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Effect of mannitol on some morphological characteristics of *in vitro stevia rebaudiana* Bertoni

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Abstract. *Stevia rebaudiana* Bertoni is a Paraguayan perennial antidiabetic herb belongs to Asteraceae family. The Leaves of stevia are about 300 times sweeter than sugar with no calories, that are the source of diterpene glycosides such as stevioside and rebaudioside A. Tissue culture is a rapid tool for propagation of stevia. In this research we studied the effect of different concentrations of mannitol on some morphological aspects of stevia under *in vitro* conditions. Axillary nodes were used as explants and they were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of mannitol (0, %1, %2, %3, %4 and %5) as an osmotic stress. The cultures were maintained for 4 weeks at a temperature of 25±2°C with a photoperiod of 16/8 hour low light/dark per day. Maximum shoot length (111.00±3.26 mm) was observed on a MS medium without mannitol and minimum shoot length (20.46±2.89 mm) was observed on a MS medium with %5 mannitol. It was observed that by increasing the concentration of mannitol in medium, shoot length, internode length, fresh weight of plants and growth rate were decreased. Maximum (54±1.45) and minimum (28.93±1.85) number of leaves was observed on a MS medium with %3 and %5 of mannitol respectively. Moreover, the results showed that osmotic stress significantly reduces the growth and yield components of *S. rebaudiana* Bertoni.

Key words: *Stevia rebaudiana* Bertoni, osmotic stress, mannitol, morphology, *in vitro* propagation.

Introduction

Stevia rebaudiana Bertoni is a perennial herb belongs to family Asteraceae. Some commonly known names are honey leaf plant, sweet chrysanthemum, sweet leaf stevia, sugar leaf and sweet weed (Ahmed et al. 2007, Jitendra et al. 2012, Hassanen & Khalil 2013). Stevia is native to South America and grows in the highlands of Amambay. It is being cultivated as commercial crop in Japan, China, Thailand, Paraguay, Brazil and certain countries of South America (Abdul Razak et al. 2014). The leaves of stevia contain glycoside diterpenes such as rebaudioside A, B, C, D, E, F, M, stevioside, steviol bioside, dulcoside A and dulcoside C (Anbazhagan et al. 2010, Mondaca et al. 2012, Singh et al. 2012, Prakash et al. 2014) which are estimated to be 200-350 times sweeter than sucrose but there are free of calories. So it has an importance to diabetic persons and diet conscious (Badran et al. 2015). The two main glycosides are stevioside and rebaudioside A that are the sweetest compounds. The powdered form stevia leaves can be added to tea and coffee, fruit juices, chewing gum and other food products that need sweet taste that possessed both hypoglycemic and body weight reducing effect without any harmful effects for diabetic patients (Gregersen et al. 2004, Anbazhagan et al. 2010).

Stevia seeds show poor germination percentage so the propagation through seeds is not effective. Stevia are conventionally propagated through cuttings, however this method cannot produce many plants. As a result, plant cell and tissue culture has been used for propagation and study the stress tolerance mechanisms in stevia under *in vitro* conditions. By this method we can produce maximum mass from a single plant in short period of time, by a limited space (Kamran Khan et al. 2014). Several researchers (Yang et al. 1981, Mukundan & Sivaram 2003, Pande & Gupta 2013, Islam & Tareq 2015) have described on the *in vitro* conditions of stevia.

Drought, high salinity and freezing impose osmotic stress on plants and effects on the growth of plants, the productivity of crops and secondary metabolites in plants (Ramakrishna & Ravishankar 2011). In some research reported that abiotic stress induced by the salts such as polyethylene glycol (PEG) and NACL, increased the concentration of steviol glycosides significantly (Gupta et al. 2014) but decreased the fresh and dry weight of stevia.

Zeng et al. (2013) reported that the total dry weight and chlorophyll contents decreased. In addition, 90-120 mM NaCl treatment decreased the content of rebaudioside A and stevioside and indicates that *S. rebaudiana* is mildly tolerant to salt stress. So salt and drought stress may be effective for optimizing the steviol glycoside composition. Mannitol is a six carbon sugar alcohol that often used in laboratories as a medium for inducing osmotic stress in plant and tissue cultures. Addition of mannitol to nutrient solution, over a period of 3-4 weeks that can be effect on gene expression, morphological and physiological characteristics and biochemical content in plants. This paper presents to study the effect of different concentration of mannitol on some morphological structures on *stevia rebaudiana* Bertoni grown under *in vitro* conditions (Pandey & Chikara 2014).

