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

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

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Received November 25, 2016; **Accepted** February 20, 2017; **Published** February 28, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.2.17

Abstract: *Stevia rebaudiana* Bertoni belongs to Asteraceae family that leaves 200-300 times sweeter than sugar. Low seed fertility is one of the most important problems in Stevia production. So, Plant tissue culture is an efficient method for mass propagation of Stevia. In this research, we studied the effect of various concentrations of nitrogen on some morphological traits of stevia under *in vitro* conditions. We used axillary nodes as explants and they were cultured on Murashige and Skoog (MS) medium containing inorganic nitrogen sources i.e. NH_4NO_3(0, 825 and 1650 mg/l), KNO_3(0, 950 and 1900 mg/l) were observed. The cultures were kept for 4 weeks at a temperature of 25±2°C with a photoperiod of 16/8 hour low light/dark each day. Maximum shoot length (89.33 mm), dry weight of plants (0.10 mg) and leaf fresh weight (0.42 mg) was observed on MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3. 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 on a MS medium without nitrogen sources. Moreover, presence of nitrogen sources increases both shooting and rooting in *Stevia rebaudiana* Bertoni.

Key words: *Stevia rebaudiana* Bertoni; Nitrogen sources; KNO_3, NH_4NO_3; Morphology; *In vitro* propagation.

Introduction

Stevia rebaudiana Bertoni belongs to the Asteraceae family, which is more than 230 shrub and sub-shrub plants (1-3).

Stevia is a native of the semi-humid subtropical regions of Paraguay and Brazil. Although Stevia has been used in Asia and Europe for centuries, but nowadays it cultivated as a suitable crop in many countries such as Korea, Mexico, United States, Tanzania and Canada (4, 5).

S. rebaudiana synthesizes more than 30 steviol glycosides in different concentrations such as rebaudioside A, B, C, D, E, F, M, stevioside, steviolbioside, dulcoside A and dulcoside C. There are reports that indicate Stevioside is a more high expression in the leaves of Stevia. Stevioside and rebaudioside A are two major sweetest glycosides in stevia (6, 7).

Steviol glycosides are about 200-300 times sweeter than sugar with zero calorie contributing to several medicinal properties such as anti-hyperglycemic, anti-microbial, anti-hypertensive, anti-oxidant and anti-cancerous and etc(8, 9).

Extract of the stevia used to be as sweetening agents, taste modifiers, and alternative sweeteners in the food industry. The powdered form stevia leaves can be added to drinks, ice cream, dairy products, table-top sweeteners, etc. that controls both hypoglycemic and body weight reducing effect (10).

Stevia propagated by seed or stem cutting. But the main problem is that Stevia has produce low seed fertility. So far, researcher do not know failing to produced enough seed. Some researcher has reported self- incompatibility as main reason. The stem cutting technique for stevia propagation has some restrictions such as low number of new plants and destruction of the donor plant (11, 12).

Overall, plant cell and tissue culture is the best way to overcome these problems, in addition there is the possibility to produce a large number of plants in short period (5, 10, 13-17).

Plant nutrients are separated into primary and secondary nutrients. Primary nutrients involve nitrogen, phosphorous and potassium, which regularly exist in fertilizers, affects plant growth by complementing plant nutrients, which allows plants to grow faster and better. Plants use primary nutrients in large quantities as they grow. Secondary nutrients such as calcium, magnesium and sulfur are also used deeply by plants, but they are generally present in the soil (1, 18).

Nitrogen is so essential because it is a main component of chlorophyll, the compound by which plants use sunlight energy to produce sugars from water and carbon dioxide. Furthermore nitrogen is the most important component of amino acids, the building blocks of proteins. In addition to nitrogen is a component of energy-transfer compounds, like ATP (adenosine triphosphate). Lastly, nitrogen is a major element of nucleic acids such as DNA. As a result without nitrogen, there would be no life as we know it (19).

Generally researchers use MS (20) medium for micropropagation of *Stevia rebaudiana* in which inorganic nitrogen sources of $NH4^+$-N and $NO3^-$-N and $NH4^+$: $NO3^-$ = 1:2 are used (1).

