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Effect of nitrogen sources on gene expression of *Stevia rebaudiana* (Bertoni) under *in vitro* conditions

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Abstract: *Stevia rebaudiana* (Bertoni) is a non-caloric sweetener belonging to Asteraceae family. Stevia compounds such as steviol glycosides (SGs) are 200 times sweeter than sugar. Stevioside and rebaudioside A are the two major steviol glycosides. Nitrogen is an essential element for plant growth and development. In this study the effects of nitrogen influenced by different concentrations of NH_4NO_3 (0, 825 and 1650 mg/l) and KNO_3 (0, 950 and 1900 mg/l) is examined in MS medium. To analysis the *UGT74G1* and *UGT76G1* genes expression, involved in the synthesis of SGs, RT-qPCR technique was performed. Data showed that there were significant differences between all media. The shoot length, seedlings dry weight and leaf fresh weight of stevia increased with applying NH_4NO_3 along with KNO_3. The highest expression of *UGT74G1* gene, was observed in plantlets grown on MS medium with 0 mg/l NH_4NO_3 and 950 mg/l KNO_3 (1.291 total lab unit) but the highest expression of *UGT76G1* gene, was observed in plantlets grown on MS medium added by 1650 mg/l NH_4NO_3 +950 mg/l KNO_3 (1.08 total lab unit). Moreover, the lowest value of *UGT74G1* gene expression were revealed in MS medium added by 1650 mg/l NH_4NO_3 +0 mg/l KNO_3 (0.80 total lab unit) and the lowest values of *UGT76G1* gene expression seen in MS medium with 0 mg/l NH_4NO_3 +950 mg/l KNO_3 (0.85 total lab unit) concentrations. The results of this study could be valuable in stevia breeding programs through glycosides biosynthesis pathways.

Key words: KNO_3; NH_4NO_3; *Stevia rebaudiana* (Bertoni); Tissue culture; *UGT74G1*; *UGT76G1*.

Introduction

The *Stevia rebaudiana* (Bertoni) (2n=22) is an herbaceous perennial shrub belonging to Asteraceae (Compositae) family and the genus stevia, native to parts of Brazil and Paraguay, is commonly known as sugar leaf. It is well-known for its low calorific value compounds *i.e.* steviol glycosides (SGs) including: Rebaudioside A, B, C, D, E, F, M, stevioside, Steviol bioside, Dulcoside A and Dulcoside C. Steviol glycosides are 250 times sweeter (1) than sucrose, considering to be the main sweetening compounds. These compounds are valuable for medicinal, food and cosmetic industries (2-10). The primary use of stevioside is in sweetening the alcoholic beverage, soju (Seon 1995; cited by (11)). In the 1970s, the group of professor Osamu Tanaka at the Hiroshima University in Japan isolated rebaudioside A, a second major sweet ent-kaurene diterpene glycoside from *S. rebaudiana* leaves and later on, six further less abundant sweet-tasting glycosides were isolated from this species mentioned above (11).

Steviol glycosides biosynthesis pathway (12) is controlled by several genes such as *CMK, MCS, HDS, HDR, GGDPS, CDPS, KS, KO, KAH, DXS, DXR, CMS, UGT, UGT85C2, UGT74G1, UGT76G1 and Sr family i.e. SrDXS, SrDXR, SrCPPS, SrKS, SrKO* (13).In this pathway, an enzyme encoded by *UGT74G1* and *UGT76G1* genes has been recognized for giving rise to glucosylate steviolbioside and stevioside to stevioside and rebaudioside A, respectively (14-18).

