Journal of Medicinal Plants and By-products (2020) 1: 1-8

Original Article

Effect of Growth Regulators on Optimization of Stevia (*Stevia rebaudiana*) Production in vitro

Bahareh Kashefi*, **Mahdi Naeimi and Elham Mazaheri**

College of Agriculture, Islamic Azad University, Damghan Branch, Damghan, Iran

Article History: Received: 05 August 2018/Accepted in revised form: 07 June 2019

Abstract

Stevia rebaudiana (Bertoni) Bertoni importance and value have been proved in the world because of its alternative potential as natural sugar and in vitro production benefits. Tissue culture of this plant was considered in order to get the best regeneration condition. In this study, proliferation of branches and rooting were measured as a factorial based on a randomized complete block design with four replications. There were two types of shoot induction hormone treatments including BAP and Kin in 0 (control sample), 0.25 and 0.5 mg/lit concentrations and two root induction hormone treatments of IBA and NAA in vitro condition. Traits were measured after 45 days on branch, leaf and root. Results showed the most branch length and root volume with 0.25 mg/lit of Kin hormone and the most root length with 0.25 mg/lit concentration of IBA. The highest rooting was related to IBA hormone in 0.5 mg/lit concentration. According tocorrelation analysis between traits, positive and significant correlation was reported. Cluster analysis grouped shoot treatment with BAP in two 0.25 and 0.5 mg/lit concentrations in one group and Kin with 0.25 and 0.5 mg/lit concentrations in another group. Also, these treatments showed the most similarity. Also, IBA root treatment with 0.25 mg/lit concentration had the most difference in comparison with other hormone treatments. According to the HPLC results, increasing of rooting hormones concentrations (NAA and IBA) leaded to leaf Stevioside increasing. Totally, hormone treatments resulted significant differences between control and treated plants. That had positive effects on plant regeneration.

Keywords: Hormone, Regeneration, Riboside, Stevioside, Tissue culture.

Introduction

One of the important biotechnology and genetic engineering issues is tissue culture. Various aspects of tissue culture are important because of these methods optimization, considering of effective reagents in secondary metabolites biosynthesis and their applications in medicinal plants breeding [1]. Stevia plant [*Stevia rebaudiana* (Bertoni) Bertoni] is resistant plant of Asteraceae family. Their leaves are small, without stem, sharp and flat and they have indentation at middle part. 8 types of glycoside with sweet properties are detected Stevia leaf tissues. Two major glycosides are Stevioside and Riboside A (Reb-A) which include 5 to 10 and 2 to 4 percentage leaves dry weight, respectively. That is reported Stevia tissues have different proportions of Stevioside in leaves, stems, roots and flowers. Leaves have the most amount of Stevioside and they are 100-300 times sweeter than sucrose [2]. Stevioside is not caloric. That is effective in reduction of sugar level in diabetic patients, treatment of hypertension and gastrointestinal diseases. Also, Stevioside is used as regime sugar [3].

Plant tissue culture is a laboratory method that means growth of separated tissues or organs of maternal plant. It leads to mass production of cells from maternal plant tissue. Cal (callus) can be used in re-production of plants directly or development of some primary and secondary metabolites [4]. Stevia seeds have too low germination percentage and its proliferation by seeds does not produce a

*Corresponding author: College of Agriculture, Islamic Azad University, Damghan Branch, Damghan, Iran
Email Address: bahareh.kashefi@gmail.com

homogenous population. Considering of these difficulties and importance of this plant in industrial and medical parts, tissue culture is a good alternative for Stevia proliferation. Stevia tissue culture are usually done by leaves, lateral buds or stems [2]. The most proliferation of lateral branch on MS medium contain 1.5 mg/lit of BAP and 0.5 mg/lit of Kin and the most root proliferation (%97, 66) was related to MS containing 0.1 mg/lit of IAA. Moktaduzzaman and Rahman [5] cultured leaf explant in MS medium. They reported, the highest number of branches (7.21) and the most leaf length (22.3 cm) were resulted from 1.8 mg/lit of BA and 0.12 mg/lit of NAA. Anbazhagan *et al*. [6] propagated Steviausing stem and leaf in MS medium with different concentration of BA, Kin and IAA. The best results were using IBA and IAA. According to other studies, BA and NAA lead to inoculation callus increasing and branches differentiation, while NAA and IBA increased root inoculation. Alhady *et al*. [7] propagated branches of Stevia in medium with different concentrations of BA. They reported the highest revival percentage (%90) and growth percentage (%100) in medium with 0.5 mg/lit BA and Kin. The largest number of reproduced branches was related to medium containing 0.5 mg/lit of Kin and 0.2 mg/lit of BAP. The most root induction (%100) was observed in medium containing 0.2 mg/lit of Kin or 0.1 mg/lit of IBA. Das *et al*. [3] reported Stevia propagation through stem tissue culture in MS medium including 2 mg/lit of kinetin. Singh *et al*. [8] showedwhile leaf parts cultured in MScontaining 1mg/lit 2,4-D and 1mg/lit of kinetin, callus was induced effectively. MS medium comprising 0.5 mg/lit of BAP and 0.3 mg/lit of NAA had best condition for root differentiation. Hassanen and Akhalili [9] reportedmost shoot induction in MS medium containing 0.2 mg/lit BAP.

