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Original Research

Investigation of different concentrations of MS media effects on gene expression and steviol glycosides accumulation in *Stevia rebaudiana* Bertoni

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Abstract: *Stevia rebaudiana* Bertoni is one of two species that contains steviol glycosides. Among steviol glycosides that extracted from leaves, stevioside and rebaudioside A are the two major and the sweetest glycosides that are about 200-300 times sweeter than sucrose with zero calories. The best method for stevia propagation is tissue culture. So, for investigation of nutrients in medium, we studied the effect of different concentrations of MS media (MS, 0.5 MS, 0.25 MS, 0 MS) on morphological traits, *UGT74G1* and *UGT76G1* genes expression and accumulation of steviol glycosides in stevia leaves. The best growth rate (0.472 mm/d) has occurred in plants grown in MS media. Also, the highest gene expression of *UGT74G1* gene (1.000 Total lab unit) was seen under MS treatment. However, the highest expression level of *UGT76G1* gene (1.701 Total lab unit) was observed at plants grown in 0 MS. The highest amount of both Stevioside and Rebaudioside A (14.23 and 8.12, respectively) were accumulated in plants under MS treatment. Obviously, dilution of MS media associated with decreasing in both expression of the intended genes and accumulation of steviol glycosides.

Key words: *Stevia rebaudiana* Bertoni; MS media; Steviol glycoside; Semi-quantitative RT-PCR; HPLC.

Introduction

Two *Stevia* species containing *S. rebaudiana* and *S. phlebophylla* from Asteraceae family are the only plant species that produce sweet steviol glycosides such as rebaudioside A, B, C, D, E, F, M, steviol bioside, dulcoside A, dulcoside C and stevioside in their leaves and small amounts in their stems (1-3). Among steviol glycosides that extracted from leaves, stevioside and rebaudioside A are two major and the sweetest glycosides that are about 200-300 times sweeter than sugar with zero calories. Thus leaves extraction of stevia plant has different applications in medicine and food industry such as anti-hyperglycemic, anti-hypertensive, anti-oxidant, anti-microbial, anti-cancerous, sweetening agents and taste modifiers (4-8).

Some studies that used labeled glucose and RT-qPCR analysis reported biosynthesis of Steviol glycoside containing MEP pathway that initiated with steviol and terminated with rebaudioside A, and different genes such as *CMS, CMK, HDS, HDR, DXS, DXR, MCS, KS, KO, KAH, GGDPS, CDPS, UGT85C2, UGT74G1* and *UGT76G1* were involved in this pathway. In addition, three of these genes such as *UGT85C2*, *UGT74G1* and *UGT76G1* that belong to family GT1 of the glycosyl transferase group involved in the synthesis of stevioside and rebaudioside A (1, 9-12).

The best method for stevia propagation is tissue culture that is done under sterile and controlled environmental conditions. Some studies reported large quantities of primary nutrients such as Nitrogen, phosphorous and potassium exist in fertilizers, and plant uses them for growing. Secondary nutrients such as calcium, magnesium and sulfur that are generally present in the soil were used deeply by plants (13-16).

Recent studies reported that the nutrients of media culture such as nitrogen could improve the plant growth during the vegetative phase and protein synthesis (17). Inorganic nitrogen sources that generally used MS medium for micropropagation of *S. rebaudiana* are NH_4^+-N and NO_3^--N and NH_4^+: NO_3^- = 1:2 (14). Some studies reported that MS medium supplemented with 2, 4-D (9.05 and 18.19 µM) and Kinetin (0 to 9.2 µM) leaded to somatic embryogenesis, and embryogenic callus formation occurred in MS medium that supplemented with 9.05 µM 2, 4-D without Kinetin (18). Another study indicated that MS medium supplemented with 0.5 mg/l BAP+2.0 mg/l Kn led to induction of multiple shoots from nodal segments that were the highest. In addition, different concentrations of IBA used for rooting and highest rooting was recorded on MS medium containing 1.0 mg/l IBA (16).

Materials and Methods

Plant materials and culture conditions

To run the project, *S. rebaudiana* Bertoni explants was provided by 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 media (19) in different concentrations: full MS (1),

Table 1. The 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

0.5 MS, 0.25 MS, 0 MS. 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 "Shoot length (mm), Root length (mm), Number of leaves, Total fresh weight (g), Total dry weight (g), Growth rate (mm/d) were recorded.