Growth, yield and quality of stevia are influenced by genetic factors, environment and nutrients available. One of the pivotal nutrients required by the plant is nitrogen that could ameliorate the growth during the vegetative phase and protein synthesis (19). Since stevia seeds have very weak germination, and the seedlings are drought sensitive and because vegetative propagation is restricted by the few number of individuals obtained from a single plant, so to overcome such barriers, micropropagation or *in vitro* culture technique i.e. tissue culture, can play a main role for mass propagation and production of genetically identical plants *of S. rebaudiana* (20). The methods of tissue culture for *S. rebaudiana* have been performed by numerous researches (21-28). Esmaeili et al. (28) corroborated that in order to optimizing the cell dedifferentiation and callus induction in stevia, medium plants supplemented with NAA of 0.5, 2,4-D of 0.0 and BAA of 0.5 was the best medium for callus induction. It was concluded also that, the best media for *Stevia rebaudiana* micropropagation was hormones-free MS medium (21). Since, examining such responses is challenging and hard to be done under field conditions, plant tissue culture techniques are essentially more properly utilized performing under sterile and controlled environmental conditions. With the aid of tissue culture techniques, it is possible to bear gameto/somaclonal variations which are the valuable tools in order to produce disease, biotic or abiotic resistant

plants and improved seed quality (29, 30). As a result of gene mutation or changes in epigenetic markers, somaclonal variations has provided a new and alternative tool to breeders for obtaining genetic variability relatively rapidly and without sophisticated technology e.g. in horticultural crops, which are either difficult to breed or have narrow genetic base (31).

There has been made reports on the effect of nitrogen on morphological, physiological and biochemical characteristics of stevia (32-35). For instance, Medina-Pérez et al. (36) assessed the effect of nitrogen fertilization and harvest time on the flavonoid composition and antioxidant properties of *Stevia rebaudiana* leaves. They showed that by means of sufficient N rate and harvest time, the bioactive compounds (i.e. stevioside and rebaudioside A and some other glycosides) levels enhanced and optimized significantly. Also nitrogen deficiency stimulated biosynthesis of bioactive phenylethanoid glycosides in the medicinal plant *Castilleja tenuiflora* Benth (36).

Although the studies found on the effect of nitrogen on gene expression of stevia plant are unknown, but on other plants are reported (37-39). For instance, Zhang et al. (39) investigated the expression levels of 13 N–P–K uptake and metabolism genes and their responses to fertilization during the flowering and berry development stages, in grapevine (*Vitis vinifera*), by using quantitative PCR. They concluded that in the particular stages where N–P–K uptake and metabolism genes were highly expressed, these genes also showed more positive responses to fertilization and the grape quality was more dramatically improved. This proved the feasibility and workability of this novel fertilization strategy.

The present work was carried out to study the effects of NH_4NO_3 and KNO_3 (nitrogen sources) on expression level of genes involved in steviol glycosides biosynthesis pathway (*UGT74G1* and *UGT76G1*) of stevia (*S. rebaudiana* Bertoni) under *in vitro* condition.

Materials and Methods

Plant materials and culture conditions

In this project, *Stevia rebaudiana* (Bertoni) explants were provided from Zagros Bioidea Co. (Razi University, Kermanshah, Iran). Axillary buds (1.5 cm in length) with two leaves were separated from the shoots and were planted on MS medium (40), with 30 g/L sucrose supplemented and different concentrations of NH_4NO_3 (0, 825 and 1650 mg/l) and KNO_3 (0, 950 and 1900 mg/l) (41).

The basal MS medium which described earlier (40), was used to cultivate *S. rebaudiana* var. Bertoni *in vitro*. The medium was solidified, and the pH was adjusted to 5.8 and then, 8 g/L agar was added and autoclaved. Each medium contained five explants and cultures were incubated at 25 ± 1°C under 16/8 h dark photoperiod provided by cool white fluorescent lamps with 3000 Lux intensity and relative humidity 72 to 75%.

RNA extraction

Total RNA of fresh leaves was extracted by RNX plus™ kit (SinaClon BioScience Co. (www.sinaclon.com) according to the manufacturer's recommendations. RNA quantification was done using Nanodrop spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, and Wilmington, USA). Whole RNA isolates had an OD260:OD280 ratio between 1.8 and 2.0. Next, the RNA quality was tested in 1.0 % agarose gel electrophoresis.

Expression analysis of *UGT74G1* and *UGT76G1* genes

The two-step semi-quantitative RT-PCR method (42) was used to determine the 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 of 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) (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), 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 2% agarose gel in TBE buffer. Four independent experiments were conducted. The amplicons were quantified by the TotalLab 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.