The purpose of this study was to investigate the effect of nitrogen on some morphological characteristics of Stevia at *in vitro* condition.

Materials and Methods

Plant material

The explants (stem nodal segments) were collected from *in vitro* propagation of *Stevia rebaudiana* Bertoni shoots at the plant tissue culture Laboratory, Zagros Bioidea Co. Razi University, Kermanshah, Iran. At first the explants were washed in running tap water for 15 minutes for surface-sterilization. Then put in Ethanol (70%) for 1 min, next those mercuric chlorides (0.2%) for 120 s were used. Finally, the explants were washed with sterilized double distilled water. Every part of this process was done under the laminar air flow (6, 21).

Culture conditions

MS medium (20) with 3% sucrose (Merck) supplemented with various concentrations of NH_4NO_3 (0, 825 and 1650 mg/l) and KNO_3 (0, 950 and 1900 mg/l) was used throughout the study. The pH of the nutrient medium was adjusted to 5.7-5.8 using 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 sterilized auxiliary nodes were put on MS medium with different concentrations of NH_4NO_3 (0, 825 and 1650 mg/l) and KNO_3 (0, 950 and 1900 mg/l). MS medium without NH_4NO_3 and KNO_3 was used as a control. 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 calculated characters such as leaf number, leaf dry weight, leaf fresh weight, total dry and fresh weight, root length and growth rate (shoot length after 4 weeks/28 days) were documented after 28 days.

Statistical analysis

In this research, each treatment contained 3 replications and in each replication 5 explants were used. The data were measured after 28 days of culture. Data were analyzed by SAS (version 9.1) and Excel (2013) software. Mean values were compared according to least significant differences test (LSD) at ($P< 0.05$ and 0.01).

Results and Discussion

The analysis of variance indicated that different concentration of NH_4NO_3 and KNO_3 on *stevia rebaudiana* Bertoni, show significantly on all studied traits at 1% level. The significance levels for measured characters for example shoot and root length (mm), leaf dry and fresh weight (mg), leaf number, total dry and fresh weight (mg) and growth rate are shown in (Table 1).

Effect of different concentrations of NH_4NO_3 and KNO_3on shoot length of Stevia

The effect of different concentrations of NH_4NO_3 and KNO_3 on some morphological traits of stevia has been shown in Figure 1. Results showed that adding NH_4NO_3 and KNO_3 to the medium, increased notably, shoot length, internode length and growth rate (shoot length after 28 days) were obviously increased (Fig. 2A, 2G).

Maximum growth rate (2.997 mm/d) was obtained in MS medium with 1650 mg/l NH_4NO_3 and 1900 mg/l KNO_3 and minimum growth rate (0.006 mm/d) was obtained in MS medium without nitrogen sources. But plants in treatment 3 to 9 were not significantly different for this character (Table 2).

The maximum shoots production was observed in the MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 (89.330 mm) after 28 days of cultivation and minimum shoot length (6.133 mm) was observed on a MS medium without nitrogen sources. But plants in 3 to 9 treatments are not significantly different about this character (Table 2).

Effect of different concentrations of NH_4NO_3 and KNO_3 on root length of Stevia

Maximum root length (25.130 mm) was observed on MS medium with 825 mg/l NH_4NO_3 and 950 mg/l KNO_3but minimum root length (6.66 mm) was observed on MS medium without nitrogen sources. Also there were not significantly differences between 3, 5, 6, 7, 8 and 9 treatments and between 1, 2 and 4 treatment about this character (Table 2).

Effect of different concentrations of NH_4NO_3 and KNO_3on fresh and dry weight of Stevia

Maximum total fresh weight (1.432 g) was observed on MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 (89.330 mm) following to 28 days of cultivation and minimum total fresh weight (0.156 g) was observed on MS medium free of nitrogen sources. Also there were not significantly difference between 3, 5 and 9 treatments about this trait (Table 2).

Maximum total dry weight (0.105 g) was observed on MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 but minimum total dry weight (0.023 g) was observed on MS medium without nitrogen sources. Also there are not significantly different between 2, 3, 4, 5 and 9 treatments and between 6 and 8 treatments about this trait (Table 2).

