J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 8 (12): 295 – 301, 2017

Stevia Rebaudiana Bertoni; Rapid Micropropagation, Stevioside Accumulation and Genetic Fidelity using ISSR Markers

Rania M. A. Aziz[1] and Kh. A. M. Khaled[2]
[1]Sugar crop Res. Inst., Agric. Res. Center, Giza, Egypt
[2]Genet. Dep. Fac. of Agric., Beni-Suef Univ., Egypt
https://orcid.org/0000-0002-3718-9148

ABSTRACT

Different regions in the world using *Stevia* rebaudiana Bertoni as sweetener crop. It is a perennial semi-shrub up to 40 cm in height. *Stevia* have versatile medicinal uses without any side effects. It could propagated by seed or by stem cutting. Although seed propagation is very common method, seed is not efficient because of poor germination, low fertility and incompatibility of the flowers. Destruction of the donor plant and decreased new plant number made stem cutting method is limited. In our study, *Stevia in vitro* propagated using nodal segments on Murashige and Skoog (MS) medium and various growth regulator concentrations [6-benzyladenine (BA), and kinetin (Kin)] The best and highest response of regenerants was in the medium contain (2.0 mg/l BA+ 0.5 mg/l Kin) which recorded (87.43), (9.07) and (4.61) for the percentage of explants, maximum total shoots number, the highest average of shoot length, respectively. Maximum average of root/shoot length and the highest percentage of root/shoot recorded in medium supplemented with 1.0 mg/l IAA (12.93, 6.89 and 98 %). The difference ratio among T1 & T2 in Constituents (%) of moisture, Fat, Ash, Crude fiber, St and Reb-A were a slight ratio thus both treated media are likely favorable in our work. Obviously, the obtained data about moisture, protein and total carbohydrate were the noticeable difference increased in T2 more than T1, these observations interpreted as evidence for the combination high ratio of protein and stevioside sweeteners might be due to the favorable media to achieve most growing performance, which reflect on dietary values. Acclimatization succeeded with a 95% survival rate of the *ex-vitro* plantlets at the hardening phase. Genetic stability of propagated plantlets compared with compared with mother plants was analyze using Inter simple sequence repeat (ISSR) markers. The present study provides report on the genetic fidelity of micropropagation for *Stevia* rebaudiana obtained from axillaries bud explants using ISSR analysis. The tissue cultured plantlets and the mother plant were identically with no variability detected.

Keywords: *Stevia rebaudiana*; Micropropagation; Stevioside; Sweetener; genetic fidelity; ISSR markers.

INTRODUCTION

The gap between sugar production and consumption in Egypt is a serious problem where an estimated 0.933 million tons. To reduce the gap between the production and consumption, the focus is on *Stevia*'s use in the food industry. *Stevia* rebaudiana (Family *Asteraceae*) is one of the 154 members of genus *Stevia* and is cultivated for its sweetening compounds. Dried *Stevia* leaves are characterized by protein content ranged between 10-20.4g/100g (Mohammad *et al*. 2007), lipid content/dry mass, ranging from 1.9 to 5.6 g/100 g of the product (Bernal *et al*., 2011), ash content ranged 6.3 to 13.1 g/100 g (Kim *et al*. 2011). *Stevia* leaves dealing with diabetic patients without any adverse effects (Summon *et al*. 2008).

Eight glycosidic diterpenes identified in *Stevia* leaves, which have sweetening properties (Tateo *et al*., 1999; Singh and Rao 2005; Abou-Arab *et al*. 2010). Stevioside (5–10% d.w.) and rebaudioside A (2–4% d.w.) are the most desired components (Dacome *et al*., 2005; Yadav *et al*., 2011), Stevioside and rebaudioside stability under wide range conditions (temperatures and pH) were estimated in different food and pharmaceutical products (Tanaka, 1988; Abou-Arab *et al.,* 2010). They do not alter the flavor and taste of a food product and are non-fermentative.