Statistical analysis

Data analyzed using Microsoft Excel, and MINITAB 18 (MINITAB Inc. USA) software systems. Collected data had a normal distribution and homogenous variances, and used directly for statistical operations. Pair-

Table 1. List of primers used in RT-PCR and house-keeping genes of the present study.

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

wise mean comparisons was performed by LSD-Fisher with critical value of P <0.05.

Results and discussion

Effect of nitrogen sources on stevia in tissue culture

Obtaining success in producing stevioside (or other steviol glycosides) in tissue culture e.g. via callus or stem-tip cultures is accomplished nowadays by many researchers. But, multiple shoot culture method using a bioreactor permit the successful propagation of *S. rebaudiana* seedlings without needing to cultivate mature plant due to its cultivation obstacles and intricacies. According to our results, maximum shoot length (89.33 mm), dry weight (0.10 mg) and leaf fresh weight (0.42 mg) of plants was observed on MS medium supplemented with 1650 mg/l NH4NO3 and 950 mg/l KNO3. Minimum shoot length (6.13 mm), root length (6.60 mm), leaf number (4.26), leaf dry weight (0.01 mg), leaf fresh weight (0.05 mg), total dry and fresh weight (0.02 and 0.15 mg) and growth rate was observed in an MS medium without nitrogen sources. Of course, in *stevia rebaudiana*, utilizing *in vitro* techniques instead of usual procedures of propagation and genetic improvement could facilitate obtaining homogenous populations free of disease, along with higher levels of the sweetening compounds, higher foliar mass, resistance to biotic/abiotic stresses, breaking of stems and branches, and another important goal is to break the negative correlation that exists between the contents of stevioside and rebaudioside A of the former and the dried leaf yield (43).

Moreover, presence of nitrogen sources makes increase both shooting and rooting of *Stevia rebaudiana (*Bertoni*)* through *in vitro* culture (Figure 1). In this subject, it is recorded (44) by optimization of callus and suspension culture by using MS basal liquid media supplemented with basal components along with NH4NO3 (24.7 mM) and KNO3 (56.4 mM) that, biomass and stevioside content of the callus and suspension culture of stevia supplemented with NH4NO3 showed maximum result, indicating a positive correlation between cell growth and steviol glycosides synthesis. As reported, the stevioside biosynthesis is a function of tissue differentiation since both *S. rebaudiana's* roots and leaves culturing lead to biosynthesize stevioside from acetate, while the final biosynthetic steps can be performed at all levels of differentiation (21).

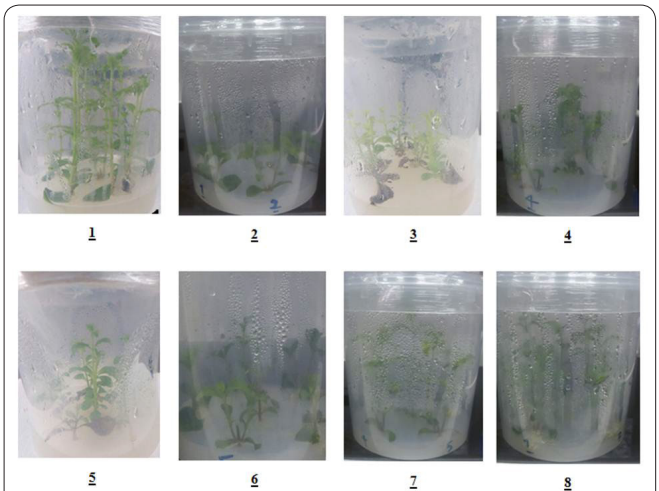

Figure 1. Shoot proliferation from nodal explants on medium (MS+ different concentration of NH_4NO_3 and KNO_3) after 28 days. 1) 1650 mg/l NH_4NO_3 +1900 mg/l KNO_3; 2) 825 mg/l NH_4NO_3 +0 mg/l KNO_3; 3) 1650 mg/l NH_4NO_3 +0 mg/l KNO_3; 4) 0 mg/l NH_4NO_3 +950 mg/l KNO_3; 5) 825 mg/l NH_4NO_3 +950 mg/l KNO_3; 6) 1650 mg/l NH_4NO_3 +950 mg/l KNO_3; 7) 0 mg/l NH_4NO_3 +1900 mg/l KNO_3; 8) 825 mg/l NH_4NO_3 +1900 mg/l KNO_3).