Materials and Methods

Plant material and culture conditions

For this study, explants of *stevia rebaudiana* Bertoni were supplied from Zagros Bioidea Co. Razi University, Kermanshah, Iran. Stem nodal segments of about 1.5 cm in length were separated from the shoots. For surface-sterilization, the explants were washed in running tap water for 15 minutes. Then put in Ethanol (70%) for 1 min, after those mercuric chlorides (0.2%) for 120 s were used. At the end, the explants were washed with sterilized double distilled water. The whole process was performed under the laminar air flow (Khalil et al 2014).

MS medium (Murashige and Skoog, 1962) with 3% sucrose (Merk) supplemented with different concentrations of mannitol (0, %1, %2, %3, %4 and %5) was used throughout the study. The pH of the nutrient medium was adjusted to 5/7-5/8 through a pH meter. Before autoclaving, 0/8% agar was added to medium for gelling it

and then autoclaved at 121°C for 20 min. The media were then placed in phytotron until use.

The sterilized auxiliary nodes were placed on MS medium with different concentrations of mannitol (0, %1, %2, %3, %4 and %5). MS basal medium was used as a control. About 5 explants were cultured on per medium. All the cultures were incubated at 25 ± 2°C under 16 h light and 8 h dark photoperiod provided by cool white fluorescent tubes with 3000 lux intensity and relative humidity 72 to 75%.

The experiment was conducted based on multi-observational CRD. The calculated traits include root length, leaf number, leaf dry weight, leaf fresh weight, total dry and fresh weight and growth rate (shoot length after 28 days as mm/d) were recorded after 28 days.

Statistical analysis

In this experiment, each treatment consisted of 3 replications and in each replication 5 explants (0bservations) per each replication were used. The data were recorded after 4 weeks of culture and analysis were calculated by using SAS (version 9.1) and Excel (2010) software. Mean values were compared according to least significant differences test (LSD) at ($P < 0.05$ and 0.01).

Results

The significance levels for measured traits, such as shoot and root length (mm), leaf number, leaf dry and fresh weight (mg), total dry and fresh weight (mg) and growth rate (mm/d) are shown in (Table 1). The analysis of variance showed that the treatments of mannitol levels as an osmotic stress differed significantly for all studied characters.

Effect of mannitol on shoot and internode length of stevia

The effect of mannitol on some morphological characteristics of stevia has been shown in Figure 1. It was observed that by increasing the concentration of mannitol in medium, shoot and internode length and growth rate were clearly decreased (Fig. 2A, 2B). The highest shoots production was found in the MS medium (111.00 mm) after four weeks of cultivation and minimum shoot length (20.46mm) was observed on a MS medium with %5 mannitol. But plants in (MS) medium with plants in (MS+%1 mannitol) medium are not significantly different about this character (Table 2).

Maximum growth rate (3.63 mm/d) was observed on MS medium and minimum growth rate (0.43 mm/d) was observed on MS+%5 mannitol.

Effect of mannitol on fresh and dry weight of stevia

It was observed that by increasing the concentration of mannitol in medium, total fresh weights were decreased (Fig. 2C) but this is not established for other weights such as total dry weight, leaf dry and fresh weight (Fig. 2D, 2E, 2F). Maximum total fresh weight (1.49 g) was observed on MS medium and minimum total fresh weight (1.36 g) was observed on MS+%5 mannitol. Also there are not significantly different between %1 and %2 (mannitol levels) medium about this character (Table 2).

Maximum leaf fresh weight (0.54 g) was observed on MS medium but minimum leaf fresh weight (0.20 g) was observed on MS+%4 mannitol. Maximum leaf dry weight (0.06

Table 1. Mean squares for effect of different concentrations of mannitol on *stevia rebaudiana* Bertoni after 4 weeks (where FW = total fresh weight; DW =total dry weight; LFW= leaves fresh weight; LDW=leaves dry weight; NL=number of leaves; RL= root length; SL= shoot length; GR = growth rate).

