Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Effect of sucrose concentrations on *Stevia rebaudiana* Bertoni tissue culture and gene expression

T. Ghorbani[1], D. Kahrizi[1*], M. Saeidi[1], I. Arji[2]

[1] Department of Agronomy and Plant Breeding,Razi University, Kermanshah, Iran
[2] Horticultural Crop Research Department, Kermanshah Agricultural and Natural Resources Research and Education Center, AREEO, Kermanshah, Iran

Correspondence to: dkahrizi@razi.ac.ir

Received June 27, 2017; **Accepted** July 19, 2017; **Published** August 30, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.8.8

Abstract: *Stevia rebaudiana* (Bert.) Bertoni is known as sweet plant which it contains a high level of steviol glycosides in the leaves. This plant has been used from centuries ago as a sweetener for tea. One of the most important steviol glycosides is stevioside that is attractive for diabetic persons. Tissue culture is the only rapid process for the mass propagation of stevia. One of the most important factors in the medium is sucrose that is a necessary for plant growth. In the present study, we use nodal segments of the stem as explants in mediums with different sucrose concentration (50 mM, 100mM and 150mM). Several morphological traits were measured in a 28 day period. Results analysis showed a significant variation between treatments. The highest growth rate, rooting and leaf production was obtained in medium with 100mM sucrose. The correlation between measured traits was significant at the 0.01 level. To investigation of *UGT74G1*, *UGT76G1, UGT85C2* and *KS* genes expression that are involved in the synthesis of SGs, RT- PCR was done with the housekeeping gene of as internal control. There were significant differences between all media. The results showed thatsucrose 100 mM containing media was more desirable than others for expression of *UGT76G1* and *UGT85C2* genes. Whereas, the best medium for expression of *UGT74G1* was sucrose 150 mM and sucrose 50 mM for *KS* gene. Totally, it seems that sucrose at a concentration of 100 mMprovides the best condition for stevia growth and steviol glycosides production.

Key words: *Stevia rebaudiana* Bertoni; Tissue culture; Sucrose; Gene expression; Semi-quantitative RT- PCR.

Introduction

Stevia rebaudiana (Bert.) Bertoni (Family: *Asteraceae*) is known as sweet plant which it contains a high level of steviol glycosides in the leaves. This plant has been used from centuries ago as a sweetener for tea.The compounds causing the sweet taste are steviol glycoside(1). One of the most important steviol glycosides is stevioside that is attractive for diabetic persons. Stevia has various properties such as antibacterial, anticandidal, antifungal, antiviral, cardio tonic (tones, balances, strengthens the heart), diuretic, hypoglycemic, vasodilator(2). Seed raising of stevia is not successful because its seeds have very low germination percentage and vegetative propagation is time and money consumer. Tissue culture is the only rapid process for the mass propagation of stevia(3).

Steviol glycosides biosynthesis pathway starts with steviol and end with rebaudioside A. This pathwayinvolved 15 genes : *DXS, DXR, CMS, CMK, MCS, HDS, HDR, GGDPS, CDPS, KS, KO, KAH, UGT85C2, UGT74G1* and *UGT76G1*. Furthermore three of these genes(*UGT85C2*, *UGT74G1* and *UGT76G1*) that involved in the synthesis of stevioside and rebaudioside A are important for us (4-6).

Carbone sources have functional role in plants development.It has been reported that sucrose entire in the metabolic pathways and it release the energy. In addition, sucrose also acts as gene regulators(7). An interesting fact regarding sucrose responsiveness is that a specific concentration of sucrose is a necessary for plant growth. It has been suggested that plants respond to changing sucrose content by undergoing morphological and anatomical variations as well as by regulating the expression of various genes via a variety of signal transduction pathways(8-10).