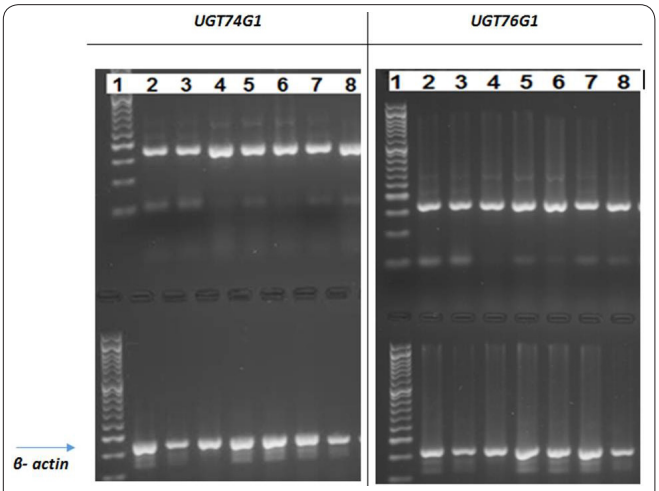

Figure 2. Semi-quantitative RT-PCR analysis of *UGT74G1* and *UGT76G1* in the leaves of *Stevia rebaudiana* (Bertoni) in plants treated with nitrogen sources for 30 days (Where, 1(MS)=1650 mg/l NH4NO3 +1900 mg/l KNO3; 2 = 825 mg/l NH4NO3 +0 mg/l KNO3; 3 = 1650 mg/l NH4NO3 +0 mg/l KNO3; 4 = 0 mg/l NH4NO3 +950 mg/l KNO3; 5=825 mg/l NH4NO3 +950 mg/l KNO3; 6= 1650 mg/l NH4NO3 +950 mg/l KNO3; 7=0 mg/l NH4NO3 +1900 mg/l KNO3; 8=825 mg/l NH4NO3 +1900 mg/l KNO3). *β-actin* was used as an internal control. The final value was the average of at least four independent experiments.

Investigation of UGT74G1 and UGT76G1 genes expression

The results of RT-qPCR (Figure 2) were normalized to the level of the housekeeping gene of β-actin in plants subjected to different concentrations of NH_4NO_3 and KNO_3. There were significant differences between all medium with different concentrations of NH_4NO_3 and KNO_3.

The highest expression of *UGT74G1,* involving the synthesis of stevioside was observed in plantlets grown on MS medium supplied by 0 mg/l NH_4NO_3 and 950 mg/l KNO_3 accounting for exactly 1.291 total lab unit (Figure 3). Also, the lowest amounts of gene expression of *UGT74G1* exhibited in MS medium supplemented with 1650 mg/l of NH_4NO_3 + 0 mg/l of KNO_3 accounting for exactly 0.8 total lab unit. Also there were not a significantly difference between 2, 4, 5, 6 and 7 (2 = 825 mg/l NH_4NO_3 +0 mg/l KNO_3; 4 = 0 mg/l NH_4NO_3 +950 mg/l KNO_3; 5=825 mg/l NH_4NO_3 +950 mg/l KNO_3; 6= 1650 mg/l NH_4NO_3 +950 mg/l KNO_3; 7=0 mg/l NH_4NO_3 +1900 mg/l KNO_3) N sources/treatments for this gene (Figure 3).

The highest expression of *UGT76G1* involving in the synthesis of rebaudioside A, was existed in plantlets grown on MS medium supplied by 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 accounting for exactly 1.083 (Figure 3). Also, the lowest amounts of gene expression of *UGT76G1* were observed in MS medium with 0 mg/l NH_4NO_3 + 950 mg/l KNO_3 accounting for exactly 0.85. Also there was not a significantly difference between 1, 2, 3, 4, 5, 7 and 8 (1=1650 mg/l NH_4NO_3 +1900 mg/l

