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Original Research

Effects of various glutamine concentrations on gene expression and steviol glycosides accumulation in *Stevia rebaudiana* Bertoni

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Abstract: *Stevia rebaudiana* Bertoni is one of the most important biologically sourced and low-calorie sweeteners that contains a lots of Steviol glycosides. Tissue culture is the best method for propagation of stevia and micro nutrients can affect both morphological traits and steviol glycosides production. In the present study, we investigated the effect of different concentrations of glutamine (10, 20, 30 and 40 g/l) on expression of *UGT74G1* and *UGT76G1* genes and stevioside and rebaudioside A accumulation in the leaves of stevia under *in vitro* conditions. The highest level of expression for *UGT74G1* (1.000 Total lab unit) was seen at plants grown in MS media without glutamine and the highest gene expression level for *UGT76G1* (1.321 Total lab unit) was observed at plants grown in 2% glutamine. Based on HPLC results, the highest amount of stevioside (22.74) was accumulated in plants which were under 3% glutamine treatment and the lowest production level of stevioside (16.19) was resulted under MS (0 glutamine) medium. The highest rebaudioside A (12.19) accumulation was observed under 2% glutamine treatment and the lowest accumulation of rebaudioside A (8.41) was seen at plants grown in MS medium.

Key words: *Stevia rebaudiana* Bertoni; Tissue Culture; Glutamine; Semi quantitative RT- PCR; HPLC.

Introduction

The wild type of *Stevia rebaudiana* Bertoni plant was found in the highlands of the Amambay at Iguac districts (a border area between Brazil and Paraguay). So, at 1964 this plant that belongs to Asteracea family, was one of the commercial cultivation in Paraguay (1-6). Since then, because of beneficial products such as versatile medicinal uses without any side effects led to focus interest towards stevia in worldwide, and it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada since 1990 (7-9).

Today the powder of stevia leaf that has several steviol glycosides such as rebaudioside A, B, C, D, E, F, M, steviol bioside, dulcoside A, dulcoside C and stevioside, are used as taste modifiers, sweetening agents and alternative sweeteners in food industry, and added to ice cream, dairy products, drinks, sherbet, table-top sweeteners for controlling hypoglycemic and body weight reduction effect (10-17).

Fifteen genes exist in the biosynthesis pathway of steviol glycosides such as *CMS, CMK, HDS, HDR, DXS, DXR, MCS, KS, KO, KAH, GGDPS, CDPS, UGT85C2, UGT74G1* and *UGT76G1* that among them *UGT85C2, UGT74G1* and *UGT76G1* genes have important roles in the synthesis of stevioside and rebaudioside A (6, 8, 12, 18-19).

Semi-quantitative reverse transcription-polymerase chain reaction (Semi-quantitative RT-PCR) is a highly sensitive and specific method used for the detection of gene expression under different experimental conditions (31).

The best method for propagating stevia with high efficiency is tissue culture that provides a condition for studying stress tolerance mechanisms (11, 20-23). Most of the studies reported the media cultures that supplied with amino acids enhanced somatic embryogenesis in many plant species and the effect on general metabolism and morphogenesis (24-27). Glutamine is known as one of the amino acids that metabolized extensively to glutamate in the supplied explant and recovery of L-glutamate as the main component suggested transport in this form. L-glutamine or other amino acids, which can be readily transferred to other amino acids and incorporated into proteins (28-29).

Das and Mandal, 2010, used glutamine for enhanced development of embryogenic callus in stevia, repor-

ted this amino acid has a vital role in the induction and maintenance of lesion and embryogenic calli without necrosis which can ultimately produce the maximum number of regenerated shoots (30).

Therefore, the present study was carried out with an objective of studying the effect of different concentration of glutamine (10, 20, 30 and 40 g/l) on some gene expression (*UGT74G1* and *UGT76G1*) and steviol glycosides (stevioside and rebaudioside A) accumulation in stevia.

Materials and Methods

Plant materials and culture conditions

In the present study, *stevia rebaudiana* Bertoni explants were obtained from Zagros Bioidea Co. Razi University, Kermanshah, Iran. Axillary buds of about 2 cm in length with two leaves were separated from the shoots. Stevia plants were propagated in MS media culture (23), with 30 g/L sucrose supplemented and different concentrations of glutamine (10, 20, 30 and 40 g/l). The pH of the medium was adjusted to 5.8 and after that 8 g/L agar was added and autoclaved. Five explants were cultured in per medium 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%.

RNA extraction

Fresh leaves total RNA was extracted using RNX plus™ kit (Cinnaclon) according to the manufacturer's protocol. Quantification of RNA was performed 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 using 2.0% agarose gel electrophoresis.