The largest number of root (7.3), root length (2.64 cm) and plant height (4.29 cm) resulted in medium with 0.1 mg/lit of IBA and 0.5 mg/lit of alar. The highest survival rate was 93.3 in greenhouse. Jamshidi Zinab *et al*. [2] were observed significant difference between shoots number, produced nodes and shoot length. Jitendra *et al*. [10] reported that most root induction in MS medium with 0.1 mg/lit of IBA. Also, Zayova *et al*. [11] were showed the highest root in MS medium containing 0.1 mg/lit of IBA. Verma *et al*. [12] cultured Stevia tissues in MS medium with BAP and Kin (0.5 mg/lit). They reported highest shoot length (3.5 cm) and leaf number (8.7) in medium with 0.5 mg/lit of GA. The most root induction and rooting beginning resulted on day of 7 in mediums with 2 mg/lit of IBA. Ahmed *et al*. [13] were done Stevia proliferation using lateral and knotted shoot.

Aim of this study was optimization of Stevia regeneration in vitro condition and determines the best hormone treatments for shoot and root induction and increase active ingredients production.

Material and Methods

This research was done in Islamic Azad university laboratory of Damghan. Stevia [*Stevia rebaudiana* (Bertoni) Bertoni] explant was obtained from medicinal plants institute of Iranian Institute of Medicinal Plants. Murashige and Skoog (MS) medium was used to cultivation of plant organs, direct regeneration and rooting. Explants were selected from shoots and leaves. Culture dishes were kept in growth chamber with 21 ± 2 °C and 16 hours of light and 8 hours of darkness. Grown explants were cut from top two leaves after 2 weeks and they were transformed to hormonal medium. Used hormones were Kin and BAP with concentrations of control, 0.25 and 0.5 mg/lit. To rooting enhancement in vitro, grown explants in shoot medium were isolated after 45 days and they were transferred to medium with three concentration (control, 0.25 and 0.5 mg/lit) of NAA and IBA. This experiment consisted of a factorial randomized complete block design with four replications.

Measured traits were included shoot, leaf and root length, diameter of shoot in cm and node number, rooting percentage, root and callus volume in terms of scoring [absence (0), small quantities (1), average value (2), good quantities (3), excellent amount (4)], active ingredients of Stevioside, Rebadiosie A and Riboside C.

Prepared Nethanol Extracts

Methanol extracts of samples were prepared to Riboside A and C and Stevioside measurement. For this purpose, stem and leaf of each treatment were dried in hot weather. Then, 0.1 g of plant powder was poured in 20 ml vials and methanol was added them. Solution was left in ultrasound device for 60 min. resulted extract was centrifuged for 15 min at 20°C (3000 g). Supernatant was isolated and that

was diluted 5 times with %20 of water and mobile phase.

Measurements Active Ingredients

Quality and quantity of active ingredients including Riboside A, Rebadiosie C and Stevioside were considered by high performance liquid chromatography device (HPLC, Waters model). Finally, 50 microliter of samples were injected to device. Properties of liquid chromatography system included Teknokroma NH2 column type, 25 cm column length, 50 µ injection volume (injector), 0.46 cm column internal diameter, WATERS-600E pump, Waters- UV 486: 297 nm detector, 1.5 ml/min flow rate, mobile phase of water-acetonitrile (20-80).

