongation Factor1 in stevia as an internal control (standard response for Real-Time PCR) was used. The cDNA samples were diluted 1:5 by using nuclease-free water, and 5 µL of cDNA was used for real-time PCR. The final volume for relative real-time PCR was 20 µL containing 4 pmol of each primer, 5 µL (diluted) of the first-strand cDNA and 1x SYBR Premix Ex Taq TM II (Takara, Japan). The initial denaturing time was 5 min., followed by 40 PCR cycles consisting of 94°C for 10s, annealing temperatures of each primer was 15 s, and 72°C for 30s. A melting curve was run after the PCR cycles followed by heating from 50 to 95°C. Proper control reaction was carried out without the reverse transcriptase treatment. For each sample, the subsequent real-time PCR reactions were performed twice under identical conditions (11).

Normalization of gene expression after Real-time PCR reaction

For the purpose of data analysis, Real-time PCR, the relative expression of the target gene in each sample were compared with control sample and CT for each sample using the Line-gene K Software. In order to normalize the expression of genes, the following formula was used. In this formula, ΔΔCt is equal to (Ct target gene minus Ct gene internal control in time x) minus (Ct target gene minus Ct gene internal control in zero time). X time in this formula means the treatment conditions and zero time in the above formula is the control samples under controlled conditions.

Expression Ratio = $2^{-\Delta\Delta Ct}$

$\Delta\Delta Ct = (Ct,Target - Ct,ef1)Time_{X} - (Ct,Target - Ct,ef1)Time_{0}$

HPLC analysis

Standard solution of Rebaudioside A was prepared in a mix of 30% water and 70% acetonitrile. To prepare calibration curve, a solution containing 100 µg/mL of standard in the ratio of 30% water and 70% acetonitrile was used. In order to extract Rebaudioside A, the *Stevia rebaudiana* leaves were pulverized and nearly 0.1 g of the powder was put in a 20-ml glass vial. Ten ml of 30% water and 70% acetonitrile mix was added to the vial which was then vortexed. Subsequently, this crude extract was sonicated for 5 minutes. The contents of the vial were centrifuged and the supernatant was diluted 10 times with 30% water and 70% acetonitrile mix. Finally, 10 microliters of the sample solution were injected into the column for analysis. The flow rate was 1 mL per minute. The HPLC device which was used is called KNAUER – smart line manager-5000. HPLC column profile contains eurospher 100-5 C18 column,

2500-KNAUER Detector, column length 250mm and measure pore 5µm. During the 14- minute period, the peak of Rebaudioside A appeared at the minute of 2.3 and the wavelength was 220 nm.

Statistical analysis

All tests were conducted in at least three independent experiments and completely randomized design and expressed as the mean values ± standard deviations. The significant differences among the means were analyzed through Duncan test at $P<0.05$ employing SPSS (SPSS Inc, Chicago IL, USA) software version 16.

Results

In this study, the effects of metal oxides Ag_2O, CrO_3, PbO, Fe_2O_3, BaO and TiO_2 on the Profiling of expression of genes involved in the biosynthesis Rebaudioside A in stevia by Real-time PCR reaction in 3 replicates with concentrations of 50, 100 and 200 µm were performed and the results were analyzed as follows**.**

Extraction of RNA from stevia Leaves

RNA extraction from stevia leaf tissue was performed. 4 extracted RNA was electrophoresed on 1% agarose gel (Fig.1). The bands of 18S rRNA and 28S rRNA were clearly observed on the gel.

Expression Profile of genes Under the influence of treatment of Ag_2O

As shown in Fig. 2, Ag_2O treatment has led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and it affects all genes and its effect on DXS, DXR, HDS, HDR, IDI and CPPS genes is more than other genes. with that difference the amount of expression at the end of the path is relatively lower while whatever going to the end of the path, the amount of expression is added. Also, in concentration 200 µm, the amount of expression is higher than other concentrations. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of Ag_2O treatment. Regarding the expression profile, the treatment Ag_2O at the beginning of the route has a greater effect on the production of the Rebaudioside A.