RNA extraction

Total RNA of fresh leaves was extracted using RNX plus™ kit (Cinnaclon) according to the manufacturer's instructions. RNA quantification was done by 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

Determine gene expression of *UGT74G1* and *UGT76G1* genes in stevia was performed by the two-step semi-quantitative RT-PCR method. For cDNA synthesis, 10 µg of total RNA was reversely transcribed with 100 UM-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) (20; Table 1). RT-PCR reactions were done 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 Reverse 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.

Electrophoresis on a 1% agarose separated The PCR products 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 MS media by the method described earlier (21). Powder of dried leaves of stevia which treated by MS media in different concentrations: 1, 0.5, 0.25, 0, was used. They were exposed to 80% methanol for 3–4 times, then dried in vacuo and defatted with hexane. Finally, the residual extract was dissolved in acetonitrile and filtered after vacuum drying. The chromatographic separation was achieved using a symmetry Xbridge amide column (4.6 × 150 mm, 3.5 µm, 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 estimation 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, the mean comparison was performed by Duncan's multiple range test with a critical value of $P < 0.05$.

Results

Effect of different media on morphological traits of stevia

Media concentration changing treatments showed significant differences in all measured traits except root length (Table 2). According to the table 3 and figure 1,

Table 2. Mean Squares of the effect of different concentration of MS media on *S. rebaudiana* Bertoni.

Source	df	Mean Squares					
		Shoot length(mm)	Root length(mm)	Number of leaves	Total fresh weight(g)	Total dry weight(g)	Growth rate
Treat	3	60.31**	2.82^{ns}	1787.88**	36.34**	0.003**	0.12**
Error	12	1.77	1.18	50.02	5.75	0.00	0.00
CV (%)		16.74	29.42	21.13	51.65	39.89	21.52

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

Table 3. Effect of different concentration of MS media on *S. rebaudiana.* Mean values within a column with the same letter are not significantly different based on the least significant difference (Duncan, p = 0.05).

Treats	Shoot length(mm)	Root length(mm)	Number of leaves	Total fresh weight(g)	Total dry weight(g)	Growth rate(mm/d)
MS	12.030^{a}	2.805^{b}	25.600^{b}	4.740^{a}	0.045^{b}	0.472^{a}
0.5 MS	9.835^{b}	3.655^{ab}	61.900^{a}	8.205^{a}	0.077^{a}	0.370^{b}
0.25 MS	6.890^{c}	3.510^{ab}	34.530^{b}	4.795^{a}	0.030^{bc}	0.237^{c}
Agar (0 MS)	3.060^{d}	4.830^{a}	11.000^{c}	0.830^{b}	0.012^{c}	0.060^{d}

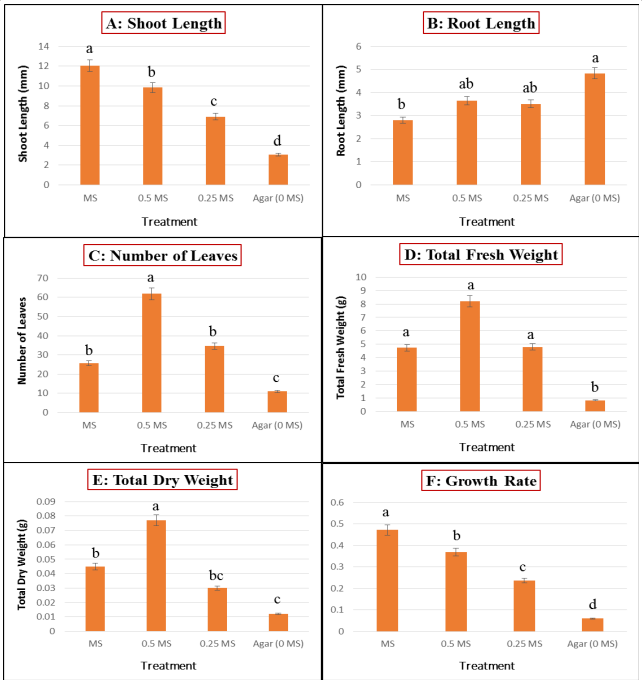

Figure 1. Effect of different concentrations of MS media on the morphological traits of *S. rebaudiana.*

the highest amount of shoot length was seen in plants grown in MS media (12.030 mm). There was a falling trend from MS to 0 MS treatment which means during media dilution, shoot growth decreased significantly (Figure 2). Interestingly, the most number of leaves was observed under 0.5 MS media (61.900) that there were significant differences with other treatments. The lowest number of leaves was due to the 0 MS media (11.000). Also, the highest total fresh weight was seen in plants grown in 0.5 MS media (8.205 g). There were no significant differences with other treatments except 0 MS (0.830 g). Naturally, the same results were observed in total dry weight which the highest amount was 0.077 g in 0.5 MS and the lowest amount was 0.12 g. However, the highest growth rate was due to MS media (0.472 mm/d) and the lowest growth rate was due to 0 MS (0.060 mm/d). There was a falling trend from MS to 0 MS media.