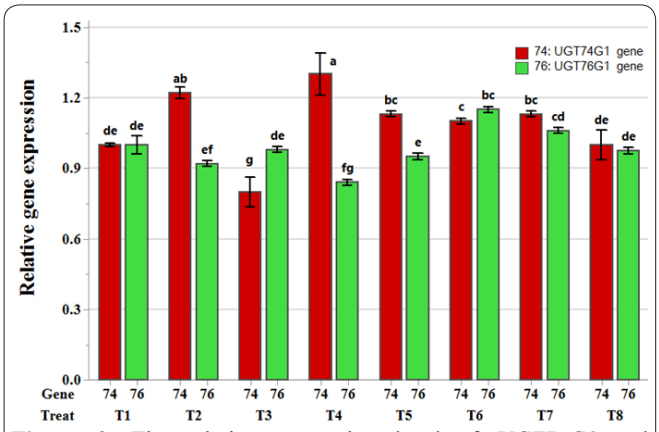

Figure 3. The relative expression level of *UGT74G1* and *UGT76G1* genes (related to *β-actin*) under different concentrations of nitrogen (where, 1(MS)=1650 mg/l NH4NO3 +1900 mg/l KNO3; 2 = 825 mg/l NH4NO3 +0 mg/l KNO3; 3 = 1650 mg/l NH4NO3 +0 mg/l KNO3; 4 = 0 mg/l NH4NO3 +950 mg/l KNO3; 5=825 mg/l NH4NO3 +950 mg/l KNO3; 6= 1650 mg/l NH4NO3 +950 mg/l KNO3; 7=0 mg/l NH4NO3 +1900 mg/l KNO3; 8=825 mg/l NH4NO3 +1900 mg/l KNO3). Values are means ± SE of four replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (LSD-Fisher test). "Treat" term denotes treatment or nitrogen concentrations in the figure.

KNO_3; 2 = 825 mg/l NH_4NO_3 +0 mg/l KNO_3; 3 = 1650 mg/l NH_4NO_3 +0 mg/l KNO_3; 4 = 0 mg/l NH_4NO_3 +950 mg/l KNO_3; 5=825 mg/l NH_4NO_3 +950 mg/l KNO_3; 7=0 mg/l NH_4NO_3 +1900 mg/l KNO_3; 8=825 mg/l NH_4NO_3 +1900 mg/l KNO_3) N sources/treatments for this gene (Figure 3). For *UGT74G1* gene, there was a decreasing trend from MS medium with 1650 mg/l NH_4NO_3 +1900 mg/l KNO_3 to medium with 1650 mg/l NH_4NO_3 + 0 mg/l KNO_3 but after that the expression raised. This decline ranged approximately from 1.00 to 0.8 for the *UGT74G1* gene (Figure 3). For *UGT76G1* gene, there was an increasing trend for MS medium with 1650 mg/l NH_4NO_3 +1900 mg/l KNO_3 to medium with 1650 mg/l NH_4NO_3 +950 mg/l KNO_3 but after that the expression decreased. This enhance was from approximately 1.00 to nearly 1.16 for *UGT76G1* gene (Figure 3).

Conclusion

Since stevioside (45) and rebaudioside A, are the main components of stevia, the most important aspect of the study on stevia is improvement the growth and stevioside and rebaudioside A contents. Thus, the aim of the studies about stevia can be raising the production of such compounds. Moreover, *UGT74G1* and *UGT76G1* genes are involved in the synthesis of stevioside and rebaudioside A, so were investigated the expression of such genes here. Beside, some other genes are reported to play role in SGs pathway *i.e.* SrDXS, SrDXR, SrCPPS, SrKS, SrKO and three glucosyltransferases namely SrUGT85C2, Sr*UGT74G1* and Sr*UGT76G1* were reported from stevia (13). They mentioned that SGs accumulated maximum in leaf tissue followed by stem and root. It is also concluded that information concerning the genetic control of the biosynthesis of SGs (like rebaudiosides D and X) should be validated *in vivo*, however, glycosylation of rebaudioside A to rebaudioside D has been demonstrated *in vitro*, mediated by the enzyme UGT91D2 (46).