Stevia plant can propagate by seed or by stem cutting. Seed is not efficient because of low fertility, poor seed germination (Miyazaki and Wantabe 1974, Mitra and Pal 2007), self-incompatibility of the flowers (Tadhani *et al*. 2006) and Stevioside level and composition varied greatly with seed planting (Nakamura and Tamura 1985). Destruction of the donor plant and decreased new plant number made stem cutting method is limited. However, pathogen accumulation slows down the vegetative propagation due to decrease the number of individuals that can obtained (Mishra *et al*., 2010). Therefore, tissue culture is the possible way to propagate Stevia plants without obstacles.

Plant tissue culture one of the most tool used for conserving and regenerating genetic resources; used for mass propagation of desired genotype for commercial production.

There are many reports explained the micropropagation of *Stevia* rebaudiana, but mass propagation need to develop new improved and efficient protocol for producing chemically characterized plantlets (Das *et al*. 2011).

Micropropagation the best method to solve those obstacles and produce large quantity of *Stevia* plantlet (mass production) in short time and producing plant to true type, free virus. Many tissues could use for *in vitro* clonal propagation like leaf (Das *et al*. 2006), shoot tip (Anbazhagan *et al*. 2010; Das *et al*. 2011) and nodal segment (Uddin *et al*. 2006 and Ahmed *et al*. 2007).

Genetic stability assessment of derived clones *in vitro* is very important step for true to type clones micropropagation (Diaz *et al.,* 2003). Molecular techniques, particularly ISSR have been proposed to be appropriate powerful tools for identification of somaclonal variation and establish genetic stability (Rahman and Rajora, 2001; Bennic *et al*., 2004; Javanmardi *et al*., 2011).

The aim of this work was to set up a micropropagation protocol and confirm the genetic fidelity of plants raised *in vitro* by ISSR technique.

MATERIALS AND METHODS

1. Plant Material, tissue culture conditions and micropropagation

Plant Material

The explants from node segments that used for multiplication collected from six months old plants of *Stevia rebaudiana* Bertoni. Experiment carried out in the greenhouse and Tissue culture lab, Sugarcane Research

Institute (SCRI), Agriculture Research Center (ARC), Giza, Egypt.

Small pieces of explants (0.5-1.0 cm long) cut and treated with of savlon for 4 min. with shaking, then washed by tap water for 20 minutes. It was sterilized superficially using 0.1% HgCl2 for 6 min and rinsed five times with double distilled water under aseptic conditions in laminar airflow cabinet. Explants inoculated aseptically on culture medium.

Tissue culture conditions and micropropagation Conditions

Segments implanted vertically on MS medium (pH adjusted to 5.8 before autoclaving at 1.06 kg cm² and 121ºC for 20 min) contains 3.0% sucrose and solidified with 1.0% agar, then incubated at temperature of 24±2°C with 16h and 2500 lux white fluorescent light photoperiod

Micropropagation

0.5- 1.0 cm segments (with a single auxiliary bud) implanted vertically on MS medium contains 3.0% sucrose and growth regulators with different concentrations as presented in Table1.

Shoot initiation and multiplication

Segments inoculated with variation in BA and Kin concentration (0.5, 1.0, 1.5, and 2.0 mg/L of BA and/or Kin 0.1, 0.2, 0.5 and 1.0 separately) on MS medium. In another way MS medium supplemented with the combined form of BA (1.5 and 2.0mg/l) and kin (0.5 and 1.0 mg/l) and BA (1.5 and 2.0 mg/l) and IBA (0.2 and 0.5mg/l) (Table1). Segments sub cultured every 21-30 days and segments from the proliferated shoots sub cultured again for additional multiple shoot induction. After five-sub culture, plantlets prepared for root initiation.

Root formation and acclimatization

Excised micro multiple shoots (3.0-3.5 cm size) were cut and placed in MS medium half –strengthen and contains 2.0% sucrose with NAA, IBA and IAA (0.2, 0.5, 1.0 and 2.0 mg/l of each auxin). Control was the MS medium without growth regulators and after three weeks of culture data recorded.