Source	df	Mean Squares							
		FW	DW	LFW	LDW	NL	RL	SL	GR
Treat	5	3.196**	0.018**	0.32**	0.003*	1267.79**	1192.94**	23203.97**	29.292**
Rep (Treat)	12	0.248**	0.002**	0.066**	0.0008**	341.03*	195.73^{ns}	530.211**	0.6150**
Error	72	0.074	0.0007	0.02	0.0002	150.45	191.31	209.63	0.21
CV (%)		28.58	26.66	36.64	31.79	33.32	79.74	21.87	22.70

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

Figure 1. Shoot proliferation from nodal explants on medium (MS+ different concentration of mannitol) after 4 weeks. A= MS; B= MS+%1 mannitol; C= MS+%2 mannitol; D= MS+%3 mannitol; E=MS+%4 mannitol; F=MS+%5 mannitol.

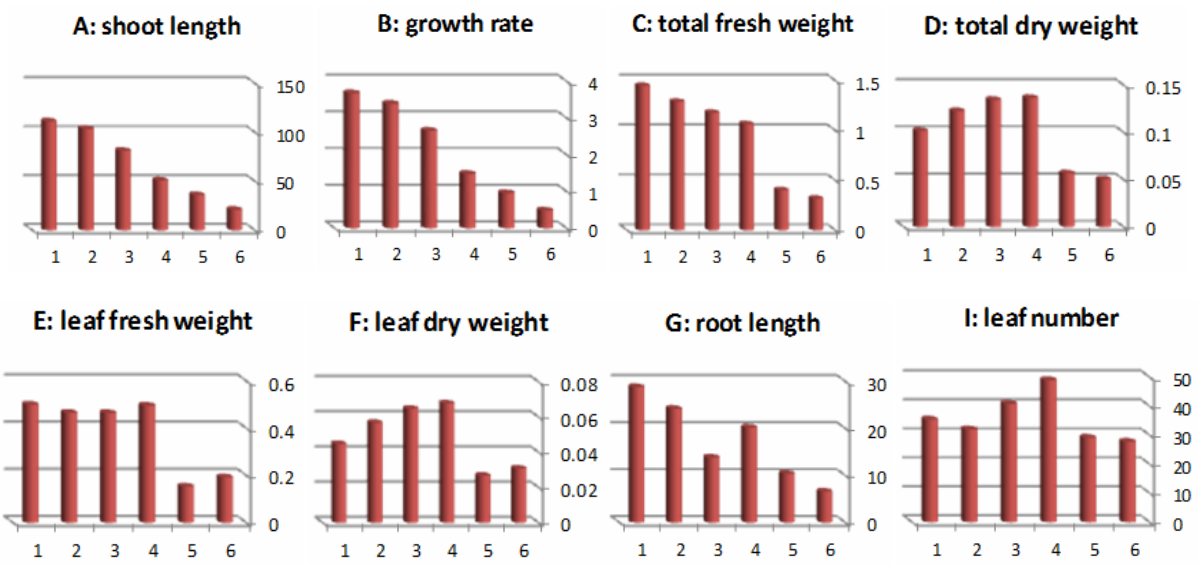

Figure 2. Effect of drought levels using mannitol concentration on studied traits. In each of the graphs, horizontal curves indicating the level of mannitol, Where, 1=control: MS (L1); 2 =%1 mannitol (L2); 3 = %2 mannitol (L3); 4 = %3 mannitol (L4); 5 =%4 mannitol (L5); 6= %5 mannitol (L6).

Table 2. Effect of different concentrations of mannitol on *Stevia rebaudiana* after 4 weeks of culture. MS : Murashige and Skoog culture as a control. (Where FW = total fresh weight; DW. =total dry weight; LFW= leaves fresh weight; LDW=leaves dry weight; NL=number of leaves; RL= root length; SL= shoot length; GR = growth rate). Mean values within a column with same letter are not significantly different based on least significant difference (LSD) at p = 0.05.

Treatments	FW (g)	DW (g)	LFW (g)	LDW (g)	LN	RL (mm)	SL (mm)	GR (mm/d)
MS (control)	1.495^{a}	0.116^{a}	0.544^{a}	0.053^{ab}	35.867^{b}	29.667^{a}	111.000^{a}	3.639^{a}
Mannitol 1%	1.297^{ab}	0.126^{a}	0.476^{a}	0.058^{a}	32.000^{b}	22.867^{a}	104.867^{a}	3.455^{a}
Mannitol 2%	1.160^{ab}	0.130^{a}	0.457^{a}	0.061^{a}	39.200^{b}	18.533^{ab}	85.533^{b}	2.740^{b}
Mannitol 3%	0.957^{b}	0.125^{a}	0.454^{a}	0.064^{a}	54.000^{a}	18.867^{ab}	46.000^{c}	1.333^{c}
Mannitol 4%	0.444^{c}	0.058^{b}	0.201^{b}	0.031^{b}	30.867^{b}	7.467^{bc}	29.333^{cd}	0.759^{cd}
Mannitol 5%	0.360^{c}	0.055^{b}	0.212^{b}	0.033^{b}	28.933^{b}	6.667^{c}	20.467^{d}	0.434^{d}