Based on studies, one the Important aspect of stevia is its potential for supersedence with sugarcane(11). Somatic embryogenesis was also obtained from floret explants cultured on MS medium supplemented with 2, 4-D (9.05 and 18.19 µM) and Kinetin (0 to 9.2 µM). On 9.05 µM 2, 4-D supplemented medium maximum without Kinetin embryogenic callus formation occurred. A multiple shoot culture was induced from nodal segments on MS medium containing half concentration of macro elements, 1% sucrose and supplemented with NAA (0.01 mg/l)(12). Jitendra*et al* showed that The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 0.5 mg/l BAP+2.0 mg/l Kn. For rooting different concentration of IBA were used and highest rooting was recorded on MS medium with 1.0 mg/l IBA. The rooted Plantlets were hardened initially in culture room conditions and then transferred to misthouse(2). In a research that carried out by Ahmed *et al*, highest rooting percentage (97.66%) was recorded on MS medium with 0.1 mg l rooted plantlets were hardened and successfully established in soil(3). Pitekelabou*et al*(2015) reported

that Sucrose appears to be the most favorable sugar to ensure the in vitro micropropagation of *Naucleadiderrichii* and the best plants' growth and rooting were obtained with sucrose. Organized tissues show a better growth and proliferation after the addition in medium an adequate source of carbon(13). Sugars enter the metabolism pathways and transformation of energy which are required for growth of cell(14). Nambiar*et al* in the study of different carbohydrate sources on proliferation of protocorm- like bodies in Dendrobium Alya Pink reported that Highest growth was recorded in PLBs supplemented with glucose, fructose and sucrose with 0.94 ± 0.55 g, 9.1 ± 0.82 g and 6.51 ± 0.52 g of PLBs respectively(15).Preethi*et al* study the effect of various carbon sources (sucrose, glucose, fructose and maltose) on *in vitro* shoot regeneration of *Stevia rebaudiana*. They reported that frequency, growth and multiplication rate were extremely influenced by the kind and concentration of carbon sources used (16).

The present study was carried out with an objective of studying the effect of different concentration of sucrose on morphological characteristics (Plant fresh and dry weight, Leaf fresh and dry weight, Leaf Number, Root Length, Shoot Length, Growth Rate), expression level of genes in steviol glycosides biosynthesis pathway (*UGT74G1*, *UGT76G1, UGT85C2* and *KS*) and the most important steviol glycosides contents (stevioside and rebaudioside A) of *S. rebaudiana* under in vitro condition.

Materials and Methods

Plant material and cultural conditions

In vitro stock plants Stevia (*Stevia rebaudiana* Bert.) were provided from Zagros Bioidea Co., Razi University Incubator. They werepropagated using single nodes of every 3-4 weeks as the regular micropropagation methods. In this study nodal explants of the same age and size were used and cultured in different sucrose concentration media with due regard to find out their sucrose concentration inducing *in vitro* response. A single nodal segments were separated (1 cm long with single leaf)and cultured in Murashige and Skoog (MS) solid media without changing the other components of the MS media(17). We used sucrose in 50, 100 and 150 mm in 3 replications and 5 explants (as 5 observations) for each replication. The pH of all the media was adjusted to 5.7. The sucrose containing media were autoclaved for 20 minutes. The cultivation was performed at 25±1°C in a well set growth room condition for 4 weeks under fluorescent lightening (Philips) of 800 Lux (16/8, D/N cycle) at the top of the culture vessels.

Morphological analysis

After 4 weeks (28 days), the morphological traits were measured. The measured traits were: Plant fresh weight (PFW), Plant Dry Weight (PDW), Leaf fresh weight (LFW), Leaf Dry Weight (LDW), Leaf Number (LN), Root Length (RL), Shoot Length (SL), Growth Rate (GR= shoot length/ days).

RNA extraction

Total RNA was isolatedfromthe 100 mg leaves from control and treated plants using RNx plus™ kit (Cinnaclon) according to the manufacturer's instructions. 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.

Expression analysis of genes

The two-step semi-quantitative RT-PCR method was used to determine gene expression of *UGT74G1, UGT76G1,UGT85C2*and*KS* genes in stevia.First-strand cDNA was synthesized using 10 µg RNA, Oligo-dt primer, M-MLV reverse transcriptase, M-MuLV buffer,dNTP and Nuclease-free Water (Viva 2-steps RT-PCR Kit, vivantis, Malaysia). *β-Actin* were used as the house-keeping (control) genes. Primers for target and *β-Actin* genes were designed using the Oligo 7 Primer Analysis Software and NCBI database to reach specific characters required for semi quantitative polymerase chain reaction (RT-PCR) [Table 1: (18)].PCR reaction was set up in a 25 µl volume of 2 µl cDNA, 0.5 µl of dNTPs, 1 µl of each primer, 0.32 µl of $MgCl_2$, 2.5 µl of 10x PCR buffer and 0.5 µl of Taq DNA polymerase. PCR reaction was performed as follows: 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.