Agar traces removed from plantlets by washing with water carefully and transplanted in a pot (5 cm) filled of sterilized soil mixture (sand and peat moss at 1:1:1 ratio) treated with 0. 1% Benlit (Fungicide), then kept under shade for 20 -25 days and placed in acclimatization room in the greenhouse for hardening at 27±2°C and 70-90% of humidity for two weeks. Five days later temperature increased from 32 to 36°C. After two weeks from hardening, transparent bags removed and low light intensity applied in greenhouse. Data recorded for s growth parameters like percentage of regenerated explant, shoots number/explants, shoot length percentage of cutting rooted, number of roots/plantlet, and root length/shoot, days to emergence of roots, and callus formation at the cutting base.

Statistical analysis

Completely randomized design was used and analysis of variance achieved using SPSS statistical program. Duncan's multiple range test used to compare differences between means at 5% level of significance.

2- Chemical analysis

The Stevia leaves were open air dried at least 12 hours per day till reach require content 6%, then extracted and purified according to Hassan *et al* (2002).

Stevioside stander preparation carried out as described by Nishiyama *et al* (1992). Moisture, protein, ash content, fat, crude and fiber determined as described in A.O.A.C. (2000).

Total soluble carbohydrates calculated according to Nishiyama *et al* (1991) by the following equation:

Total soluble carbohydrates = 7.66+ 0.96 Stevioside content

Table 1. Growth regulators concentrations among the different stage

Growth regulator	Growth stage		
	Shoot initiation	Shoot multiplication	Root formation
BA	0.5 mg 1.0 1.5 2.0		
Kin	0.1 0.2 0.5 1.0		
BA + Kin		1.5 + 0.5	
BA + Kin		1.5 + 1.0	
BA + Kin		2.0 + 0.5	
BA + Kin		2.0 + 1.0	
BA + IBA		1.5 + 0.2	
BA + IBA		2.0 + 0.5	
IBA			0.2 0.5 1.0 2.0
IAA			0.2 0.5 1.0 2.0
NAA			0.2 0.5 1.0 2.0

2. Molecular Studies and Genetic Fidelity:

Genomic DNA extraction

Stevia leaves (young) were used to extract DNA, two sample groups were collected; the group control (untreated plants) and the treated group (treated with growth retardant). DNA extracted using the CTAB-method modified and described by Khaled and Esh (2008). DNA quality was determined visually on 0.8 % agarose gel. DNA concentration quantified and adjusted to 50 ng/µl.

ISSR-PCR amplification conditions PCR reaction:

Fifteen sets of ISSR primers synthesized by HVD Corporation, Germany used for the PCR reaction (Table 2). PCR Amplification performed for 42 cycles using MJ 200CT according to condition described in Khaled *et al.* 2015 as follow:

4 min at 94 °C for Denaturation, 40 cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 10 min, then hold at 4°C. The amplification products were separated by in 1.4% Agarose with 1× TBE and stained by ethidium bromide

Data analysis

The patterns of ISSR primers analyzed to determine the genetic variances between different Stevia rebaudiana genotypes. The fragments scored as present (1) or absent (0) and Dice similarity determined as described by Sneath and Sokal (1973)

Table 2. Primers name and sequences with annealing temperatures used in this study.

No.	Primer Name	Sequence	Annealing temp
1	UBC807	AGAGAGAGAGAGAGAGT	55
2	UBC 808	AGAGAGAGAGAGAGAGC	55
3	UBC811	GAGAGAGAGAGAGAGAC	55
4	UBC 817	CACACACACACACACAA	55
5	UBC 825	ACACACACACACACACT	55
6	844A	CTCTCTCTCTCTCTCTAC	52
7	844B	CTCTCTCTCTCTCTCTGC	52
8	17898 A	CACACACACACAAC	48
9	17898 B	CACACACACACAGT	48
10	HB 10	GAGAGAGAGAGACC	55
11	HB 12	CACCACCACGC	54
12	HB 13	GAGGAGGAGGC	54
13	TE	GTGGTGGTGGTGAC	52
14	BEC	CACACACACACACATC	55
15	HAD	CTCCTCCTCCTCAC	52