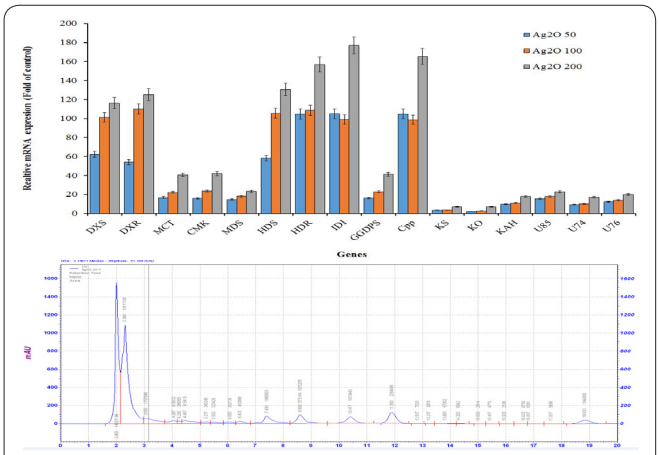

Figure 2. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of Ag_2O.

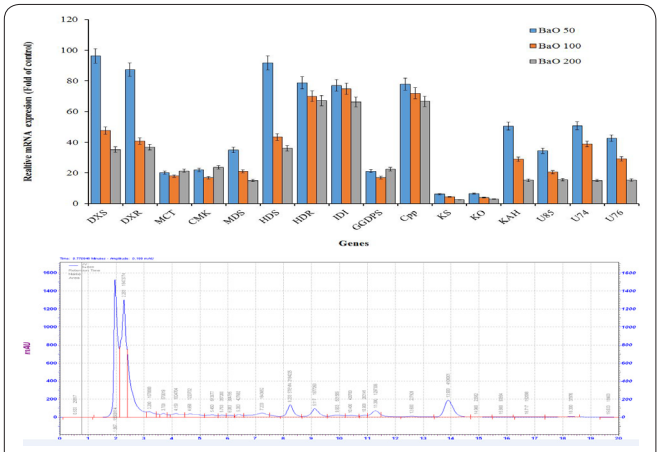

Figure 3. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of BaO.

Transcriptional profiling of genes Under the influence of treatment of BaO

As shown in Fig. 3, BaO treatment has led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A. expression profile treatment BaO shows that treatment BaO uniformly leads to increased expression of genes. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of BaO treatment. expression profile treatment BaO shows that treatment BaO uniformly leads to increased expression of genes.

Transcriptional profiling of genes involved in steviol glycoside biosynthesis as function of CrO_3

As shown in Fig. 4, CrO_3 treatment has led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and it affects all genes and its effect on HDR, IDI and CPPS genes is more than other genes. according to the expression profile, it was found that the best concentration for using this treatment was 100 µm. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of CrO_3 treatment.

Expression Profile of genes Under the influence of treatment of Fe_2O_3

As shown in Fig. 5, Fe_2O_3 treatment has led to an

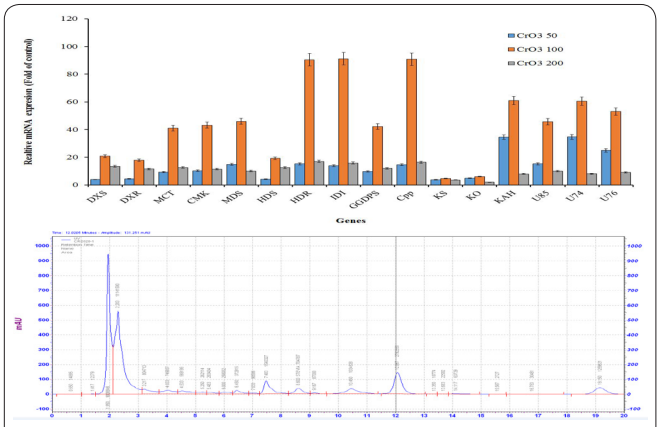

Figure 4. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of CrO_3.

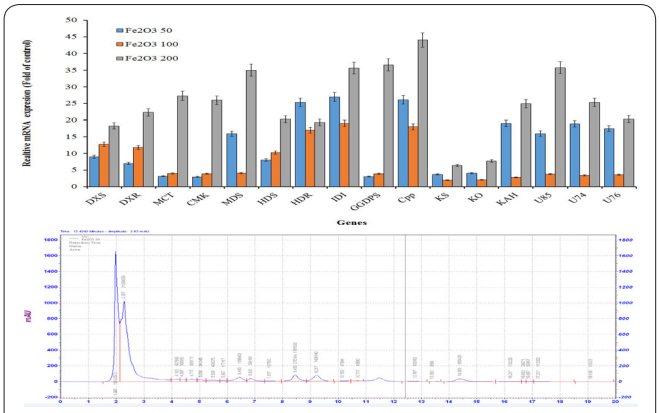

Figure 5. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of Fe_2O_3.

increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and it affects all genes and its effect on KO and KS genes is fewer than other genes. according to the expression profile, it was found that the best concentration for using this treatment was 200 µm. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of Fe_2O_3 treatment.