After that The PCR products were separated by electrophoresing on a 2% agarose gel. Three independent experiments were conducted. PCR bands were quantified using Total Lab TL120 v2009 software (19). Which delivers quantitative estimates of the amplicon band intensities by changing them into corresponding numerical values. The expression levels of *UGT74G1*, *UGT76G1*, *UGT85C2* and *KS* were normalized relative to the amount of *β-Actin* expression.

Statistical analysis

The collected data were analyzed using SPSS (Ver. 19) software. The data on different parameters were evaluated using ANOVA and mean differences were separated at the 1 % level of significance.

Results

The plantlets grown in each treatment was compared after 4-week period. The tested plants showed a variable

Table 1. List of primers used in RT-PCR and house-keeping genesin *Stevia rebaudiana.*

Gene	Primer sequence 5′→ 3′ (forward/reverse)	Amplicon length (bp)	Accession number
UGT74G1	AATCGGGCCAACACTTCCAT/ TCGGGTCCATGTTTCACCAG	174	AY345982
UGT76G1	GACCAACAACCGCCAAGTTC/ CCCAAGAACCCATCTGGCAA	185	AY345974
UGT85C2	TTCCACACGTTCGATGAGTT/ TGAAGCCACTGGAAACACTC	191	AY345978
KS	AACGCTTACGTGTCATTTGC/ CTACCGCGTTTAATTTGCCT	196	AF097310
β-Actin	TTGCCCTGAGGTTCTGTTCC/ ATCCGGTCAGCAATACCAGG	171	AF548026

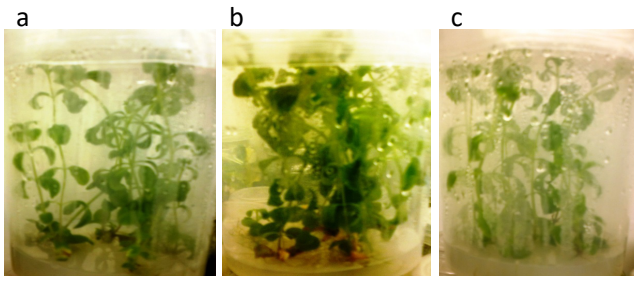

Figure 1. *Stevia rebaudiana*proliferation under different concentrations of sucrose. a, b, c: 50, 100 and 150 mMsucrose respectivelyin MS media under*invitro* conditions, 28 days after culture.

response to different sucrose concentration (Fig. 1).

PFW, PDW, LFW, LDW, LN, SL and GR showed differences at the 1 % level of significance (Table 2).

Effect of sucrose concentrations on shoot length and growth rate of stevia

The highest shoot production was found in sucrose 100 mM (75.067 mm) and the lowest amount of it was observed in sucrose150 mM- media (42.40 mm). Also the maximum growth rate was observed in sucrose 100 mM (2.68 mm/d), and the lowest amount of them was found in sucrose150 mM- media (1.519 mm/d) (Table 3, Fig. 2.).

Effect of sucrose concentrations on wet and dry weight of stevia

The maximum plant fresh weight was observed in sucrose 100 mM (0.689 gr), and the minimum plant fresh weight was observed in sucrose 50 mM- media (0.350 gr). The maximum plant dry weight was seenin sucrose 100 mM (0.093 gr), and the minimum plant dry weight was seenin sucrose 50 mM- media (0.036 gr). The maximum leaf fresh weight was observed in sucrose 100 mM (0.275 gr), and the minimum leaf fresh weight was observed in sucrose 150 mM- media (0.180 gr). The maximum leaf dry weight was observed in sucrose 100 mM (0.048 gr), and the minimum leaf dry weight was observed in sucrose 50 mM- media (0.021 gr) (Table 3, Fig. 2.).