(1) Riboside A: [Ws/W] x [Aa/As] x 100
(2) Stevioside: [Ws/W] x Ast x [0.83/As] x100
(3) Riboside C: [Ws/W] x ACx [0.98/As] x 100

Ws: standard weight of Riboside A (mg) in standard solution
W: weight of plant sample
As: area under standard peak of Riboside A
Aa: area under peak of Riboside A in unknown sample
Ast: area under peak of Stevioside in sample
Ac: area under peak of Riboside C in unknown sample

Statistical Analysis

Resulted data were statistical analyzed using SAS software. Mean comparison was done by Duncan's test. Grouping was performed in %1 and %5 levels.

Results and Discussion

Variance analysis results of different traits with shooting treatments have shown in table 1. Significant difference between these traits indicated various effect of applied treatments. Duncan's test was used to mean comparison of traits.

Treatments were divided into two groups for node number trait. BAP hormonal treatment had higher mean in two group and they were placed in a separate group compared to other treatments. Kin treatment did not show significant difference between control and other two groups (Table 2).

According results, Kin treatment of 0.25 mg/lit had significant difference compared to other treatments. Mean comparison of shooting treatments effect on leaf length have shown that most and lowest means were related to BAP 0.25 and Kin 0.5 mg/lit, respectively. Other treatments had no significant difference with mentioned treatment (Table 2).

Mean comparison results of hormonal treatments effect on root length have shown that BAP treatment 0.25 mg/lit (2.68 cm) had significant difference compared to other treatments. BAP treatment of 0.5 mg/lit for this trait was in second place and that has shown significant difference in comparison with control. Also, different hormonal treatments had positive effect on root volume. Kin treatment of 0.25 mg/lit had the most effect on root volume with mean of 2.40. Unlike leaf length trait, Kin treatment had more effect on root volume trait (Table 2).

Callus mean comparison has shown that 0.5 mg/lit of BAP with average of 1.60 was most amount for this trait. That had great difference with other treatments.

Table 1 Analysis of variance on different *Stevia rebaudiana* (Bertoni) Bertoni traits under shooting treatments

Sources of variation	Freedom degree	Node Number	Shoot length	Leaf Length	Root Length	Root volume	Callus
Treatment	4	17.172 **	0.891**	0.182 **	0.2758 **	0.653 **	1.483 **
Error	15	0.855	0.1465	0.0573	0.0203	0.0546	0.0086
Coefficient of Variation (CV%)		9.46	3.03	10.68	0.27	11.57	13.89

Significant at 1% level **

Table 2 Comparison of average on different *Stevia rebaudiana* (Bertoni) Bertoni traits under shooting treatments

Treatment	Node Number	Shoot length	Leaf Length	Root Length	Root volume	Callus
Control	7.85 b	12.53 b	2.15 ab	2.03 c	1.35 b	0.15 d
0.25 Kin	9.05 b	13.45 a	2.25 ab	2.21 bc	2.4 a	0.2 d
0. 5 Kin	8 b	12.3 b	2.05 b	2.08 bc	2.25 a	0.45 c
0.25 BAP	11.98 a	12.55 b	2.6 a	2.68 a	2 a	0.95 b
0. 5 BAP	12 a	12.33 b	2.15 ab	2.37 b	2.1 a	1.6 a

The least amount of mean was related to control sample and other treatments were placed in separate groups (Table 2). Variance analysis of different traits with rooting treatments has shown significant difference at %1 level (Table 3).

Results of node number have determined that 0.5 mg/lit of IBA treatment had the highest mean. That has considered in a separate group compared to other treatments (Table 4).

Mean comparison of rooting hormones effect on branch length has shown that 0.25 mg/lit of IBA (14.6 of node number) had the most impact and that was placed in separate group. NAA and IBA treatments with 0.5 mg/lit concentration were placed in another group and they had significant difference compared to other treatments. NAA treatment of 0.25 mg/lit and control samples had no significant difference (Table 4).

Mean comparison of root hormones treatment on leaf length has indicated significant difference between control and other treatments. Hormonal treatment of IBA with 0.25 mg/lit concentration had the most average compared to other treatments. IBA and NAA treatments in level of 0.25 mg/lit had more mean than 0.5 mg/lit.

Results of root length mean comparison have determined that 0.25 mg/lit of NAA treatment had the most effect (3.10 cm). Concentration increasing of NAA from 0.25 to 0.5 mg/lit reduced root length. Mean comparison of root volume have shown that there was no significant difference between hormone treatments. Both two level in NAA hormone treatment had more amount compared to IBA treatment (Table 4).