RESULTS AND DISCUSSION

1-Shoots multiplication

Explants incubated on MS medium contains BA (0.5, 1.0, 1.5, 2 mg/l) and/or kin (0.1, 0.2, 0.5 and 1.0 mg/l); and BA with IBA (0.2 and 0.5 mg/l). Shoots multiplication emerged after four weeks directly from cultured explants auxiliary nodes. Regenerants quality and quantity have best and highest response with MS medium contains (2.0 mg/BA + 0.5 mg/l Kin). The highest values recorded for the percentage of explants (87.43), number of total shoots (9.07) and highest average length of shoot (4.61), followed by the treatment (1.5 mg/BA + 1.0 mg/l Kin) which is recorded (75.53,5.53 and 3.58) at the same trait, respectively (Table 3). In the other hand, mineral nutrients are being as the basic component of culture media play a vital role in rapid growth of tissue, the extent and the quality of morphogenesis of tissue. Similar type of results has been got early works in the same species i.e. *Stevia rebaudiana* (Handro and Ferreira, 1989; and Ahmed *et.al*., 2007).

Table 3. Growth regulators Effect

Growth regulators mg/l	Shoot multiplication % per explants	Shoot/ culture	Average length of shoots
Control	24.12^{M}	2.020^{L}	1.20^{I}
BA 0.5	37.43^{K}	2.493^{IJ}	2.87^{D}
BA 1.0	48.87^{HI}	3.577^{G}	2.61^{EFG}
BA 1.5	52.10^{H}	3.915^{F}	2.31^{FG}
BA 2.0	68.97^{D}	6.403^{B}	3.43^{BC}
Kin 0.1	41.20^{J}	2.623^{I}	2.59^{EFG}
Kin 0.2	55.43^{G}	3.219^{H}	2.48^{EFG}
Kin 0.5	72.10^{CD}	3.966^{F}	2.28^{FG}
Kin 1.0	63.80^{E}	4.922^{D}	2.96^{C}
BA 1.5 + Kin 0.5	68.87^{D}	5.059^{D}	2.69^{E}
BA 1.5 + Kin 1.0	75.53^{B}	5.845^{C}	3.58^{B}
BA 2.0 + Kin 0.5	87.43^{A}	9.421^{A}	4.61^{A}
BA 2.0 + Kin 1.0	60.60^{F}	4.426^{E}	2.68^{E}
BA 1.5 + IBA 0.2	27.10^{L}	2.24^{JK}	1.34^{H}
BA 2.0 + IBA 0.5	50.10^{HIJ}	3.454^{GH}	2.17^{G}
Mean	55.58	3.919	2.65

Proliferation of multiple shoots of *Stevia rebaudiana* was induced in the presence of used hormones. On the other hands, Kin has less effect on inducing multiple shoots than BA.

This result is in consistent with finding of Ummi *et al*., (2014) and Soliman *et al* (2014). They mentioned that increasing in BA up to 2 mgl−1 (individually or in combination with Kin) led to increase shoot multiplication rate, while shoot multiplication rate began to decline when BA increased above 2 mgl−1. Silva *et al*. (2009) found that Kin alone had no effect on *in vitro* multiplication of *Vriesea scalaris*. On other hands, Sharuti *et al*. (2011) mentioned that Kin has more effect as multiple shoot inducer when combined with BA.

2-Root induction

For root initiation, multiplication shoots cultured on MS medium half strengthen and contains IAA, IBA and NAA individually (0.2, 0.5, 1.0 and 2 mg/l). As presented in Table 4, IAA, NAA and IBA had positive effect on root induction; it had a positive effect on roots/shoot numbers and average length of root/shoot Steven *et al*., (1992). The maximum No. of root formation/shoot, root/shoot length and the highest root/shoot percentage recorded in medium contains 1 mg/l IAA (12.93, 6.89 and 98%), followed by the medium supplemented with 1 mg/l IBA which recorded 10.77, 5.85 and 85%, respectively at the same trait. Soliman *et al.* 2014 found similar type of results among three Auxins tested; they found that IAA was superior for rooting in comparison to IBA.