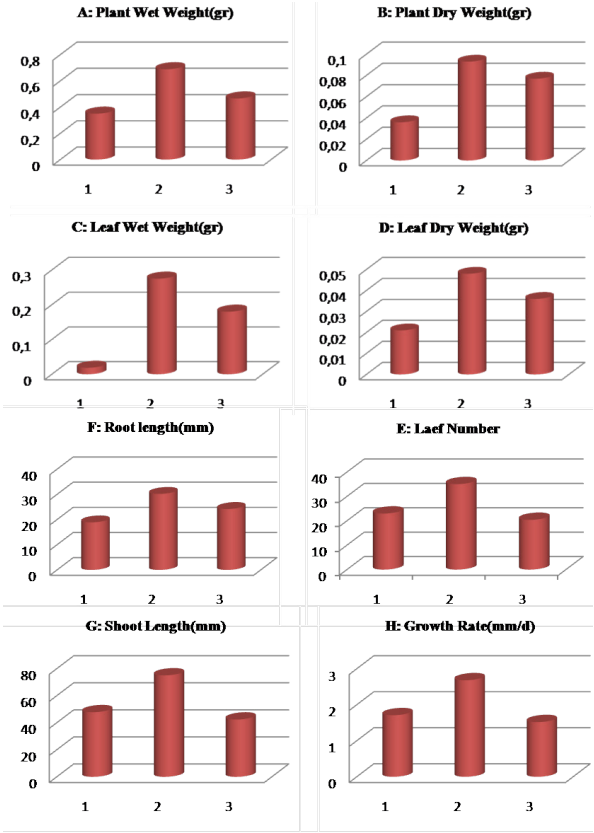

Figure 2. Mean performances of *Stevia rebaudiana*morphological traits (A - H) in different sucrose concentration (Treatments: 1(50 mM), 2 (100 mM), 3 (150 mM)) containing MS media. Data was collected 28 days after culture.

Effect of sucrose concentrations on root length and leaf number of stevia

The highest root length was found in sucrose 100 mM (30.20 mm) and the lowest amount of it was observed in sucrose 50 mM- media (18.90 mm). The maximum leaf number was observed in sucrose 100 mM (35.067), and the minimum leaf number was observed in sucrose 150 mM- media (20.40) (Table 3, Fig. 2.).

Correlation coefficient

There was significant correlation at 0.01 level

Table 2. F value of different morphological traits of *Stevia rebaudiana*and three sucrose concentrations.Where Degree of freedom (df), Plant Fresh Weight (PFW), Plant Dry Weight (PDW), Leaf Fresh Weight (LFW), Leaf Dry Weight (LDW), Leaf Number (LN), Root Length (RL), Shoot Length (SL), Growth Rate (GR= shoot length/ days).

S.O.V.	df	F Value							
		PFW	PDW	LFW	LDW	LN	RL	SL	GR
Treatment (T)	2	10.53^{**}	28.48^{**}	7.76^{**}	26.27^{**}	9.33^{**}	2.48^{ns}	12.30^{**}	12.18^{**}
Ee (Replication * T)	6	2.49^{*}	6.71^{**}	2.48^{*}	5.31^{**}	5.24^{**}	1.02^{ns}	0.74^{ns}	0.74^{ns}
Es (Error)	36								
Total	45								

**, *, ns significant (P<0.01), significant (P<0.05) and non-significant, respectively.

Table 3. Mean value of sucrose concentration on influence to different growth traits in *Stevia rebaudiana*. Data after 28-day period culture.Where Plant Fresh Weight (PFW), Plant Dry Weight (PDW), Leaf Fresh Weight (LFW), Leaf Dry Weight (LDW), Leaf Number (LN), Root Length (RL), Shoot Length (SL), Growth Rate (GR= shoot length/ days).