Variance analysis of callus trait has determined significant difference between various hormones. NAA treatment with 0.5 mg/lit had the most amount of callus (mean of 2.3) and that was placed in separate group. Treatments of NAA (0.25 mg/lit) and IBA (0.5 mg/lit) were in one group and IBA treatment with 0.5 and 0.25 mg/lit were in another group. Control with least amount of callus was placed in separated group. Results have shown that concentration increasing of hormones from 0.25 to 0.5 mg/lit leaded increment in mentioned trait. Rooting hormone treatment in lowest and highest amount resulted 10 and 23 times increasing, respectively. Mean comparison of various treatments effect on root number have shown that NAA treatment (0.25 mg/lit) with average of 8.05 had significant difference with control and 0.5 mg/lit of IBA (Table 4).

Mean comparison of shoot diameter have defined that IBA treatment (0.5 mg/lit) with average of 1.61 cm had the most mean. IBA treatment with 0.25 mg/lit concentration had significant difference compared to other treatments and that was placed in separated group. Control treatment with average of 0.33 cm had the lowest amount for this trait (Table 4).

Mean comparison of rooting trait have shown that there was significant difference between hormone treatments and control. IBA treatment with 0.5 mg/lit concentration had the most rooting percentage with average of 2.76 (Table 4).

Percentage Calculation of Riboside A, Riboside C and Stevioside:

In treated leaf: Addition of IBA hormone with different concentrations (0.25 and 0.5 mg/lit) in leaf leaded to Riboside A reduction about 0 concentration. NAA addition was resulted its reduction in leaf (Table 5).

Apply of IBA hormone (0.25 mg/lit) leaded reduction of Riboside C concentration to 0. Reduction of this substrate was shown by two IBA concentrations. 0.25 and 0.5 mg/lit of NAA hormone have resulted Riboside C incensement and reduction in leaf, respectively (Table 5).

IBA hormone has caused Stevioside concentration increasing. Addition of different concentrations of IBA and NAA hormones has leaded Stevioside incensement in the leaf (Table 5).

In treated stems: IBA hormone addition with 0.25 and 0.5 mg/lit concentrations in shoot has caused Riboside A reduction to 0 concentration. Therefore, IBA increasing has resulted reduction of Riboside A. 0.25 and 0.5 mg/lit of NAA have leaded to Riboside A increasing and reduction in shoot, respectively (Table 6).

Applying of IBA in shoot with 0.25 and 0.5 mg/lit concentrations have caused Riboside C concentration to 20.19% and 2.13%, respectively.

Table 3 Analysis of variance on different *Stevia rebaudiana* (Bertoni) Bertoni traits under rooting treatments

Sources of variation	Freedom degree	Node number	Shoot length	Leaf length	Root Length	Root volume	callus	Root number	Rooting percentage	Shoot diameter
Treatment	4	2.773 **	10.671 **	1.446 **	0.3473 **	0.935 **	2.58 **	2.838 **	0.9148 **	1.299 **
Error	15	0.2226	0.1236	0.0583	0.0371	0.116	0.05	0.498	0.1151	0.005
Coefficient of Variation (CV%)		6.48	2.92	10.06	7.07	13.62	17.88	9.97	13.89	6.85

Significant at 1% level **

Table 4 Comparison of average on different *Stevia rebaudiana* (Bertoni) Bertoni traits under rooting treatments

Treatment	Node Number	Shoot length	Leaf Length	Root Length	Root volume	Callus	Root number	Rooting percentage	Shoot diameter
Control	6.85 b	10.48 c	1.38 b	2.28 b	1.7 b	0.1 d	6.25 b	1.6 b	0.33 d
0.25 IBA	6.7 b	14.65 a	2.83 a	2.7 a	2.6 a	1 c	7.05 ab	2.7 a	0.54 c
0. 5 IBA	8.7 a	11.78 b	2.45 a	2.78 a	2.45 a	1.3 bc	6.25 b	2.76 a	1.61 a
0.25 NAA	6.8 b	10.9 c	2.85 a	3.1 a	2.85 a	1.55 b	8.05 a	2.6 a	1.42 b
0. 5 NAA	7.35 b	12.25 b	2.5 a	2.76a	2.9 a	2.3 a	7.8 ab	2.55 a	1.3 b

Table 5 The percentage of Riboside A, C and Stevioside in treated leaf with NAA and IBA hormones