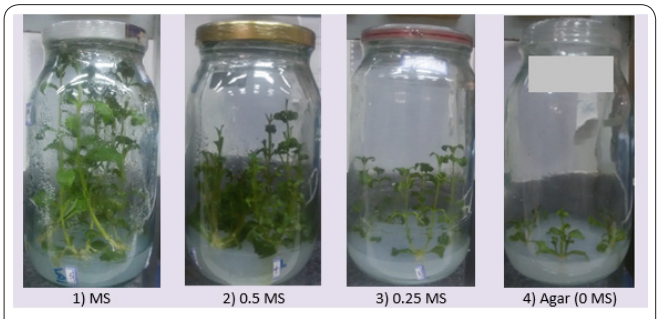

Figure 2. Effect of different concentrations of MS media on *S. rebaudiana* shoot length under in-vitro situation.

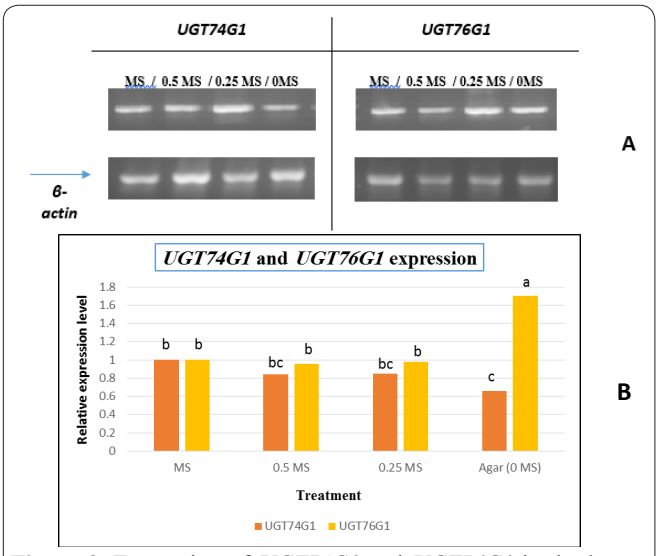

Figure 3. Expression of *UGT74G1* and *UGT76G1* in the leaves of *S. rebaudiana* Bertoni under different concentrations of MS media. (a) Semi-quantitative RT-PCR analysis of *UGT74G1* and *UGT76G1* in plants treated with different concentrations of MS media. *β-actin* was used as an internal control.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

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 MS media. According to the results which have been shown in Figure 3, although the highest gene expression was seen under MS treatment (1.000 Total lab unit), there were no significant differences between various treatments in term of *UGT74G1* gene expression. However, the highest expression level of *UGT76G1* gene was observed at plants grown in 0 MS (1.701 Total Lab unit) which was different significantly with other treatments. The lowest gene expression for *UGT76G1* was seen in plants treated with 0.5 MS media (0.957 Total Lab unit) whereas no significant differences with MS and 0.25 MS media.

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 MS media showed significant differences between the amounts of two intended steviol glycosides. As it has been shown in Figure 4 and 5, the highest amount of Stevioside was accumulated in plants

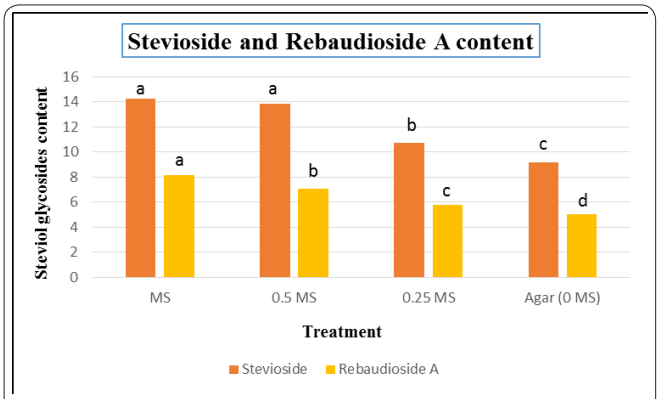

Figure 4. Steviol glycosides contents in leaf tissues of *S. rebaudiana* subjected to the different concentration of MS media.

under MS treatment with no significant differences from 0.5 MS media. However, the lowest production level of stevia was observed at plants grown in 0 MS media. In term of Rebaudioside A accumulation, the highest amount was due to MS media and the lowest amount was seen in plants which were treated by 0 MS media. Interestingly, there was a falling trend for both intended steviol glycosides from MS to MS media.