According to callus formation from the cut portion of the shoot, it was noticed callus initiation at only the concentration of 1 mg/l (IAA), 0.1, 0.2, 0.3 mg/l (NAA) and 0.2, 0.5 and 1 mg/l (IBA). Figure 1 showed the stages of shoots and root multiplications.

The acclimatization process was successful and 95% of the plants survived when put under temperature 36°C and exposed to shade and bright light conditions.

Chemical analysis of *Stevia* leaves from the most optimum media treatments

Analysis of *Stevie* leaf showed that it contains protein, carbohydrates and crude fibers (Table 4). Similar nutritional composition found to consider as risk reducer and wellness promotor for some diseases, these reports mentioned by de Oliveira *et al.* (2011).

Table 4. IAA, IBA and NAA concentration and their effect on root formation

Concentrations of auxins (mg/l)	roots/ shoot no.	root/ shoot length	root/ shoot %	Days of growing roots
Control	4.80^{L}	2.11^{HIJ}	56^{J}	6-14
IAA 0.2	7.31^{FGH}	4.80^{D}	73^{D}	6-14
IAA 0.5	8.2^{DE}	5.60^{B}	89^{B}	7-14
IAA 1.0	12.93^{A}	6.89^{A}	98^{A}	8-12
IAA 2.0	6.11^{J}	5.21^{C}	70^{E}	7-15
IBA 0.2	7.01^{G}	3.11^{FG}	69^{EF}	8-13
IBA 0.5	7.85^{E}	3.62^{E}	84^{C}	8-13
IBA 1.0	10.77^{B}	5.85^{BC}	85^{C}	8-12
IBA 2.0	6.48^{H}	2.79^{G}	65^{G}	8-12
NAA 0.2	5.94^{K}	2.31^{HI}	58^{I}	8-14
NAA 0.5	6.22^{IJ}	3.19^{F}	62^{H}	8-12
NAA 1.0	8.51^{C}	4.76^{D}	67^{F}	8-12
NAA 2.0	6.29^{IJ}	2.47^{H}	46^{K}	7-14
Mean	7.57	4.05	70.92	

Data analysis in Table 5 show that the difference ratio among T1 & T2 in Constituents (%) of moisture, fat, Ash, Crude fiber, St and Reb- A were a slight ratio thus both treated media are likely favorable in our work. Obviously, the obtained data about moisture, protein and total carbohydrate were the noticeable difference increased in T2 more than T1, these observations interpreted as evidence for the combination high ratio of protein and stevioside sweeteners might be due to the favorable media to achieve most growing performance, which reflect on dietary values. Our data agree with data obtained by Mishra *et al*. (2010) who found an increased in water retention capacity of *Stevia* leaf, that could be beneficial and due to high protein content. Moreover, increasing results associated by highest amount ratio in two majors of stevioside sweeteners (St and Reb- A) these results are in line with those (Dacome *et al*., 2005; Yadav *et al*., 2011). As general, *in vitro* propagation could be a vital protocol for commercial propagation and product with high nutritional values.

Table 5. Chemical analysis of *Stevia* leaves from the optimum media treatments (on dry weight basis).

Constituents (%)	T(1)	T(2)
Moisture	6.60^{B}	7.30^{A}
Protein	11.50^{B}	12.55^{A}
Fat	2.56^{B}	2.90^{AB}
Ash	10.80^{ABC}	10.20^{B}
Crude fiber	15.50^{B}	17.70^{A}
Total carbohydrate	70.54^{B}	73.05^{A}
Total Soluble carbohydrate	8.047^{A}	8.098^{A}
St	0.403^{BC}	0.457^{AB}
Reb- A	0.120^{BCD}	0.134^{A}

Figure 1. Micropropagation of *Stevia* (*Stevia* rabaudiana) stages.