Treatment	Concentration	Riboside A			Riboside C			Stevioside		
		Retention time	Area	Percentage	Retention time	Area	Percentage	Retention time	Area	Percentage
Standard		11.037	5902.308	0	9.14	1039.729	**0**	8.17	8243.793	0
Control	0	10.87	144.812	2.453	9.08	83.466	7.867	0	0	0
IBA	0.25	0	0	0	0	0	0	8.24	78	1
IBA	0.5	0	0	0	8.9	43.793	4.128	8.49	80.569	0.811
NAA	0.25	0	0	0	8.71	171.795	16.193	8.34	71.963	0.725
NAA	0.5	10.82	11.407	0.193	9.36	18.552	1.749	8.45	179.953	1.812

Table 6 The percentage of Riboside A, C and Stevioside in treated stems with NAA and IBA hormones

Treatment	Concentration	Riboside A			Riboside C			Stevioside		
		Retention time	Area	Percentage	Retention time	Area	Percentage	Retention time	Area	Percentage
Standard		11.037	5902.308	0	9.14	1039.729	0	8.17	8243.793	0
Control	0	10.49	11.501	1.195	8.89	22.451	2.116	8.14	52.286	0.526
IBA	0.25	0	0	0	8.84	214.274	20.197	8.49	87.654	0.883
IBA	0.5	0	0	0	9.5	22.625	2.133	8.25	14.015	0.141
NAA	0.25	11.49	58.796	0.996	9.5	3.583	0.338	8.12	95.041	0.957
NAA	0.5	11.07	8.162	0.138	9.59	30.175	2.844	8.45	22.905	0.231

Considering controlling sample, IBA hormone addition in different concentrations has leaded to Riboside C increment (Table 6). 0.25 and 0.5 mg/lit of NAA treatment have caused Riboside C reduction and increment in shoot, respectively.

IBA treatment with 0.25 mg/lit concentration has resulted to Stevioside increment and 0.5 mg/lit of that reduced Stevioside (Table 6). 0.25 and 0.5 mg/lit of NAA have caused Stevioside increment and reduction in shoot, respectively.

Discussion

Stevioside was the first compound which was identified and applied as a sweetener in food products. The stevia glycosides are the compounds responsible for the sweet taste. The leaves of stevia are the source of steviol glycosides such as stevioside and rebaudioside, which have 250–300 times the sweetness of sugar [14].

In vitro culture method is only technique to obtain quality growth of plant sample [15]. Das *et al*. [3] indicated that plant hormone is necessary for all growth stages in stevia. Also, results suggested that MS medium with kin for proliferation but MS media with auxins had an adverse effect on rooting. Results of this research showed that the highest rooting was related to IBA hormone in 0.5 mg/lit concentration. Ferreira and Handro [16] have declared that lack of significant traits related to rooting in this plant was because of easy rhizogenic. Many researchers have proved that auxin facilitated rooting in plants with auxin cofactors (but they have low auxin). Auxin has no significant effect on plants rooting with high auxin and auxin cofactor. Results of this study have shown that rooting cofactors or auxin in Stevia plant were high and there was significant difference between treatments. Jamshidi Zinab *et al*. [2] have declared that different concentrations of IAA growth regulators effect on proliferation, number and shoot length. While, rooting study in 3 concentrations of IAA have shown that root numbers, root length and rooting percentage have not significant difference. As NAA hormone treatment (0.25 mg/lit) have significant effect on root numbers and length, that can be concluded this hormone treatment have more impact compared to IAA treatment. Razak *et al*. [17] observed highest shoot formation and rooting on a MS medium with 0.5 mg/L BAP + 0.25 mg/L Kin and MS medium containing 1.0 mg/L IBA, respectively.

Administration of different phytohormone enhances and accelerates the production of in vitro plants with good agronomical traits and steviosides content in leaves [18]. Studied indicated that the leaves contain maximum levels of stevizide than other parts [19, 20] and they have the highest type and levels of secondary metabolites [21]. Singh and Dwivedi [22] reported significant increase in the content of stevizide in tissue culture plants compared to natural conditions and it was claimed that the production of stevizide depends on the tissue and age of the plant.

