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Original Research

Effect of KH_2PO_4 on gene expression, morphological and biochemical characteristics of *stevia rebaudiana* Bertoni under *in vitro* conditions

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Abstract: *Stevia rebaudiana* is one of the most important biologically sourced and low-calorie sweeteners Bertoni that has a lot of steviol glycosides. Tissue culture is the best for propagation of stevia and micro nutrients can affect both morphological traits and steviol glycosides production. Therefore, the effect of different concentrations of KH_2PO_4on stevia growth factors and gene expression had been studied by tissue culture methods, RT-PCR and HPLC. According the results, bud numbers had increased significantly in MS + 0.034 mMKH_2PO_4 media and the highest measured length was seen in plants grown under MS + 0.034 mM KH_2PO_4 treatment. Also, the highest growth rate (1.396 mm/d) was observed in MS + 0.034 mMKH_2PO_4.The best concentration of KH_2PO_4 for expression of *UGT74G1* was 0.00425mMand the best one for *UGT76G1* expression was 0.017mM. Interestingly, the best media for both stevioside and rebaudioside A accumulation was 0.017mM KH_2PO_4containing media. There was positive correlation between the best media for gene expression and the best one for steviol glycosides production.

Key words: Stevia *rebaudiana* Bertoni; Tissue Culture; KH_2PO_4; RT- PCR; HPLC; *UGT74G1*; *UGT76G1*.

Introduction

Stevia rebaudiana Bertoni is one of the most important biologically sourced and low-calorie sweeteners that has a lot of Steviol glycosides such as rebaudioside A, B, C, D, E, F, M, steviol bioside, dulcoside A, dulcoside C and stevioside in leaves, that leads to known as "Sweet Leaf" or "Honey Leaf" (1-4). This herbal plant belongs to genus Stevia from Asteraceae family and is native to parts of Brazil and Paraguay. The steviol glycosides specially rebaudioside A and stevioside that are extracted from leaves because of some properties such as zero calorie, anti-microbial, anti-hypertensive, anti-hyperglycemic, anti-cancerous, anti-oxidant have several applications in medicine (5-7).

Recent study reported among different genes such as *CMS, CMK, HDS, HDR, DXS, DXR, MCS, KS, KO, KAH, GGDPS, CDPS, UGT85C2, UGT74G1* and *UGT76G1* that involved steviol glycoside biosynthesis pathway, *UGT85C2*, *UGT74G1* and *UGT76G1*behave in a region selective manner and propose a modified pathway for the synthesis of rebaudioside A from steviol (8-9).

Among different methods for propagating Stevia, tissue culture is the best method with high efficiency that can overcome to problems of traditional methods (11-15).

Recent study reported genetic factors, nutrient accessibility and environment are the most important factors that have effect on Growth, yield, and quality of stevia (16). Another study reported agricultural operations, use of organic fertilizers in a technicalway and water management effect on active constituents specifically stevioside in *Stevia*herb (17-18).

Inorganic nutrient that used in media composition of plant tissue culture divided to macro and micro nutrients. Macro nutrients containing phosphorus, potassium, calcium, magnesium and sulphur are essential for plant cell and tissue culture. Phosphorus that found in meristematic and other fast growing tissue, is an essential element required in respiration and photosynthesis and it effects on plant maturation and root growth. Potassium phosphate is one of the main sources of phosphorus that used in plant tissue culture media (19). The $H_2PO_4^-$and HPO_4^{2-} are two forms of phosphorus, the primary and secondary orthophosphate anions, were observed in plants by active process (20).

Another study showed that phosphorus in MS medium influenced the growth of seedlings of *A. blanchetiana* cultured, and 2.5 mM concentration of phosphorus in MS medium provided the highest production of fresh and dry weight in both shoot and root system (21) Therefore the present study was carried out with an objective of studying the effect of KH_2PO_4on morphological characteristics (bud number, root number and shoot length), expression level of genes in steviol glycosides biosynthesis pathway (*UGT74G1* and *UGT76G1*) and the most important steviol glycosides contents (stevio-

side and rebaudioside A) of *S. rebaudiana* under *in vitro* conditions.

Materials and Methods

Plant materials and culture conditions

To the present study, *stevia rebaudiana* Bertoni explants were provided from Zagros Bioidea Co. Razi University, 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 (22), with 30 g/L sucrose supplemented and different concentrations of KH_2PO_4 (0, 0.034, 0.017, 0.0085 and 0.00425 mM). The pH of the medium was adjusted to 5.8 and after that 8 g/L agar was added and 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 evaluations

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) (23; Table 1). RT-PCR reactions were performed for the targets and house-keeping gene. 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). 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 KH_2PO_4 by the method described earlier (24). Powder of dried leaves of stevia which treated by different concentrations of KH_2PO_4 (0, 0.034, 0.017, 0.0085 and 0.00425 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 Duncans multiple range test with critical value of $P < 0.05$.