Sucrose concentration	Morphological traits							
	PFW(gr)	PDW(gr)	LFW(gr)	LDW(gr)	LN	RL(mm)	SL(mm)	GR(sl/day)
50 mM	0.35^{b}	0.04^{b}	0.018^{b}	0.02^{b}	23.00^{b}	18.90^{b}	47.80^{b}	1.71^{b}
100mM	0.69^{a}	0.09^{a}	0.27^{a}	0.05^{a}	35.06^{a}	30.20^{a}	75.06^{a}	2.68^{a}
150mM	0.47^{b}	0.07^{a}	0.18^{b}	0.04^{a}	20.40^{b}	24.20^{b}	42.40^{b}	1.52^{b}

Table 4. Pearson correlation between morphological traits in *Stevia rebaudiana*. Where Plant Fresh Weight (PFW), Plant Dry Weight (PDW), Leaf Fresh Weight (LFW), Leaf Dry Weight (LDW), Leaf Number (LN), Root Length (RL), Shoot Length (SL), Growth Rate (GR= shoot length/ days).

	PFW	PDW	LFW	LDW	LN	RL	SL	GR
PFW	1.000							
PDW	0.848**	1.000						
LFW	0.920**	0.738**	1.000					
LDW	0.880**	0.968**	0.825**	1.000				
LN	0.748**	0.604**	0.751**	0.690**	1.000			
RL	0.654**	0.452**	0.573**	0.516**	0.475**	1.000		
SL	0.791**	0.570**	0.813**	0.670**	0.543**	0.581**	1.000	
GR	0.790**	0.570**	0.813**	0.670**	0.542**	0.581**	1.000**	1

**. Correlation is significant at the 0.01 level (2-tailed).

between all of the traits (Table 4). According to the Pearson correlation table, the highest correlation was seen between growth rate and shoot length (1.000).

"The study on effect of carbohydrate source on embryogenesis in *Zea mays L.* anther culture" was done, and showed that sucrose or a high concentration of maltose was found to be necessary for embryogenesis in anther culture of maize(20), the importance of sucrose in tissue culture supports our result in the present study. In the experiment that carried out on potato, sucrose was the most effective carbon source for high-level induction of the genes(21). This result confirms the idea of sucrose necessity in culture media.

Gene expression study

Expression of four genes, *UGT74G1*, *UGT76G1*, *UGT85C2* and *KS* that are involved in the synthesis of steviol glycoside was studied by semi quantitative RT-PCR technique. The results were normalized to the level of the housekeeping gene of *β-actin* in plants subjected to different concentrations of sucrose (Fig. 3). Based on the histogram in Fig. 4, there were significant differences between media with different sucrose concentrations. According to the results, the highest level of *UGT74G1* gene expression was occurred in plant grown on sucrose 150 mM containing media (0.942Total lab unit) and the lowest level was observed in sucrose 100mM containing medium (0.726 Total lab unit). The highest expression amount of *UGT76G1*was observed in sucrose 100 mM medium (0.964 Total lab unit) and its expression decreased in other media insofar as the lowest level of expression occurred in sucrose 150 mM medium accounting for 0.736 Total lab unitwith no significant differences from sucrose 50 mM.Interestingly, there were same results for *UGT85C2* gene. The highest amount of expression was seen in sucrose 100 mM media (1.267 Total lab unit) while the lowest gene expression level was observed in sucrose 150 mM media (0.925 Total lab unit). In contrast, *KS* gene expression betided in its highest value in sucrose 50 mM media amounting to 0.849 Total lab unit and in its lowest value accounting for 0.752 Total lab unit in sucrose 100 mM medium. It seems that sucrose 100 mM containing media was more desirable than others for *UGT76G1* and *UGT85C2* genes expression. Whereas, the best medium for expression of*UGT74G1* was sucrose 150 mM and sucrose 50 mM for*KS* gene expression.