3-Molecular Studies and Genetic Fidelity

Fingerprinting using ISSR profiles of culture regenerates and donor plants did to confirm the stability and genetic fidelity of propagated plantlets (Table 6 and Fig. 2a and b).

DNA was isolated and PCR-based ISSR technique used to test both propagated and mother plant for genetic fidelity. Out of 15-screened ISSR primers, only five primers produced clear and distinct bands.

A total of 29 fragments were visualized across the mother and regenerated plants ranged from 455 to 1767 bp, with 5.8 bands/primer. The total numbers of bands, which were developed by these five primers, i.e. UBC807, UBC811, BEC, HAD and TE were 7, 6, 8, 4 and 4 respectively.

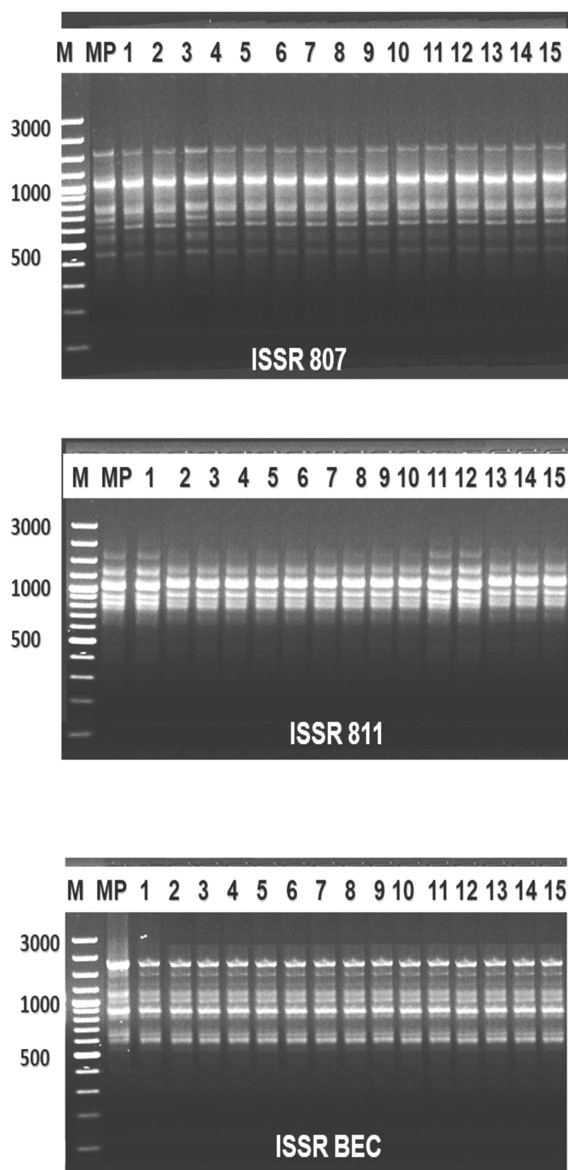

Figure 2a. Electrophoresis separation patterns of ISSR-PCR products (as revealed on 0.8% agarose gel) using primers 807, 811 and BEC. Lane M: 1Kb plus DNA ladder marker. MP: mother plant and Lane's 1 to 15 represented *Stevia* rebaudiana genotype treated medium with growth regulators, respectively.