Table 3. Correlation coefficient among traits under overall treatments. (Where FW = total fresh weight; DW. =total dry weight; LFW= leaves fresh weight; LDW=leaves dry weight; NL=number of leaves; RL= root length; SL= shoot length; GR = growth rate)

Traits	FW(g)	LFW(g)	DW(g)	LDW(g)	LN	RL(mm)	SL (mm)	GR (mm/d)
FW	1							
LFW	0.971**	1						
DW	0.856*	0.920**	1					
LDW	0.763	0.877*	0.970**	1				
LN	0.279	0.482	0.623	0.737	1			
RL	0.982**	0.965**	0.799	0.727	0.310	1		
SL	0.964**	0.878*	0.749	0.616	0.027	0.924**	1	
GR	0.963**	0.876*	0.747	0.614	0.022	0.922**	1.000**	1

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

g) was observed on MS+%3 medium and minimum leaf dry weight (0.03 g) was observed on MS+%4 mannitol (Table 2). Maximum total dry weight (0.13 g) was observed on MS+%2 medium but minimum total dry weight (0.05 g) was observed on MS+%5 mannitol. Also there are not significantly different between (MS, MS+%1, MS+%2, MS+%3 mannitol) and between (MS+%4, MS+%5 mannitol) about this character (Table 2).

Effect of mannitol on root length of stevia

Maximum root length (29.66 mm) was observed on MS medium but minimum root length (6.66 mm) was observed on MS+%5 mannitol. Also there are not significantly different between (MS, MS+%1 mannitol) and between (MS+%2, MS+%3 mannitol) about this character (Table 2). In addition Simple correlation coefficients among all traits were estimated (Table 3).

Discussion

In this experiment, we found that there are significant differences among total fresh weight with leaf dry weight, so because of dried leaves of stevia are used, fresh weight is im-

portant for us. This results show that, drought stress effect on morphological and physiological characteristics. These results are in agreement with Badran et al. (2015) and Guangxi & Yan (2012).

Pandey & Chikara (2014) reported that by increasing the concentration of NaCl and mannitol (25, 50, 75 and 100mM) in medium, significant decrease in shoot number, shoot length, root number, root length, shoot/root ratio, leaf number, leaf fresh weight, stem dry weight, root dry weight, shoot dry weight and leaf dry weight were observed, that is agreement with us. In addition, they reported that in this experiment, rate of proline accumulation was increased and protein content was decreased.

Badran et al. (2015) used four levels of polyethylene glycol (0.0, 10,000, 20,000, 30,000 ppm) as drought stress and reported that survival % and number of shoots should be measured as the important factors associated with stevioside content in *stevia rebaudiana*. They showed that all drought stress reduced fresh and dry weight, which is agreement with us.

Hajihashemi & Ehsanpour (2013) reported that polyethylene glycol (0% - 6% w/v) reduced fresh and dry weight, chlorophylls, carotenoids, this result is similar to our funding.

Some research about salinity and drought stress on stevia indicated that the amount of protein decreases with increasing drought stress time (Guangxi & Yan 2012).

It is concluded that a biotic stress induced by the drought increased the concentration of steviol glycosides such as stevioside content significantly. So we can use these conclusions for future research, in order to increase the steviol glycosides in *stevia rebaudiana* Bertoni.

References

Abdul Razak, U.N.A., Ong, C.B., Yu, T.S., Lau, L.K. (2014): *In vitro* Micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. Brazilian Archives of Biology and Technology 1: 23-28.

Ahmed, M.B., Salahin, M., Karim, R., Razvy, M.A., Hannan, M.M., Sultana, R., Hossain, M., Islam, R. (2007): An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. American-Eurasian Journal of Scientific Research 2(2): 121-125.

Anbazhagan, M., Kalpana, M., Rajendran, R., Natarajan, V., Dhanavel, D. (2010): In vitro production of Stevia rebaudiana Bertoni. Emirates Journal of Food and Agriculture 22 (3): 216-222.