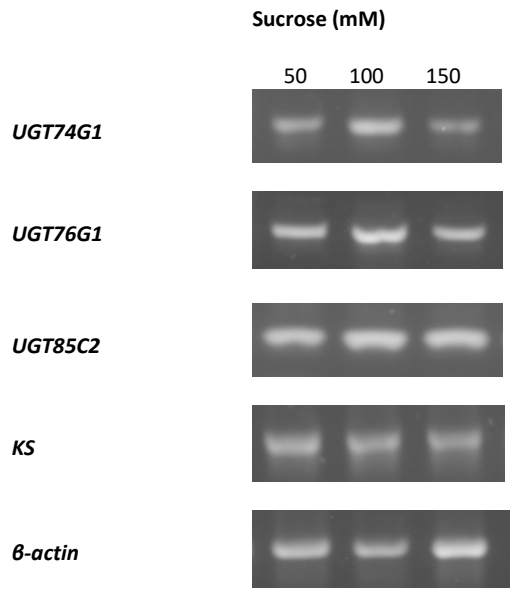

Figure 3. Expression of *UGT74G1*, *UGT76G1*, *UGT85C2* and *KS* in the leaves of *stevia rebaudiana*Bertoni under different concentrations of sucrose. Semi-quantitative RT-PCR analysis of *UGT74G1, UGT76G1*, UGT85C2 and *KS* in plants treated for 28 days. *β-actin* was used as an internal control. The final value was the average of at least three independent experiments. Only the best pictures are shown.

Discussion

The experiment supports our intention that it is very important to study the sugar effects at low nitrate MS media with due regard to optimum plantlet growth and their subsequent effect may be of good information

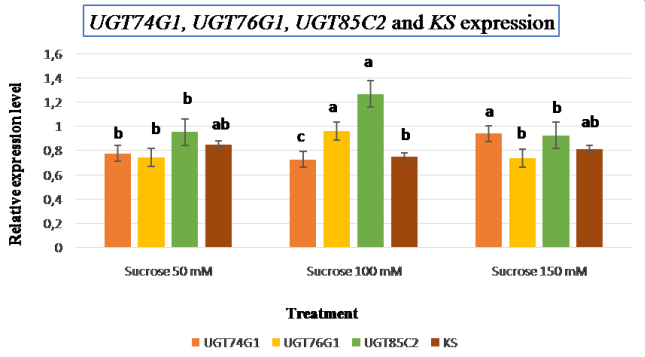

Figure 4. The relative expression level of *UGT74G1, UGT76G1, UGT85C2 and KS* (related to *β-actin*) under different concentrations of sucrosein *Stevia rebaudiana*. Values are means ± SE of three replications and bars indicate SE. Columns with different letters indicate significant differences at P = 0.05 (Duncans test).

in the refinement of tissue culture media. Sucrose is a prime carbon source of stevia micropropagtion and influence of developing vigor plantlets but it was not fully explain the performances of other disaccharides or monosaccharide as far as optimum stevia tissue culture is concerned. There is a general agreement in the literature that sucrose is required in the medium for stevia tissue culture(4, 14-15, 17, 20-22).

This study was done according to this fact that a specific concentration of sucrose is a necessary for plant growth and the suggestion that plants respond to changes sucrose content by undergoing morphological and anatomical variations as well as by regulating the expression of various genes via a variety of signal transduction pathways.

The importance of plant tissue culture composition has been reported previously (23-25).

Finally the best concentration of sucrose was 100 mM for Stevia growth in tissue culture condition. It is necessary to study the performances of other disaccharides or monosaccharide to find the optimum Stevia tissue culture media.

Acknowledgments

I would like to thank Miss. Matin Ghaheri (M. Sc. of Biotechnology, Razi University, Kermanshah, Iran) for her kindly helps in doing this research. Thanks to Zagros Bioidea Co., Razi University Incubator for all supports.

References

1. Madan S, Ahmad S, Singh G, Kohli K, Kumar Y, Singh R, Garg M. *Stevia rebaudiana* (Bert.) Bertoni-a review. Indian J Nat Prod Resour. 2010; 1: 267-286.

2. Jitendra M, Monika S, Ratan SD, Priyanka G, Priyanka S, Kiran DJ. Micropropagation of an Anti diabetic Plant-Stevia rebaudiana Bertoni,(Natural Sweetener) in Hadoti Region of South-East Rajasthan. India ISCA J Biol Sci. 2012*;* 1: 37-42.

3. Ahmed MB, Salahin M, Karim R, Razvy MA, Hannan MM, Sultana R, Hossain M, Islam R. An efficient method for in vitro clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh American-Eurasian. J Sci Res 2007; 2: 121-125.

4. Brandle J, Telmer P. Steviol glycoside biosynthesis. Phytochem 2007; 68: 1855-1863.

5. Khan SA, Rahman L, Verma R, Shanker K. Physical and chemical mutagenesis in *Stevia rebaudiana:* variant generation with higher UGT expression and glycosidic profile but with low photosynthetic capabilities. Acta Physiol Plant 2016; 38: 1-12.