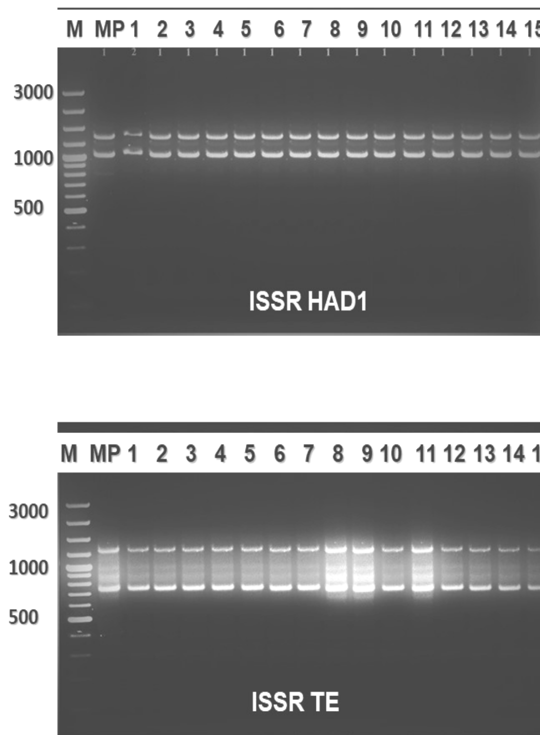

Figure 2b. Electrophoresis separation patterns of ISSR-PCR products (as revealed on 0.8% agarose gel) using primers HAD1 and TE. Lane M: 1Kb plus DNA ladder marker. MP: mother plant and Lane's 1 to 15 represented *Stevia* rebaudiana genotype treated medium with growth regulators, respectively.

Table 6. Primers and amplification products of ISSR-PCR used for the checking identity of propagated plants of *Stevia* rebaudiana.

Primer Name	Sequence	scorable loci per primer	Size range (bp)
807	$(AG)_8$ T	8	436-1530
811	$(GA)_8$ C	8	436-1530
TE	GT $(GGT)_3$ GAC	5	309-1288
BEC	$(CA)_7$ TC	9	609-1795
HAD	CT $(CCT)_3$ CAC	5	822-1530
Total		33	
Average		6.6	

All bands were monomorphic, the regenerated plants were identical, and like the mother plant, these results confirming that *in vitro*-raised plants have true-to-true type nature. Among the used primer, the highest number of bands (9 bands) produced by primer BEC while the lowest produced by primers TE and HAD (5 bands) (Table 6 and Fig. 2a and b).

Our results were in harmony with Hemant *et al*., (2013) who evaluate *in vitro* propagated *Stevia rebaudiana* Bert for genetic fidelity using ISSR and found that all the ISSR bands from propagated plants were monomorphic and identical to mother plants, confirming the genetic fidelity among them.

CONCLUSION

In conclusion, the present study provides report on the genetic fidelity of micropropagation [*Stevia* rebaudiana] obtained from axillaries bud explants using ISSR analysis. No variability was detected among the tissue cultured plantlets and the mother plant; hence we concluded that the protocol developed could be effectively used for rapid micropropagation can be produced without the risk of genetic instability and can be used for commercial production of chemically characterized *Stevia* using the protocol used in this study. The study is increasingly important given the increasing global demand for highly effective natural sweeteners is constantly increasing and commercial utilization of [*Stevia rebaudiana*] without much risk of genetic in stability.

REFERENCES

A.O.A.C (2000). Association of official Analytical Chemists official methods of analysis. 26 th Ed. A.O.A.C, Inter,Washington,D.C.,USA.

Abou-Arab A.E., Abou-Arab A. A., Abu-Salem M. F. (2010). Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia* rebaudiana Bertoni plant. African Journal of Food Science 4 (5): 269- 281

Ahmed MB, Salahin M, Karim R, Razvy MA, Hannan MM, Sultana R, Hossain M and Islam R(2007). Method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia* rebaudiana *bertoni*) in bangladesh. American-Eurasian Journal of Scientific Research 2: 121-125.

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

Bennici A., Anzidei M. and Vendramin G. 2004. Genetic stability and uniformity of *Foeniculum vulgare* Mill. Regenerated plants through organogenesis and somatic embryogenesis plant Sci. 166:221-227.