Badran, A.E., Abd Alhady, M.R.A., Hassan, W.A. (2015): *In Vitro* Evaluation of Some Traits in *Stevia rebaudiana* (Bertoni) under drought stress and their relationship on stevioside content. American Journal of Plant Sciences 6: 746-752.

Gregersen, S., Jeppesen, P.B., Holst, J.J., Hermansen, K. (2004): Antihyperglycemic Effects of Stevioside in Type 2 Diabetic Subjects. Metabolism 53(1): 73-76.

Guangxi, R., Yan, S. (2012): The Effects of Drought Stress on the Photosynthetic Parameters and Dry Leaf Yield of Stevia rebaudian Bertoni. Advanced Materials Research 4786: 518-523.

Gupta, P., Sharma, S., Saxena, S. (2014): Effect of Salts (NaCl and Na2CO3) on Callus and Suspension Culture of Stevia rebaudiana for Steviol Glycoside Production. Applied Biochemistry and Biotechnology 172: 2894-2906.

Hajihashemi, S., Ehsanpour, A.A. (2013): Influence of exogenously applied paclobutrazol on some physiological traits and growth of stevia rebaudiana under in vitro drought stress. Biologia 68: 414-420.

Hassanen, S.A., Khalil, P.M.A. (2013): Biotechnological Studies for Improving of Stevia (*Stevia rebaudiana* Bertoni) in vitro Plantlets. Middle-East Journal of Scientific Research 14 (1): 93-106.

Islam, S.A.M.N., Tareq, S.A.M. (2015): In vitro cloning and stem cutting of stevia (*Stevia rebaudiana* Bertoni) for mass propagation in Chittagong, Bangladesh. The International Journal of Biotechnology 4(3):14-19.

Jitendra, M., Monika,S., Ratan, S.D., Priyanka, G., Priyanka, S., Kiran, D.J. (2012): Micropropagation of an Anti diabetic Plant - Stevia rebaudiana Bertoni, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India. ISCA Journal of Biological Sciences 1(3): 37-42.

Kamran Khan, M., Misra, P., Sharma, P., Shukla, P.K., Ramteke, P.W. (2014): Effect of adenine sulphate on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. Journal of Medicinal Plant Research 8(13): 543-549.

Khalil, S.A., Zamir, R., Ahmad, N. (2014): Selection of suitable propagation method for consistent plantlets production in *Stevia rebaudiana* (Bertoni). Saudi Journal of Biological Sciences 21(6): 566-73.

Mondaca, R.L., Gálvez, A.V., Bravo, L.Z., Hen, K.A. (2012): Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chemistry 132: 1121-1132.

Mukundan, U., Sivaram, L. (2003): In vitro culture studies on *Stevia rebaudiana*. *In Vitro* Cellular and Developmental Biology 5: 520-523.

Murashige, T., Skoog, F. (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. Journal of Plant Physiology 15:473–497.

Pande, S.S., Gupta, P. (2013): Plant tissue culture of *Stevia rebaudiana* (Bertoni): A review. Journal of Pharmacognosy and Phytotherapy 5(1): 26-33.

Pandey, M., Chikara, S.K. (2014): In vitro regeneration and effect of abiotic stress on physiology and biochemical content of *Stevia rebaudiana* 'Bertoni' Journal of Plant Science and Research 1(3): 113.

Prakash, I., Markosyan, A., Bunders, C. (2014): Development of Next Generation Stevia Sweetener: Rebaudioside M. Foods (3): 162-175.

Ramakrishna, A., Ravishankar, G.A. (2011): Influence of abiotic stress signals on secondary metabolites in plants Plant Signaling and Behavior 6: 1720-1731.

Singh, S., Garg, V., Yadav, D., Beg, M.N., Sharma, N. (2012): *In-vitro* antioxidative and antibacterial activities of various parts of *stevia rebaudiana* (Bertoni). International Journal of Pharmacy and Pharmaceutical Sciences 4(3): 468-473.

Yang, Y.W., Hsing, Y.I., Chang W.C. (1981): Clonal propagation of *stevia rebaudiana* Bertoni through axillary shoot proliferation *in vitro*. Bot. Bull, academia Sinica 22:57-62.

Zeng, J., Chen, A., Li, D., Yi, B., Wu, W. (2013): Effects of salt stress on the growth, physiological responses, and glycoside contents of *Stevia rebaudiana* Bertoni. Journal of Agricultural and Food Chemistry 61(24):5720-6.