6. Richman A, Swanson A, Humphrey T, Chapman R, McGarvey B, Pocs R, Brandle J. Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of Stevia rebaudiana. Plant J 2005; 41: 56-67.

7. Guleria P, Kumar V, Yadav SK. Effect of sucrose on steviol glycoside biosynthesis pathway in Stevia rebaudiana. Asian J Plant Sci 2011; 10: 401-409.

8. Koch K. Carbohydrate-modulated gene expression in plants. Annu Rev Plant Biol 1996; 47: 509-540.

9. Loreti E, De Bellis L, Alpi A, Perata P. Why and how do plant cells sense sugars?Ann Bot 2001; 88: 803-812.

10. Smeekens S. Sugar-induced signal transduction in plants. Annu Rev Plant Biol 2000; 51: 49-81.

11. Ramesh. K, Singh V, Megeji NW. Cultivation of Stevia [*Stevia rebaudiana* (Bert.) Bertoni]. Adv Agron2006; 89: 137-177.

12. Nepovim A, Vanek T. In vitro propagation of Stevia rebaudina plants using multiple shoot culture. Planta Med 1998; 64: 775-776.

13. Mazinga K, Mario G, Baboy L, Useni S, Nyembo K, Kasongo L, Van K. Effets des sources de carbone (sucrose, glucose), et des doses de saccharose sur l'induction de la rhizogénèse chez l'hybride FHIA-01 (AAAB) du bananier en culture *in vitro*. J Appl Biosci 2015; 73: 5991-6001.

14. Gauchan D. Effect of different sugars on shoot regeneration of maize (*Zea mays* L.). KUSET.2012; 8: 119-124.

15. Nambiar N, Tee C, Maziah M. Effects of organic additives and different carbohydrate sources on proliferation of protocormlike bodies in'Dendrobium'Alya Pink plant. Omics J 2012*;* 5: 10-18.

16. Preethi D, Sridhar T, Naidu C. Carbohydrate concentration influences on *in vitro* plant regeneration in *Stevia rebaudiana*. J Phytol 2011; 3: 61-64.

17. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 1962; 1: 473-497.

18. Marone M, Mozzetti S, De Ritis D, Pierelli L, Scambia G. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biol Proced Online 2001; 3: 19-25.

19. Chivers JE, Gong W, King EM, Seybold J, Mak JC, Donnelly LE, Holden NS, Newton R. Analysis of the dissociated steroid RU24858 does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids. MolPharmacol 2006; 70: 2084-2095.

20. Ismaili A, Mohammadi PP. Effect of genotype, induction medium, carbohydrate source, and polyethylene glycol on embryogenesis in maize (*Zea mays* L.) anther culture. Acta Physiol Plant 2016; 38, 1-8.

21. Rhee Y, Hwang KR, Cho S, Lee M, Kil EJ, Choi S, Hahn BS, Kim D, Auh CK, Lee S. Expression analysis of D-type cyclin in potato (Solanum tuberosum L.) under different culture conditions. Acta Physiol Plant 2016; 38: 1-12.

22. Bondarev N, Reshetnyak O, Nosov A. Peculiarities of diterpenoid steviol glycoside production in vitro cultures of Stevia rebaudiana Bertoni. Plant Sci 2001; 161: 155-163.

23. Esmaeili F, Kahrizi D, Mansouri M, Yari K, Kazemi N, Ghaheri M. Cell dedifferentiation in Stevia rebauiana as a pharmaceutical and medicinal plant. J Rep Pharma Sci 2016; 5(1): 12-17.

24. Akbari F, Arminian A,Kahrizi D,Fazeli A. Effect of nitrogen sources on some morphological characteristics of in vitro *stevia rebaudiana*Bertoni. Cell MolBiol2017; 63(2): 107-111.

25. Ghaheri M, Kahrizi D,BahramiGh.. Effect of mannitol on some morphological characteristics of *in vitrostevia rebaudiana*Bertoni. BiharianBiologist 2017(online first): art. 151415.