Results and discussion

Effect of different concentrations of KH_2PO_4 on Stevia morphological traits

All measured traits of Stevia plants drown under different concentration of KH_2PO_4 treatment, showed significant differences. Traits was included bud number and bud length (both after 15 days and 30 days), germination rate and growth rate (Table 2). The mean comparison results were shown in Table 3 and Figure 1 and Figure 2. According to the results, the largest number of buds (2.80) in the first measurement was seen under MS + 0.034 mM KH_2PO_4 treatment while the lowest number of buds (1.20) in this stage was observed in MS media

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. ANOVA table of the effect of different concentration ofKH_2PO_4 on *stevia rebaudiana*Bertoni. Where Bud N1. = Bud Number after 15 days, Bud N2 = Bud Number after 30 days, Bud L1= Bud Length after 15 days, Bud L2= Bud Length after 30 days.

		Mean Squares					
Source	df	Bud N1	Bud N2	Bud L1 (mm)	Bud L2 (mm)	Germination Rate	Growth Rate(mm/d)
Treat	4	7.360*	34.160**	415.312*	7167.975*	0.555*	4.863*
Error	20	21.600	32.000	548.392	12726.089	0.931	10.365

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

Table 3. Effect of different concentrations of KH_2PO_4on *Stevia rebaudiana.* MS culture withoutKH_2PO_4as the control. (Where BudN1. = Bud Number after 15 days, Bud N2 = Bud Number after 30 days, Bud L1= Bud Length after 15 days, Bud L2= Bud Length after 30 days) Mean values within a column with the same letter are not significantly different based on the least significant difference (LSD, p = 0.05).

	Treats	Bud N1	Bud N2	Bud L1(mm)	Bud L2(mm)	Germination Rate	Growth Rate (mm/d)
1	0 $mMKH_2PO_4$ (MS)	1.2000^{b}	2.2000^{b}	2.3000^{c}	5.9000^{b}	0.7060^{a}	0.1180^{b}
2	MS + 0.00425 $mMKH_2PO_4$	1.8000^{ab}	2.8000^{b}	4.4000^{bc}	24.7000^{ab}	0.3640^{b}	0.6700^{ab}
3	MS + 0.0085 $mMKH_2PO_4$	2.4000^{ab}	3.6000^{b}	11.3500^{ab}	45.0500^{a}	0.2880^{b}	1.1220^{ab}
4	MS + 0.017 $mMKH_2P_O4$	2.0000^{ab}	3.0000^{b}	5.7000^{abc}	36.7500^{ab}	0.3300^{b}	1.0340^{ab}
5	MS + 0.034 $mMKH_2PO_4$	2.8000^{a}	5.6000^{a}	12.8400^{a}	54.7240^{a}	0.4460^{ab}	1.3960^{a}

(control). In the second measurement of buds was seen that bud numbers had increased significantly in MS + 0.034 mM media (5.60) whereas the lowest number (2.20) of buds was belonged to MS media. In addition, the highest measured length was seen in plants grown under MS + 0.034 $mMKH_2PO_4$treatment in both stages of measurement (12.840mm and 54.7240mm respectively). On the other hand, the highest germination rate (0.7060) was belonged to plants grown in MS media while there was no significant differences between MS and MS + 0.034 $mMKH_2PO_4$media. Interestingly, the highest growth rate (1.396mm/d) was observed in MS + 0.034 $mMKH_2PO_4$and the lowest growth rate was seen in MS media.

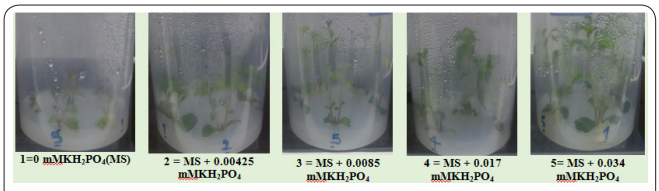

Figure 1. Shoot proliferation from nodal explants on medium (MS+ different concentration of KH_2PO_4) after 30 days. (Where, 1=0 $mMKH_2PO_4$(MS); 2 = MS + 0.00425$mMKH_2PO_4$; 3 = MS + 0.0085$mMKH_2PO_4$; 4 = MS + 0.017$mMKH_2PO_4$; 5= MS + 0.034$mMKH_2PO_4$).