Expression analysis of *UGT74G1* and *UGT76G1* genes

The two-step semi-quantitative RT-PCR method had been 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 intended and *β-Actin* genes were designed using the Oligo 7 Primer Analysis Software and to obtain specific characters required for semi-quantitative polymerase chain reaction (RT-PCR) (31; 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 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.

The products of PCR were separated by electrophoresis on a 1% agarose gel in TBE buffer. Four independent experiments were conducted. The amplicons had been 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 levels of *UGT74G1* and *UGT76G1* expression were normalized relative to the amount of *β-Actin* expression.

HPLC analysis

The accumulation of stevioside and rebaudioside A were estimated in the leaves of stevia treated by different concentration of glutamine by the method described earlier (32). Dried leaves powder of stevia grown under different treatments of glutamine (10, 20, 30 and 40 g/l) was applied to extracting by 80% methanol for 3–4 times and after that dried in vacuo and defatted with hexane and the residual extract was vacuum dried. Then, the obtained extract was dissolved in acetonitrile and filtered. The chromatographic separation was obtained by 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 volume of injection was 10 µl with a flow rate of 0.8 ml/min. The stevioside and rebaudioside A accumulation had been measured using three independent replicates.

Statistical analysis

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

Results

According to the figure 1, expression of *UGT74G1* and *UGT76G1* in the leaves of stevia had been changed under different concentrations of glutamine. The highest level of expression for *UGT74G1*was seen at plants grown in MS media without any added glutamine (1.000 Total Lab unit) that there were not significant differences between MS media and other treatments except 2% glutamine which caused the lowest level of expression for *UGT74G1* (0.707 Total Lab unit). However, the highest gene expression level for *UGT76G1* was observed at plants grown in 2% glutamine (1.321 Total Lab unit) which had differences significantly with MS

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

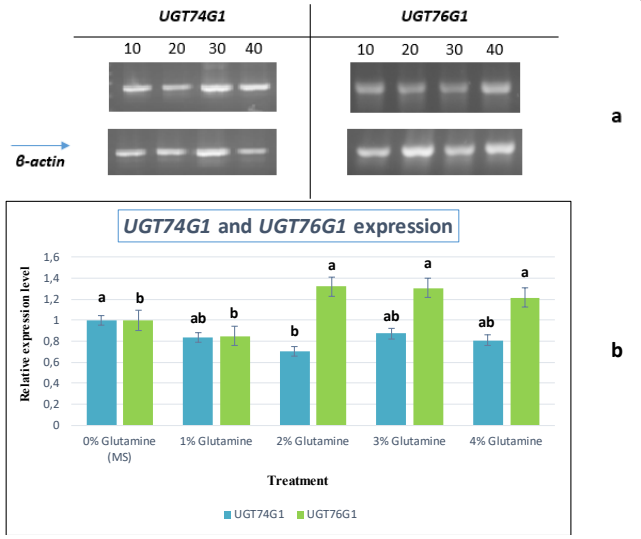

Figure 1. Expression of *UGT74G1* and *UGT76G1* in the leaves of *stevia rebaudiana* Bertoni under different concentrations of glutamine. (a) Semi-quantitative RT-PCR analysis of *UGT74G1* in plants treated with different concentrations of glutamine for 30 days. *β-actin* was used as an internal control. The final value was the average of at least three 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 glutamine. 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 glutamine) and 1% glutamine treatments (it showed the lowest expression: 0.850 Total Lab unit).

Based on HPLC results (figure 2 and 3), the highest amount of stevioside was accumulated in plants which were under 3% glutamine treatment that there was no significant difference with 2% glutamine treatment. The lowest production level of stevioside resulted under MS (0 glutamine) medium. Obviously, there was a rising trend from 0 glutamine to 3% glutamine treatment. On the other hand, the highest rebaudioside A accumulation was observed under 2% glutamine treatment. There were no significant differences with 1% and 3% glutamine. The lowest accumulation of rebaudioside A was seen at plants grown in MS medium which had no significant differences with 4% glutamine added medium.

Discussion

Glutamine is one of the most amino acids in plants. Furthermore, glutamine is the main donor for the synthesis of protein, nucleotides, amino acids and other nitrogen-containing compounds in plants. So, Glutamine can efficiently support the growth of *Stevia rebaudiana*. In this study, the changes some of the steviol glycosides contents (stevioside and rebaudioside A) and the expression levels of some steviol glycosides biosynthetic genes (*UGT74G1* and *UGT76G1*) were investigated under different glutamine treatment.