Transcriptional profiling of genes Under the influence of treatment of PbO

As shown in Fig. 6, PbO treatment has led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and it affects all genes and its effect on DXR, HDS, HDR, IDI and CPPS genes is more than other genes. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of PbO treatment. Regarding the expression profile, the treatment PbO at the beginning of the route has a greater effect on the production of the Rebaudioside A.

Transcriptional profiling of genes involved in steviol glycoside biosynthesis as function of TiO_2

As shown in Fig. 7, TiO_2 treatment has led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and it affects all genes and its effect on HDR, IDI and CPPS genes is more than other genes. According to the expression profile, it was found that the best concentration for using

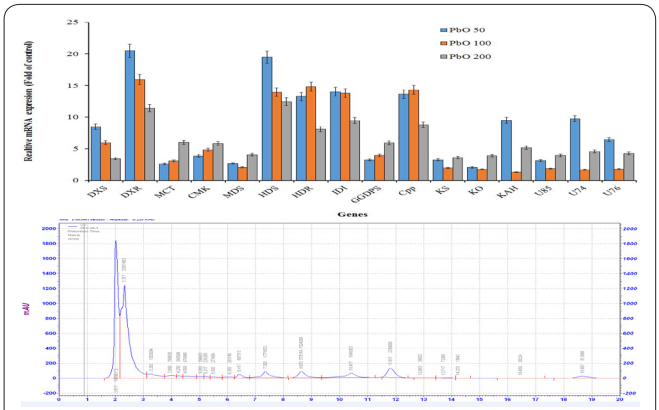

Figure 6. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of PbO.

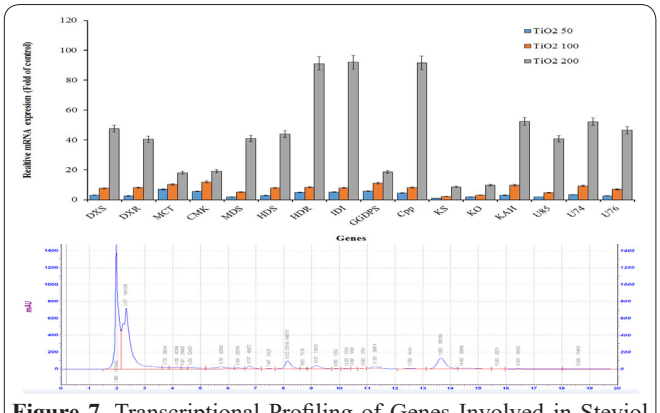

Figure 7. Transcriptional Profiling of Genes Involved in Steviol Glycoside Biosynthesis (top) and RebaudiosideA production (bottom) in *Stevia rebaudiana* Bertoni as function of TiO_2.

this treatment was 200 µm. the peak indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of TiO_2 treatment. The expression profile of the treatment TiO_2 showed that increased concentrations had a significant effect on the expression of genes.

The results of the mean comparison test

The results of Duncan's test at the level of 0.05 showed in treatments Ag_2O and PbO there are no significant differences in the concentrations used. about treatments BaO and TiO_2 there were significant differences between concentrations of 50 µm with 100 µm and 200 µm concentrations. Also in treatment CrO_3, concentration of 100 µm with concentrations 50 and 200 µm there is a significant difference at the level of 0.05. But in treatment Fe_2O_3 there was a significant difference between all three concentrations used and the highest mean related to concentration 200 µm (Table 2).

Discussion

In this study, the effects of metal oxides Ag_2O, CrO_3, PbO, Fe_2O_3, BaO and TiO_2 on the Profiling of expression of genes involved in the biosynthesis Rebaudioside A in stevia by Real-time PCR reaction in 3 replicates with concentrations of 50, 100 and 200 µm were performed. In recent years, many original and effective genes involved in the biosynthesis of steviol glycosides have been diagnosed and their properties have been studied, however, there are still unanswered questions in a series of special routes. For example, pathway biosynthetic rhamnosyl and xylosyl is not still clear (12). In 2013, Mandal and partners examined the effect of inoculation arbuscular mycorrhizal fungi (AMF) species Rhizophagus fasciculatus in the the root of stevia in terms of biomass increase and the production of secondary metabolites. The results show that increasing the amount of biomass and the production of secondary metabolites is strongly influenced by the increase in photosynthesis in plant. Regarding the fact that in the inoculated plants the amount of mineral absorption such as potassium, iron, magnesium nitrate increases and P uptake by the production of the enzyme phosphatase is quadruple, plant growth and photosynthesis rate increases as well. As a result, the amount of biomass will increase and production of secondary metabolites is tripled in control mode (non-inoculated with AMF). In addition, Mandal and

Table 2. The results of the means comparison by Duncan test at P<0.05 employing SPSS (SPSS Inc, Chicago IL, USA) software version 16.