Maximum leaf fresh weight (0.428 g) was observed

Table 1. Mean squares for effect of different concentration of NH_4NO_3 and KNO_3on *stevia rebaudiana* Bertoni after 28 days (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 Squares							
Source	**df**	**FW**	**DW**	**LFW**	**LDW**	**NL**	**RL**	**SL**	**GR**
Treat	8	0.400**	0.002**	0.033**	0.001*	168.360**	142.350**	2027.800**	2.820**
Error	18	0.026	0.0003	0.003	0.0005	11.584	26.584	153.45	0.154
CV (%)		21.15	27.51	22.45	35.05	15.23	28.53	18.01	16.72

Table 2. Mean comparison for effect of different concentrations of NH_4NO_3 and KNO_3 on *Stevia rebaudiana* after 28 days of culture. MS culture without NH_4NO_3 and KNO_3as 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	GR (mm/d)	FW (g)	DW (g)	LFW (g)	LDW(g)	RL (mm)	SL (mm)	NL
1	0 mg/l NH_4NO_3+ 0mg/l KNO_3	0.006^{c}	0.156^{a}	0.023^{e}	0.056^{d}	0.012^{a}	6.600^{c}	6.133^{c}	4.267^{c}
2	825 mg/l NH_4NO_3+ 0mg/l KNO_3	1.647^{b}	0.586^{d}	0.053^{cd}	0.221^{c}	0.026^{a}	11.130^{bc}	50.470^{b}	17.670^{b}
3	1650 mg/l NH_4NO_3+ 0mg/l KNO_3	2.380^{a}	0.796^{cd}	0.074^{bc}	0.318^{b}	0.045^{a}	22.800^{a}	69.80^{ab}	23.730^{a}
4	0 mg/l NH_4NO_3+ 950mg/l KNO_3	2.680^{a}	0.604^{d}	0.047^{cde}	0.209^{c}	0.018^{a}	11.670^{bc}	78.870^{a}	21.870^{ab}
5	825 mg/l NH_4NO_3+ 950mg/l KNO_3	2.840^{a}	0.912^{bc}	0.063^{bcd}	0.272^{bc}	0.030^{a}	25.130^{a}	80.330^{a}	25.330^{a}
6	1650 mg/l NH_4NO_3+ 950mg/l KNO_3	2.930^{a}	1.432^{a}	0.105^{a}	0.428^{a}	0.041^{a}	24.730^{a}	89.330^{a}	27.070^{a}
7	0 mg/l NH_4NO_3+ 1900mg/l KNO_3	2.837^{a}	0.523^{d}	0.044^{de}	0.202^{c}	0.039^{a}	24.530^{a}	83.200^{a}	27.340^{a}
8	825 mg/l NH_4NO_3+ 1900mg/l KNO_3	2.747^{a}	1.099^{b}	0.090^{ab}	0.315^{b}	0.040^{a}	17.530^{ab}	78.730^{a}	27.400^{a}
9	1650 mg/l NH_4NO_3+ 1900mg/l KNO_3	2.997^{a}	0.749^{cd}	0.069^{bcd}	0.320^{ab}	0.082^{a}	18.530^{a}	82.260^{a}	26.400^{a}