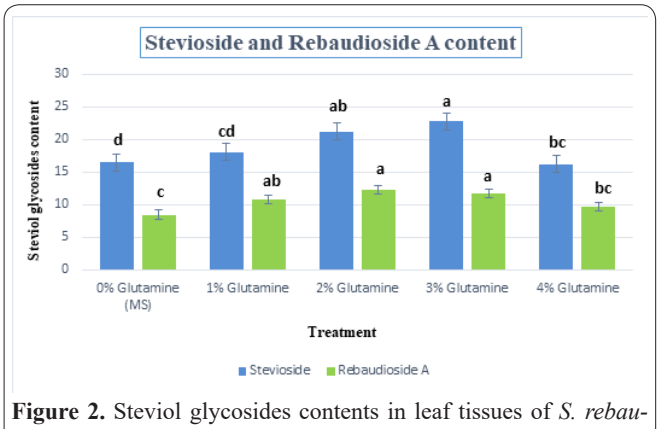

Figure 2. Steviol glycosides contents in leaf tissues of *S. rebaudiana* subjected to different concentration of glutamine.

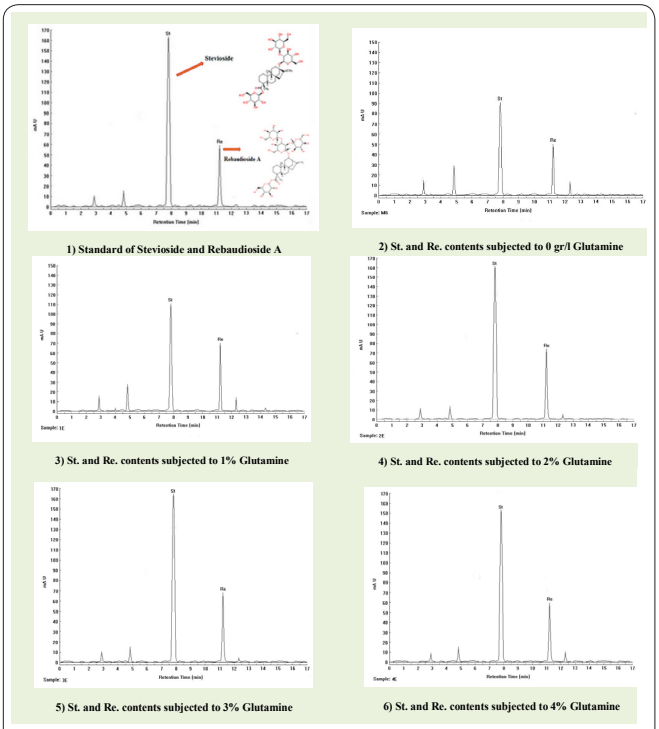

Figure 3. Representative HPLC chromatograms for quantification of stevioside and Rebaudioside A in methanolic extract of *S. rebaudiana* leaf tissues.

As it was shown, gene expression of plants grown in media culture containing glutamine amino acid had a various trend. In addition, in particular concentrations of glutamine, expression of the intended genes increased significantly. This fact that media cultures that supplied with amino acids have important effects on plant growth and metabolites production was mentioned in several researches (24-27).

Thilakavathy and Jagadeesan, 2017, studied the caulogenesis and chlorophyll content in *Stevia rebaudiana* by using glutamine in the culture medium and reported that total biomass and chlorophyll content increased by using 100 mg/l glutamine in the medium. Also, they reported that presence of glutamine in the medium favoured an increase in shoots bud number per explants (33).

Das and Mandal, 2010, investigated a new protocol to accelerate the production of embryogenic calli for *in vitro* plant regeneration of stevia. They used two different types of amino acids (Tryptophan and Glutamine) in different concentration and three different types of additives (yeast extract, casein hydrolysate and potato extract) for this aim. They found that glutamine (50 mg/l) and casein hydrolysate (100 mg/l) produced healthy calli for producing a large number of regenerated shoots. They reported that glutamine played a vigorous role in the induction and maintenance of necrosis free embryogenic calli (30).

Kan et al., 2015, studied the effect of glutamine on the expression of key transcription factor genes

involved in nitrogen and stress responses in rice roots and reported that glutamine induced the expression of at least 35 genes involved in metabolism, transport, signal transduction, and stress responses within 30 min. Furthermore, they reported that glutamine may also has a function as a signalling molecule to regulate gene expression in different plants which is supported by this results (34). Medicinal plants have been explained previously (35).

According to the reports, the most important goal of researches about stevia is increasing the steviol glycosides contents which cause the sweet taste of it. So, optimizing the culture media to obtain the highest level of steviol glycoside accumulation is an important target. In the present study, we defined the best concentration of glutamine for the highest level of both gene expression and steviol glycosides accumulation in *Stevia rebaudiana*.

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