Treatment / Concentrations	Ag_2O	BaO	CrO_3	PbO	Fe_2O_3	TiO_2
50 µm	1.4173±0.13 A	1.6956±0.11 A	2.3835±0.65 B	3.6382±0.75 A	3.9647±0.84 B	6.9647±0.76 A
100 µm	1.4943±0.32 A	0.9388±0.14 B	3.6395±0.62 A	3.2156±0.66 A	2.6773±0.9 C	1.6463±0.83 B
200 µm	1.5601±0.34 A	0.9469±0.12 B	2.1942±0.53 B	2.7946±0.71 A	5.8573±0.78 A	1.1862±0.74 B

partners also examined the effect of inoculated AMF on stevia in terms of the increase in Rebaudioside A and stevioside production. Their results showed that the amount of stevioside and Rebaudioside A production in the inoculated plants will be doubled and 1.5 times greater than control mode, and this increased concentration could be the result of the following issues. First, AMF inoculation resulted in better absorption of phosphorus in stevia roots and given that phosphorus is a key element in the MEP path (13), it can increase the level of transcription of genes 1-deoxy-d-xylulose 5-phospate synthase (DXS), 1-deoxy-d-xylulose 5-phospate reductoisomerase (DXR), (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) (14). Second, the increase in the amount of sugar in the plant through glycosylation (15) will eventually lead to an increase in the amount of stevioside and Rebaudioside A in Stevia. In fact, metal oxides insinuate an oxidative stress by the release of metal ions or free radicals into MS culture medium (16). The role of metal oxides and antioxidants naturally present in plants is to help plant cells to cope with an oxidative stress (17).

Plants produce different kinds of UDP-dependent glycosyltransferases (UGTs) which leads to the catalysis of reactions leading to formation of secondary metabolites (18). UGTs usually act as a substrate and through glycosylation reactions and changes in hydrophilic molecules, the chemical properties and positions in tricellular leads to changes in zeobiotic and cells homeostasis. So far, 733 different UGTs have been identified in living organisms. In *Arabidopsis thaliana* 120 different UGTs have been isolated and identified (19). There are various types of UGTs in stevia that will ultimately lead to the production of the final product called Rebaudioside A. Other UGTs are involved in different Rebaudioside A and dalcosides pathways. For example, UGT76G1 is more conducive to the production of Rebaudioside C and dalcoside A and UGT74G1 more lead to the production of Rebaudioside F and D (20). Also, previous studies showed the important role of UGTs genes in regulating steviol glycoside contents (8,15). *CPPS*, *HDS* and *GGDPS* are widely divergent group of enzymes that transfer a sugar residue from an activated donor to an acceptor molecule (8). In stevia, *CPPS*, *HDS* and *GGDPS* were proposed to be involved in the production of Rebaudioside A, which were unique in the plant world because of their intense sweetness and high concentration in the leaf tissue (21). 161 uni-genes are found so far that were predicted to encode UDP-glycosyltransferases, including *CPPS*, *HDS* and *GGDPS*, which have been reported to be involved in the Rebaudioside A biosynthetic pathway. generally, the 16 major genes involved in the production of Rebaudioside A main role in this project were studied. the results of the study pertaining to the expression pattern of these genes showed that metal oxides have led to an increase in the expression of the regulatory genes involved in biosynthesis of Rebaudioside A and according to the expression profile, it was found that its effect on DXR, HDS, HDR, IDI and CPPS genes is more than other genes. The peak HPLC indicated for stevioside and Rebaudioside A represents an increase in the production of this active ingredient under the influence of all treatments.

In general, the expression profile of these genes and the results of HPLC show that whatever going to the end of the pathway of production of Rebaudioside A, the activity of the enzymes increases under the influence of these treatments, and eventually a greater amount of Rebaudioside A will be produced. This process shows that metal oxides will have a significant effect on the biosynthesis of Rebaudioside A. Also, about the peak of the HPLC samples examined, as seen the peak of the Rebaudioside A is higher than that of stevioside, which indicates the greater effect of metal oxides on the main effective material of this plant. In general, the results of this study showed that the use of metal oxides leads to increase stevioside and Rebaudioside A, in view of the fact that the purpose of cultivation this plant is to produce more of these main effective materials, this can be used from this project in other related studies.