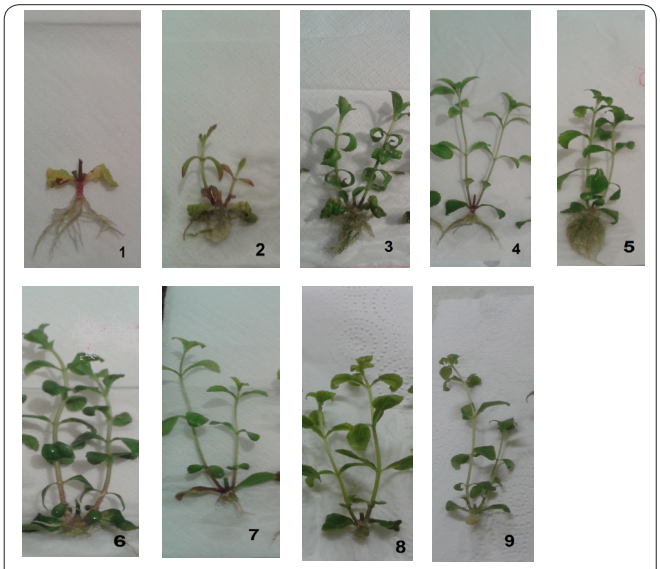

Figure 1. Shoot proliferation from nodal explants on medium (MS + different concentration of NH_4NO_3 and KNO_3) after 28 days. (Where, 1 = NH_4NO_3 and KNO_3 free; 2 = 825 mg/l NH_4NO_3; 3 = 1650 mg/l NH_4NO_3; 4 = 950 mg/lKNO_3; 5=825 mg/l NH_4NO_3 +950 mg/l KNO_3; 6= 1650 mg/l NH_4NO_3 +950 mg/l KNO_3; 7= 1900 mg/l KNO_3; 8 = 825 mg/l NH_4NO_3 + 1900 mg/l KNO_3; 9 = 1650 mg/l NH_4NO_3 +1900 mg/l KNO_3).

on MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 but minimum leaf fresh weight (0.056 g) was observed on MS medium without nitrogen sources.

Maximum leaf dry weight (0.082 g) was observed on MS medium with 1650 mg/l NH_4NO_3 and 1900 mg/l KNO_3 and minimum leaf dry weight (0.012g) was observed on MS medium without nitrogen sources (Table 2).

Furthermore Simple correlation coefficients among all traits were estimated (Table 3).

This results show that, different concentration of NH_4NO_3 and KNO_3 effect on morphological and physiological characteristics. These results are in agreement with Aladakatti et al. (2012), Yadav et al. (2013), Kumar et al. (2013), and Ibrahim et al. (2008).

In this research, we observed that there are significant differences among total fresh weight with leaf dry weight (0.681**), so because of dried leaves of stevia are used, total fresh weight is important for us (Table 3).

Yadav et al. (2013) were cultured the shoots of *Stevia rebaudiana* on MS medium supplemented with various concentrations and combinations of different auxins α-NAA (1.0 and 2.0 mg/l) and IAA (0.5 - 5.0 mg/l) and cytokinines, BAP (0.5-5.0 mg/l), kinetin (0.3-5.0 mg/l) alone and in combination BAP (3.0-5.0 mg/l) and then were inoculated aseptically on MS medium with NH_4NO_3 and KNO_3, Kinetin (1.0 mg/l) and BAP (5.0 mg/l) for induction of shoots. Results showed that existence of nitrogen sources increases both shooting and rooting in stevia. However less concentration of NH_4NO_3 (14 N/l) is required as compared with KNO_3 (400 N/l) for better induction and multiplication of shoot and root, which is agreement with us (1).

Kumar et al. (2015) cultured *in vitro* shoots on MS

Table 3. Correlation coefficient among studied 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	GR(mm/d)	FW(g)	DW(g)	LFW(g)	LDW(g)	RL(mm)	SL(mm)	NL
GR	1							
FW	0.738**	1						
DW	0.521**	0.734**	1					
LFW	0.692**	0.829**	0.681**	1				
LDW	0.490**	0.644**	0.877**	0.632**	1			
RL	0.692**	0.884**	0.796**	0.890**	0.713$_{**}$	1		
SL	0.733**	0.832**	0.709**	0.978**	0.637**	0.898**	1	
NL	0.464*	0.660**	0.578**	0.636**	0.584**	0.603**	0.601**	1