Tamura *et al*. [23] showed the function of the sweetening compounds in the leaf tissue of the stevia plant can vary according to the propagation method but other study indicated that stevizide content isn't depend to type of propagation. Reports showed plant growth regulators can be affected shoot proliferation and root induction of Stevia rebaudiana. The maximum content of stevioside (8.18%) at half MS medium with 0.2 mg/L IBA and 2 mg/L active carbon [14]. According to the HPLC results of this study, increasing of rooting hormones concentrations (NAA and IBA) leaded to leaf Stevioside increasing. Komatsu *et al*. [24] have declared lower amount of Stevioside in MS medium compared to 1/2 MS. Salts in MS medium may cause reduction of Stevioside leaf. Karim *et al*. [25] obtained 9.6% stevioside in the leaf from tissue culture and suggested that the method can be used for commercial production.

Unfavorable relationship between medium ability and active ingredients accumulation could be actually described that full nutrient media mostly elevate initial cellular growth and metabolism and in some cases prevent differentiation of morphological and biochemical tissue in invitro of stevia [26]. The research about some stevia metabolites in invivo and invitro conditions showed that total 55 and 70 chemical compounds were obtained in the extract of tissue culture and natural condition, respectively which among them 39 compounds in tissue culture and 54 ones in natural condition was exclusive. Also, the results identified that there were eight similar compounds of essential oil among two conditions and 41 and 164 compounds in the natural and tissue culture, respectively. Among them 33 and 156 compounds in invitro and invivowere unique [27].

HossiniHashemzadeh and Mahbali Pour [1] analyzed Stevia extract by HPLC and they reported

that methyljasmonate and salicylic acid treatments have significant effect on Stevioside content. Tahmasi *et al*. [28] indicated that maximum levels of rebaudiosideA and stevioside in invitro culture were obtained 48h with 60 mg/L and 96h with 90 mg/L salicylic acid usage, respectively. Rafiq *et al*. [29] reported that there was no significant difference between the approximate composition of carbohydrate and protein content in the leaves extract from natural and tissue culture conditions.

Conclusion

Treated plants had significant difference compared to control plants due to hormone effects on Stevia regeneration. Among rooting treatments, NAA hormone with 0.25 mg/lit concentration had most root length and number. There was no significant difference for rooting trait between different treatments but they had high difference with control sample. Also, Kin treatment (0.25 mg/lit) among shoot hormones treatments had the highest shoot length. The most leaf length in shoot hormone treatment was BAP with 0.25 mg/lit. IBA and NAA (0.25 mg/lit) had most average among root hormone treatments. As a result, leaf length was reduced with rooting and shooting hormone treatments. There was significant difference between rooting and shooting treatments for callus induction trait. Callus induction was increased in two mentioned treatments. As hormone concentration increasing has leaded to 10 times increment (in most cases 23 times increasing). According to results of HPLC diagrams, IBA and NAA increment have leaded to Stevioside increasing in leaf but that did not follow any rules in other cases.