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References

1. Soejarto DD. Botany of Stevia and Stevia rebaudiana. Stevia: the genus Stevia. Medicinal and aromatic plants: industrial profiles, 2002; 19: 18-39.

2. Bridel, M. and Lavielle R. Sur le principe sucre des feuilles de kaa-he-e (Stevia rebaundiana B). Academie des Sciences Paris Comptes Rendus, 1931; 192: 1123-1125.

3. Dacomi E, Mobrien C, Scannell AGM, Arendt EK. Evaluation of antioxidant activity of some plant extracts and their application in biscuit. Journal of Food Chemistry and Nutrition, 2005; 90: 118-121.

4. Chaturvedula VSP, Upreti M, Prakash I. Structures of the novel α-glucosyl linked diterpene glycosides from Stevia rebaudiana. Carbohydrate research, 2011; 346(13): 2034-2038.

5. Rita Elkins MH. Stevia natures sweetener. Woodland publishing, Inc, 1997; 160: 1-29.

6. Kumar H, Singh K, Kumar S. 2C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase from Stevia rebaudiana Bertoni is a functional gene. Molecular biology reports, 2012, 39(12), 10971-10978.

7. Veau B, Courtois M, Oudin A, Chénieux J C, Rideau M, Clastre M. Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in Catharanthus roseus. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 2000; 1517(1): 159-163.

8. Kumar H, Kiran K, Suphla BG, Vijay K, Sanjay K. A comprehensive analysis of fifteen genes of steviol glycosides biosynthesis pathway in Stevia rebaudiana (Bertoni), 2012, Gene. 492 (1):276-284.
9. Jain Anil, Ruud Bolle, Sharath Pankanti. Biometrics: personal identification in networked society, 2006, Vol. 479: Springer Science and Business Media.
10. Nicot N, Jean-François H, Lucien H, Daniele E. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany, 2005; 56 (421):2907-2914.
11. Livak, Kenneth J. and Thomas D Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods, 2001; 25 (4):402-408.
12. Ceunen, S. and Geuns J. M. Steviol glycosides: chemical diversity, metabolism, and function. Journal of natural products, 2013; 76(6): 1201-1228.
13. Mandal S, Evelin H, Giri B, Singh V P, Kapoor R. Arbuscular mycorrhiza enhances the production of stevioside and Rebaudioside¬ A in Stevia rebaudiana via nutritional and non-nutritional mechanisms. Applied soil ecology, 2013, 72, 187-194.
14. Cordoba E, Salmi M, Leon P. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. Journal of Experimental Botany, 2009; 60: 2933–2943.
15. Richman A, Swanson A, Humphrey T, Chapman R, McGarvey B, Pocs R, Brandle J. Functional genomics uncovers three glucosyltransferase involved in the synthesis of the major sweet glucosides of Stevia rebaudiana. Plant Journal, 2005, 41, 56–67.
16. Dimkpa CO, McLean JE, Latta DE, Manangon E, Britt DW, Johnson WP, Boyanov MI, Anderson, AJ. CuO and ZnO nanoparticles: phytotoxicity, metal speciation and induction of oxidative stress in sand-grown wheat. Journal of Nanoparticle Research, 2012; 14, 1125.
17. Klaper R, Crago J, Barr J, Arndt D, Setyowati K, Chen J. Toxicity biomarker expression in daphinids exposed to manufactured nanoparticles: changes in toxicity with functionalization. Environ. Pollut, 2009, 157, 1152e1156.
18. Gachon CM, Langlois-Meurinne M, Saindrenan P. Plant secondary metabolism glycosyltransferases: the emerging functional analysis. Trends in plant science, 2005; 10(11): 542-549.
19. Osmani S A, Bak S, Møller B L. Substrate specificity of plant UDP-dependent glycosyltransferases predicted from crystal structures and homology modeling. Phytochemistry, 2009; 70(3): 325-347.
20. Houghton-Larsen J, Hicks PM, Naesby M, Ostergaard TT, Hansen J, Dalgaard Mikkelsen M, Halkjaer HE, Simon E. Recombinant production of steviol glycosides, 2013, Patent WO 2013/022989 (February 14, 2013).
21. Chen J, Hou K, Qin P, Liu H, Yi B, Yang W, Wu W. RNA-Seq for gene identification and transcript profiling of three Stevia rebaudiana genotypes. BMC genomics, 2014; 15(1): 571.