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

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

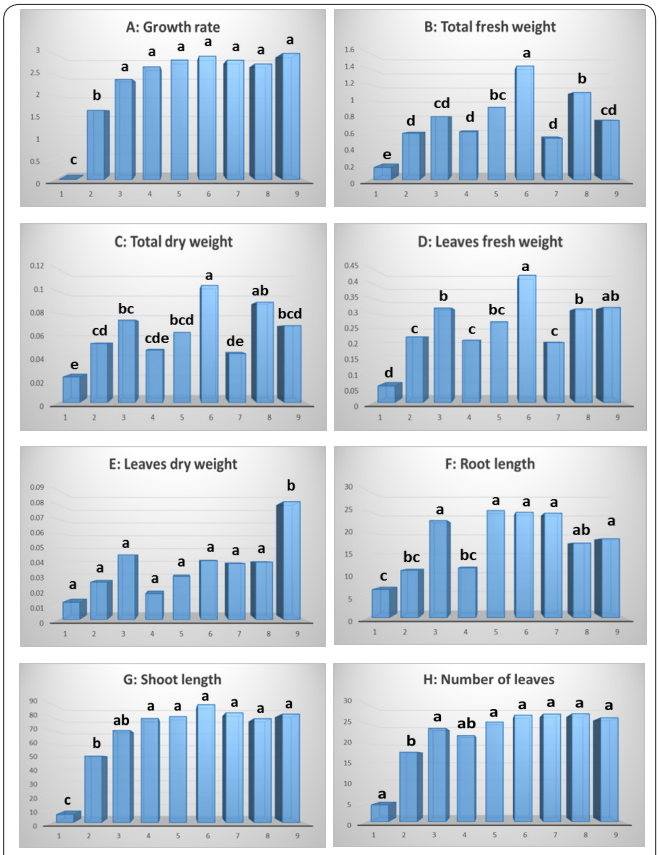

Figure 2. Effect of different concentrations of NH_4NO_3 and KNO_3 on *Stevia rebaudiana* after 28 days of culture. In each of the graphs, horizontal histogram indicating the trait numbers (Where, 1 = NH_4NO_3 and KNO_3 free; 2 = 825 mg/l NH_4NO_3; 3 = 1650 mg/l NH_4NO_3; 4 = 950 mg/lKNO_3; 5=825 mg/l NH_4NO_3 +950 mg/l KNO_3; 6= 1650 mg/l NH_4NO_3 +950 mg/l KNO_3; 7= 1900 mg/l KNO_3; 8 = 825 mg/l NH_4NO_3 + 1900 mg/l KNO_3; 9 = 1650 mg/l NH_4NO_3 +1900 mg/l KNO_3), (A: Growth rate, B: Total fresh weight, C: Total dry weight, D: Leaves fresh weight, E: Leaves dry weight, F: Root length, G: Shoot length, H: Number of leaves)

medium fortified with BAP (5.0 mg/l) along with 2,4-D (0.1 mg/l). After four weeks multiple shoots were transferred on modified MS medium having NH_4NO_3 (14-56 N mg/l), KNO_3 (100-400 N mg/l) with BAP (0.5-5.0 mg/l). After 12 weeks cultures result showed maximum multiplication of shoots on MS medium with NH_4NO_3 (14 N mg/l), KNO_3 (400 N mg/l) with BAP (5.0 mg/l) (2).

In 2012 Aladakatti et al. researched about the effect of nitrogen, phosphorus and potassium levels on growth and yield of stevia (*Stevia rebaudiana* Bertoni.). The results show that nitrogen (400 kg ha^{-1} N2) recorded the highest B:C (3.01) which was on par with nitrogen N2 i.e., 300 kg ha^{-1} (2.93), but significantly higher than N1 i.e., 200 kg ha^{-1} (2.66). Interactions of N, P and K were not significant (22).

We concluded that MS medium with 1650 mg/l NH_4NO_3 and 950 mg/l KNO_3 was the best medium to have maximum total fresh and dry weight, leaf fresh and dry weight and shoot length (Fig. 1 number 6). However the plants in MS medium without nitrogen sources were yellowish and they did not grow (Fig. 1 number 1). So stevia need nitrogen sources for growing well.

Acknowledgments

We would like to thank Miss Matin Ghaheri (M. Sc. of Biotechnology, Razi University, Kermanshah, Iran) for her kindly helps in doing this research. Special thanks to Zagros Bioidea Co. Lab, Razi University Incubator for support of this research project.