The application of more nitrogen sources may be a reason to increase the expression level of these genes because the main target of application of nitrogen fertilizers are green tissues like leaves. In this case, researches indicated that nitrogen sources are main factors affecting plant growth and modulate the leaf senescence (27). It is reported also that top growth and root growth of *Tifblue'rabbiteye* blueberry were superior with NH-4NO3 application, and that fertilizers containing the NH4-N form, are essential for optimum growth (47). It is also reported that the accumulation of steviol glycosides (SVglys) is known to be affected by the method of propagation, day length, and agronomic practices such as irrigation and fertilization (48-50). As mentioned before, Tavarini and Sgherri and Ranieri and Angelini (50) concluded that by applying sufficient N rate and harvest time, the stevioside and rebaudioside and some other glycosides levels of stevia enhanced and optimized significantly. Similarly, it is also reported (5) that abiotic stresses like drought, declined and had a negative effect on the content of SVglys and transcription of SVglys biosynthetic genes and also a sufficient irrigation of stevia was required to obtain a high content of SVglys. According to the results, stevia growth is impacted by nitrogen sources. Actually, presence of nitrogen sources increases both shooting and rooting in stevia (Figure 1). As shown, the best final production of stevia was in MS medium with suitable nitrogen sources (32), although, for *S. rebaudiana*, the comprehensive effects of mineral nutrition on plant physiology, growth, and SVgly production remain poorly understood (51). However, in order to investigate the relevance of nitrogen (N) supply for leaf yield and for SVgly concentrations in leaves, which are the two major components of *S. rebaudiana* productivity, it is reported (51) that N content in leaves was negatively correlated with SVgly content. Also they expressed that the increased SVgly accumulation compensates for the negative effect on biomass production suggests that adequate SVgly productivity per plant may be achieved with relatively low fertilization. Moreover, it is concluded that the applying foliar nutrients [mainly KNO3 and Ca(NO3)2] led to an enhancement in chlorophyll, nitrogen, and potassium content in the leaves but not in SVgly content (52).

Although, there were not any reports on the effect of nitrogen on gene expression of stevia, but the effect of other nutrition's sources like sucrose on gene expression of stevia is reported (53), where reporting that sucrose at 100 mM concentration in the media was more desirable than other factors for expression of *UGT76G1* and *UGT85C2* genes. Whereas, the best medium for expression of *UGT74G*1 was the sucrose at 150 mM and sucrose 50 mM for KS gene. In total, they stress that sucrose at a concentration of 100 mM, provides the best condition for stevia growth and steviol glycosides production. Moreover, in this regard, Koch (54) reported that carbohydrates cause increasing or decreasing gene expression through the signaling or their role as substrate. However the same carbohydrate may have a different effect on various species (9). In addition, Guleria et al. (18) suggest that sucrose might be acting as an enhancer of transcriptional trigger to the genes of steviol glycoside biosynthesis pathway that could positively manipulate the production of steviol glycosides.

Our results suggest that both the growth and the ex-

pression level of *UGT74G1* and *UGT76G1* genes were affected by different concentration of nitrogen sources. But the effect of nitrogen sources, even sufficient or deficiency, on the genes expression of plants has been studied less. In this case, Jian et al. (55), while studying the effects of N fertilizers on the expression of N metabolic genes of grape, using qRT-PCR and semi-quant itative RT-PCR techniques witnessed the increased expression of five N metabolic genes, after foliar applied different types of N fertilizers. They concluded that N fertilizers could induce glutamate dehydrogenase gene expression, which catalyzes transamination to regulate the catabolism and anabolism of intracellular carbon and N flows.

Similarly, Lu et al. (56) concluded that in spite of the impact and determining role of nitrogen and precise management of its sourced fertilizers, little is known at the gene expression level and the response of field crops to different amounts and forms of nitrogen fertilizer. They showed using expressed sequence tag (EST)-based wheat microarrays, that gene expression is significantly influenced by the amount and form of nitrogenous fertilizer, and that specific genes have surprisingly different expression levels in the grain endosperm when nitrogen is supplied and many of the genes showing differential expression are known to participate in nitrogen metabolism and storage protein synthesis. Thus, specific gene expression is diagnostic for use of organic sources of nitrogen fertilizer. According to such reports, fertilizers like nitrogen sources used in this experiment play great roles in the management of morphology, physiology and more important in the metabolic and genetic pathways of stevia, which could be considered especially for stevia glycosides production and enhancement in breeding programs.

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