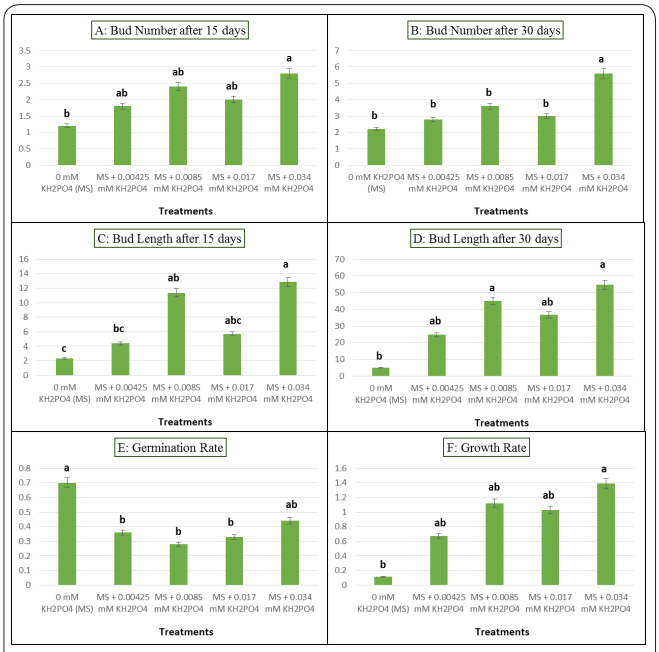

Figure 2. Effect of different concentrations of KH_2PO_4 on *Stevia rebaudiana*after 15 and 30 days of culture.

Investigation of *UGT74G1* and *UGT76G1* genes expression

The results of RT- PCR were normalized to the level of the housekeeping gene of *β-actin* in plants subjected to different concentrations of KH_2PO_4 (Figure 3). There were significant differences between different treatments.The highest amount of *UGT74G1* gene expression was occurred in plants grown under MS +

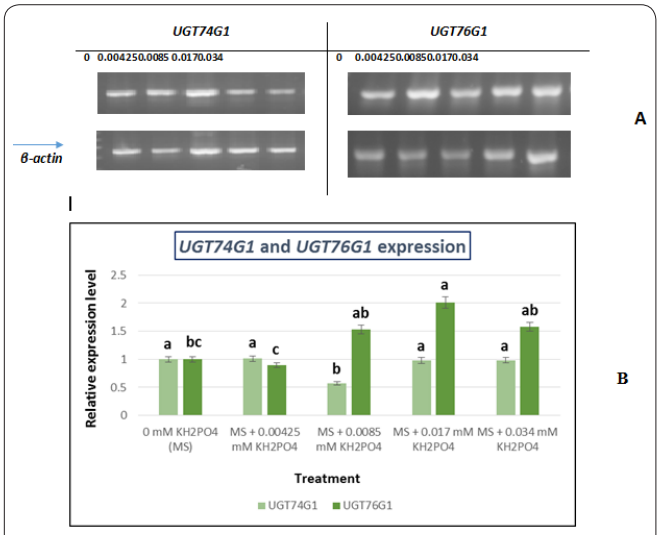

Figure 3. Expression of *UGT74G1* and *UGT76G1* in the leaves of *Steviarebaudiana*Bertoni under different concentrations of KH_2PO_4. (a) Semi-quantitative RT-PCR analysis of *UGT74G1*and *UGT76G1* in plants treated with different concentrations of $KH_2PO_4$for 30 days. *β-actin* was used as an internal 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 KH_2PO_4. Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncans test).

0.00425 mM KH_2PO_4 treatment which had no significant differences with other media except MS + 0.0085 mM KH_2PO_4. There was no determined trend for added KH_2PO_4 to MS media. However, the highest gene expression for *UGT76G1* was seen under MS + 0.017 mM KH_2PO_4 which had no significant differences with MS + 0.0085 mM KH_2PO_4 and MS + 0.034 mM KH_2PO_4. As regards, the lowest *UGT76G1* gene expression was belonged to plants grown under MS + 0.00425 mM KH_2PO_4.