References

1. Yadav P, Kumari P, Arya A, Tripathi S, Kumar S. Effect of nitrogen sources on rooting of *in vitro* culture of *Stevia rebaudiana* Bertoni. J Biotechnol 2013; 4: 41-46.
2. Kumar S, Yadav P, Kumari P, Tripathi S, Arya A. Effect of nitrogen on micropropagation of *Stevia rebaudiana* Bertoni. J Dev Res 2013; 8: 023-029.
3. 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.
4. Verma R, Gena DD, Lal Jat B. *In vitro* propagation of *Stevia rebaudiana* Bertoni (a sweeting plant). J Pharm Res 2016; 5: 666-685.
5. Abdul Razak UNA, Ong CB, Yu TS, Lau LK. *In vitro* Micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. Braz Arch Biol Technol2014; 1: 23-28.
6. Ghaheri M, Kahrizi D, Bahrami Gh. Effect of mannitol on some morphological characteristics of in vitro *stevia rebaudiana* Bertoni. Biharian Biologist 2015; (online first): art. 151415.
7. Mondaca RL, Gálvez AV, Bravo LZ, Hen KA. *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. J Food Chem 2012; 132: 1121-1132.
8. Soufi S, D'Urso G, Pizza C, Rezgui S, Bettaieb T, Montoro. Steviol glycosides targeted analysis in leaves of *Stevia rebaudiana* (Bertoni) from plants cultivated under chilling stress conditions. J Food Chem 2016; 190: 572–580.
9. Debnath M. Propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. J Med Plant Res 2008; 2: 45–51.
10. Anbazhagan M, Kalpana M, Rajendran R, Natarajan V, Dhanavel D. In vitro production of *Stevia rebaudiana* Bertoni. Emir J food agric 2010; 22: 216-222.
11. Raina R, Bhandari SK, Chand R, Sharma Y. Strategies to improve poor seed germination in *Stevia rebaudiana*, a low calorie sweetener. J Med Plants Res 2013; 7: 1793-1799.
12. Savita S, Sheela K, Sunanda S, Shankar A, Ramakrishna P. *Stevia rebaudiana*–a functional component for food industry. J Hum Ecol 2004; 15: 261–264
13. Kamran Khan M, Misra P, Sharma P, Shukla PK, Ramteke PW. Effect of adenine sulphate on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. J Med Plants Res 2014; 8: 543-549.
14. Das A, Gantait S, Mandal N. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. Int J Agric Res 2011; 6: 40-48.
15. Hwang SJ. Rapid in vitro propagation and enhanced stevioside accumulation in *Stevia rebaudiana* Bert. J Plant Biol 2006; 49: 267-270.
16. Jain P, Kachhwaha S, Kothari SL. Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* Bertoni by using high copper levels in the culture medium. Sci Hort 2009; 119: 315-319.
17. Mukundan U, Sivaram L. In vitro culture studies on *Stevia rebaudiana*. J In Vitro Cell Dev Biol 2003; 5: 520-523.
18. Ma L, Shi Y. Effects of potassium fertilizer on physiological and biochemical index of *Stevia rebaudiana* Bertoni. J Energy Procedia 2011; 5:581-586.
19. Sinclair TR, Horie T. Leaf Nitrogen, Photosynthesis, and Crop Radiation Use Efficiency: A Review. J Crop Sci 1989; 29: 90-98.

20. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. J Plant Physiol 1962; 15:473–497.
21. Khalil SA, Zamir R, Ahmad N. Selection of suitable propagation method for consistent plantlets production in *Stevia rebaudiana* (Bertoni). Saudi Journal of Biological Sciences. Saudi J Biol Sci. 2014; 21: 566–573.
22. Aladakatti YR, Palled YB, Chetti MB, Halikatti SL, Alagundagi SC, Patii PL, Patii VC, Janawade AD. Effect of nitrogen, phosphorus and potassium levels on growth and yield of stevia (*Stevia rebaudiana* Bertoni.). J Agric Sci 2012; 25: 25-29.
23. Ibrahim I. A, Nasr MI, Mohammedm BR, El-Zefzafi MM. Nutrient factors affecting in vitro cultivation of *Stevia rebaudiana*. J Sugar Tech 2008; 10:248-253.