References

1. Hossini Hashemzadeh H, Mahbali Pour N. The effect of methyl jasmonate and salicylic acid on the content of Stevioside in *Stevia rebaudiana* Bertoni under in vitro conditions. 13th Iran. Crop Sci Con and 3rd Iran. Seed Sci and Tech. Con. 2014.
2. Jamshidi ZA, Ghanbari AR, Sharafi Y. Bina F. Micropropagation of *Stevia rebaudiana* Bertoni via *in vitro*. 3rd Iran Agri Biot Con. 2012.
3. Das A, Gantait S, Mandal N. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. Int J Agric Res. 2011;6:40-48.
4. Vyas SP, Dixit VK. Pharmaceutical biotechnology. CBS. 2010.
5. 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. Am. Eurasian J Sci Res. 2007;2:121-125.
6. Moktaduzzaman M, Rahman SMM. Regeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. Biotech. 2009;8:449-455.
7. 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.
8. Alhady MRAA. Micropropagation of *Stevia rebaudiana* Bertoni: A new sweetening crop in Egypt. Glob. J Biotechnol Biochem. 2011;6:178-182.
9. Singh N, Yadav K, Kumari S. Metabolic changes during differentiation in callus cultures of *Stevia rebaudiana* (Bertoni). J Phytol. 2011;3:63-67.
10. Hassanen SA, AKhalili RM. Biotechnological studies for improving of stevia (*Stevia rebaudiana* Bertoni) In vitro plantlets. Middle-East J Sci Res. 2013;14:93-106.
11. Jitendra M, Monika S, Ratan SD, Priyank G, Priyank S, Jayraykiran D. Micropropagation of an antidiabetic plant- *Stevia rebaudiana* of south-east rajasthan, india, ISCA J biolo sci. 2012;1:37-42.
12. Zayova E, Stancheve I, Mgeneva M, Petrova M, Dimitrova L. Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins, Turk Boil. 2013;37:106-113.
13. Verma S, Kuldeep Y, Singh N, Optimization of the protocols for surface sterilization, regeneration and acclimatizeation of *Stevia rebaudiana* Bertoni, American-Eurasian. J Agric Environ Sci. 2011;11:222-227.
14. Mehrafarin A, Etminan AR, Delkhosh B, Golrokhan M. Assessment of Stevioside Content, Shoot Proliferation, and Root Induction of *Stevia rebaudiana* Bertoni under In vitro Conditions. J Med Plants. 2016;15:98-110.
15. Singh M, Saharan V, Dayma J, Rajpurohit D, Sen Y, Sharma A. In vitro Propagation of *Stevia rebaudiana* (Bertoni): An Overview. Int J Curr Microbiol App Sci. 2017;6:1010-1022.
16. Ferreira CM, Handro W. Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. Plant Cell Rep. 1988;7:123-126.
17. Razak UNAA, Ong CB, Yu TS, Lau LK. In vitro Micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. Braz Arch Biol Technol. 2014;57:23-28.
18. Jain P, Kachhwaha S, Kothari SL. Biotehcnology and metabolic engineering of *Stevia rebaudiana* (Bert.) Bertoni: Perspective and Possibilities. Int J Life Sc Bt Pharm. Res. 2014;3:1-23.
19. Ladygin VG, Bondarev NI, Semenova GA, Smolov AA, Reshetnyak OV, Nosov AM. Chloroplast ultrastructure, photosynthetic apparatus activities and

production of steviol glycosides in *Stevia rebaudiana* in vivo and in vitro. Biol Plant. 2008;52:9-16.

20. Woelwer-Rieck U, Lankes C, Wawrzun A, Wu¨st M. Improved HPLC method for the evaluation of the major Steviol glycosides in leaves of *Stevia rebaudiana*. Eurasian J Food Res Technol. 2010; 231: 581–588.
21. Kumari M, Chandra S. Phytochemical studies and estimation of major steviol glycosides in varied parts of *Stevia rebaudiana*. Int J Pharm Pharm Sci. 2015;7:62-65.
22. Singh P, Dwivedi P. Two-stage culture procedure using thidiazuron for efficient micropropagation of *Stevia rebaudiana*, an antidiabetic medicinal herb. 3 Biotech. 2014;4:431-437.
23. Tamura Y, Nakamura S, Fukui H, Tabata M. Comparison of Stevia plants grown from seeds, cuttings and stem tip cultures for growth and sweet diterpene glycosides. Plant Cell Rep. 1984;3:180-182.
24. Komatsu K, Nozaki W, Takemura M. Nakaminami M. Production of natural sweetener. ChemAbstr. 1976; 84, 169.
25. Karim MdZ, Uesugi D, Nakayama N, Hossain MM, Ishihara K, Hamada H. Identification of Stevioside Using Tissue Culture-Derived Stevia (*Stevia rebaudiana*) Leaves. Biochem. 2015;8:33-37.
26. Fadel D, Kintzios S, Economou AE, Moschopoulou G, Constantinidou HIA. Effect of different strength of medium on organogenesis, phenolic accumulation and antioxidant activity of spearmint (*Mentha spicata* L.). The Open Hort. J. 2010;3:5- 31.
27. Malekirad M, Nasr N, Kazemitabar SK. Study on some Stevia (*Stevia rebaudiana* Bertoni) metabolites in the invitro and invivo conditions using GC- MS. J Med Plants Biotechnol. 2016;2:41- 54.
28. Tahmasi S, GaroosiGh A, Ahmadi J, Farjaminezhad R. Effect of salicylic acid on stevioside and rebaudioside a production and transcription of biosynthetic genes in in vitro culture of *Stevia rebaudiana*. Iran J Genet Plant Breed. 2017;6:1-8.
29. Rafiq M, Muhamamd MU, Mangrio S, Naqvi HA, Qarshi IA. In vitro clonal propagation and biochemical analysis of filed established *Stevia rebaudiana* Bertoni Pak J Bot. 2007;39:2467-2474.