Discussion

According to the literature the most important aspect of the researches about stevia is the amount of steviol glycosides and the effect of different nutrients on that. So, the main target of the studies can be increasing these sweetener components production. Based on the results, stevia growth is impacted by changes in media nutrients. Obviously, dilution of MS media associated with decreasing in both expressions of the intended genes and accumulation of steviol glycosides. Also, the measured morphological traits almost had been regressive under subtilized MS media. Many studies had been carried out and showed that nutrients have a significant impact on various stages of plants development (22-24).

Also, recent researches reported nutrients of media culture such as nitrogen could improve the growth during the vegetative phase and protein synthesis (17). Inorganic nitrogen sources that generally used MS medium for micropropagation of *S. rebaudiana* are $NH4^{+}$-N and $NO3^{-}$-N and $NH4^{+}$: $NO3^{-}$ = 1:2 (14). Another study showed MS medium that supplemented with 0.5 mg/l BAP+2.0 mg/l Kn led to induction of multiple shoots from nodal segments that were the highest. In addition, different concentrations of IBA used for rooting and highest rooting was recorded on MS medium containing 1.0 mg/l IBA (16).

In 2017 Akbari et al. studied about the effect of nitrogen sources on some morphological characteristics and gene expression of *S. rebaudiana* Bertoni under in vitro conditions. Their results showed that existence of nitrogen sources increases both shooting and rooting in stevia. Also the highest expression of *UGT74G1* was observed in plantlets grown on MS media without NH_4NO_3 and 950 mg/l KNO_3 but the highest expression of *UGT76G1* was observed in plantlets grown on MS media with 1650 mg/l NH_4NO_3 +950 mg/l KNO_3 (25-26). In 2017 Fallah et al. investigated about the effects of salinity and osmotic stress caused by different concentration of NaCl on morphological traits, genes expression and

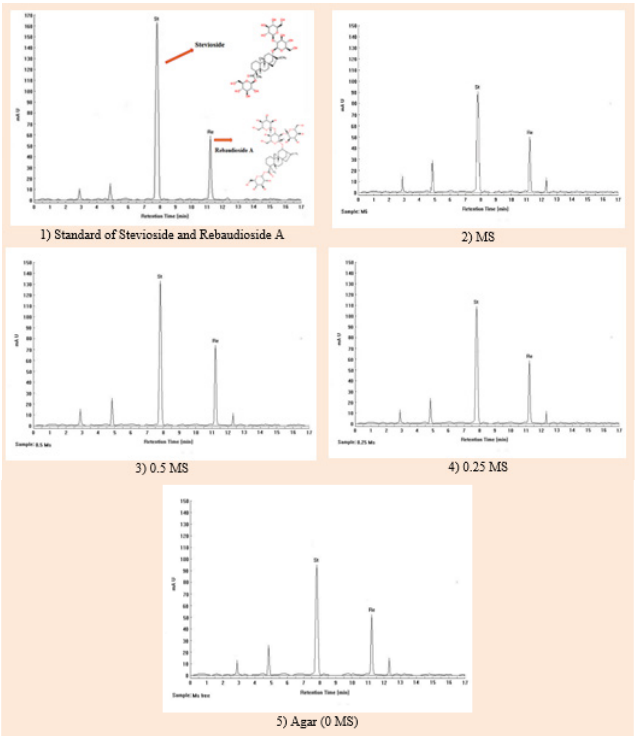

Figure 5. Representative HPLC chromatograms for quantification of stevioside and Rebaudioside A in methanolic extract of *S. rebaudiana* leaf tissues under different concentrations of MS media treatment.

amount of steviol glycosidws under *in vitro* conditions. As they said, with increasing salinity, the values of all studied morphological traits decreased and the highest expression of *UGT74G1* and *UGT76G1* was observed in plantlets grown on MS media (without NaCl) but the lowest amounts of gene expression of these genes were seen in MS media with 60 mM NaCl (27).

Kahrizi et al, 2017, observed the effect of different concentrations of KH_2PO_4 on stevia morphological characteristics and gene expression by tissue culture methods, RT-PCR and HPLC. They reported that the best concentration of KH_2PO_4 for expression of *UGT74G1* was 0.00425 mM KH_2PO_4 and the best one for *UGT76G1* expression was 0.017 mM KH_2PO_4 (28).

Finally, it had been clear that MS media is optimum for stevia growth and expression of *UGT74G1* and *UGT76G1* There was a positive correlation between the best media for gene expression and the best one for steviol glycosides accumulation.

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