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 KH_2PO_4 showed significant differences between the amounts of two intended steviol glycosides. The highest amount of stevioside had been accumulated in plants grown in MS + 0.017 mM KH_2PO_4 media which had no significant differences with MS + 0.0085 mM KH_2PO_4 and MS + 0.034 mM KH_2PO_4. It is noticeable that the results are consistent with *UGT76G1* gene expression outputs. Also, the highest accumulation of rebaudioside A was observed in MS + 0.017 mM KH_2PO_4 media which had no significant differences with other media except MS.HPLC chromatograms obtained from all the samples showed the peaks corresponding to standard stevioside and rebaudioside A marker compounds (Figure 4 &5).

The most important aspect of the studies about stevia is the amount of steviol glycosides and the effect of different nutrients on that. So, the aim of the studies can be enhancing these sweetener components production. According to the results, stevia growth is affected by changing in media nutrients. Obviously, increasing in KH_2PO_4 associated with enhancing in both expression of the intended genes and accumulation of steviol glycosides. Also, the measured morphological traits were improved under increased KH_2PO_4 media. Many studies had been carried out and showed that nutrients have a significant impact on various stages of plants development (19-21). Finally, it had been clear that the best concentration of KH_2PO_4 for expression of *UGT74G1* was 0.00425mM and the best one for *UGT76G1* expression was 0.017mM. Interestingly, the best media for both stevioside and rebaudioside A accumulation was 0.017mM KH_2PO_4 containing media. There was positive correlation between the best media for gene expression and the best one for steviol glycosides production.

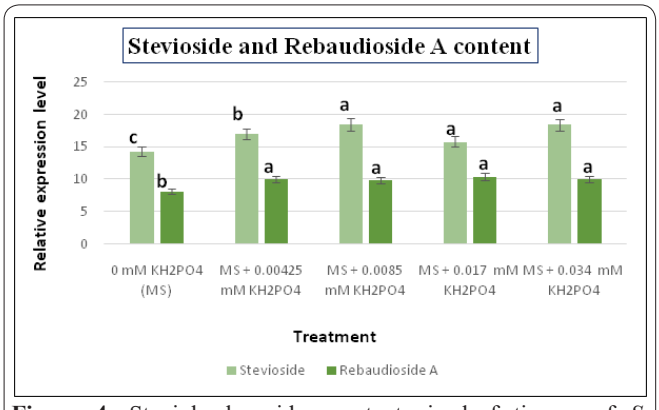

Figure 4. Steviol glycosides contents in leaf tissues of *S. rebaudiana* subjected to different concentration of KH_2PO_4.

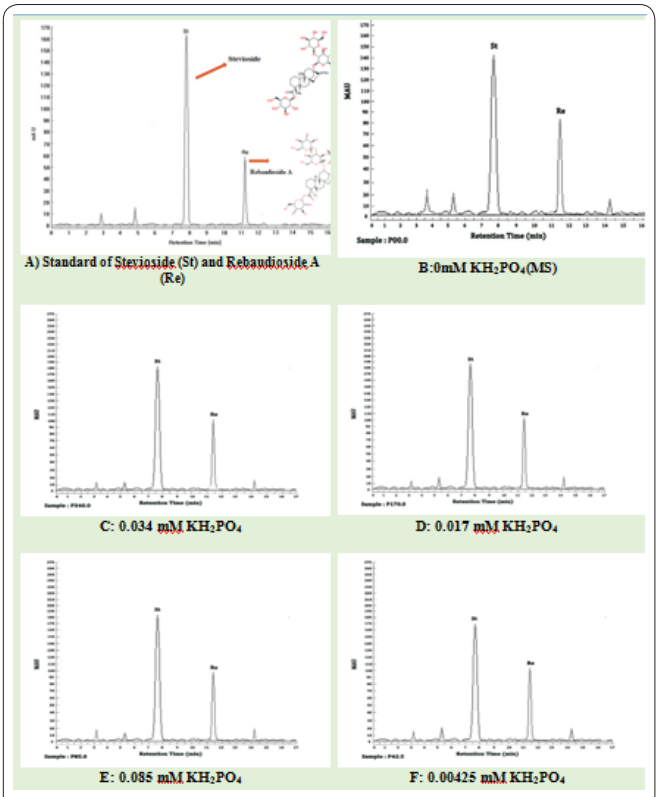

Figure 5. Representative HPLC chromatograms for quantification of stevioside and rebaudioside A in methanolic extract of *S. rebaudiana*leaf tissues. A: Standard of Stevioside (St) and Rebaudioside A (Re); B: 0 mM KH_2PO_4 (MS); C: 0.034 mM KH_2PO_4; D: 0.017 mM KH_2PO_4; E: 0.085 mM KH_2PO_4; F: 0.00425 mM KH_2PO_4.

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