Bernal, J., Mendiola, J., Ibǚez, E., & Cifuentes, A. (2011). Advanced analysis of nutraceuticals. Journal of Pharmaceutical and Biomedical Analysis, 55, 758–774.Bharani, A., Ganguly, A., & Bhargava, K. (1995). Salutary effect of Terminalia arjuna in patients with severe refractory heart failure. International Journal of Cardiology, 49, 191–199.

de Oliveira, A. J. B., Gonçalves, R. A. C., Chierrito, T. P. C., dos Santos, M. M., de Souza, L. M., Gorin, P. A. J., ... & Iacomini, M. (2011). Structure and degree of polymerisation of fructooligosaccharides present in roots and leaves of Stevia rebaudiana (Bert.) Bertoni. Food Chemistry, 129(2), 305-311.

Dacome, A. S., da Silva, C. C., da Costa, C. E. M., Fontana, J. D., Adelmann, J., da Costa, S. C. (2005). Sweet diterpenic glycosides balance of a new cultivar of *Stevia*rebaudiana (Bert) Bertoni: isolation and quantitative distribution by chromatographic, spectroscopic and electrophoretic methods. Process Biochem. 44, 3587–3594.

Das, A. Gantait, S and Mandal, N. (2011) Micropropogation of an Elite Medicinal Plant (*Stevia* rebaudiana Bertoni) International Journal of Agriculture Research, Vol. 6, No. 1, , pp. 40-48.

Das, K., Dang, R., Rajasekharan, P.E. (2006) Establishment and maintenance of callus of *Stevia* rebaudiana Bertoni under aseptic environment. *Nat.Prod. Rad.*,5(5): 373-373.

Diaz S., Pire C. and Ferre J. 2003. Identification of *Phoenix dactylifera* L.varieties based on amplified fragment length polymorphism (AFLP) markers. Cell MolBiolLett 8:891-899.

Handro W, Ferreira CM. (1989) *Stevia* rebaudiana (Bert.) Bertoni: Production of natural sweeteners. In: Bajaj YPS, editor. Biotechnology in agriculture and forestry. Berlin: Springer-Verlag; 1989. p. 468-487.

Hassan, M. I, Zeitoun, A. M, Zeitoun, M. A. M and Moussa, M. M (2002). Effect of harvesting time drying extraction and purification methods on sweeteners from *Stevia* rebaudiana (Bertoni). J.Agric.Sci. Mansoura Univ, 27, 223-339.

Hemant Lata, Suman Chandra, Natascha Techen, Yan-Hong Wang, Ikhlas A. Khan (2013). Molecular Analysis of Genetic Fidelity in Micropropagated Plants of *Stevia* rebaudiana Bert. Using ISSR Marker *American Journal of Plant Sciences*, 4, 964-971.

Javanmardi, N., Bagheri, A., Moshtaghi, N., Sharifi, A., and Hemati Kakhki, A. (2011). Identification of Safflower as a fraud in commercial saffron using RAPD/SCAR. Journal of Cell and Molecular Research, 3(1):31-37.

Khaled A. M. K. and A. M. H. Esh (2008). High quality genomic DNA impurities-free from sugar crops and other plant tissue. Proc. Inter. Confer. (IS-2008) Al Arish, Egypt, September 11-14.

Kim, I., Yang, M., Lee, O., & Kang, S. (2011). The antioxidant activity and the bioactive compound content of *Stevia* rebaudiana water extracts. LWT – Food Science and Technology, 44, 1328–1332.

Mishra, P., Singh, R., Kumar, U., Prakash, V. (2010). *Stevia* rebaudiana – A magical sweetener. Global J. Biotechn. Biochem., 5, 62–74.

Mitra,A and Pal,A (2007) "*In vitro* Regeneration of *Stevia* rebaudiana (Bert.) from Nodal Explants," Journal of Plant Biochemistry and Biotechnology, Vol. 16, No. 1, , pp. 59-62. doi:10.1007/BF03321930

Miyazaki Y, Wantenabe H. (1974) Studies on the cultivation of *Stevia* rebaudiana Bertoni on the propagation of plant. *J Trop Agric.* 17: 154-157.

Mohammad, M., Mohammad, U., Sher, M., Habib, A., Iqbal,A. (2007). *In vitro* clonal propagation and biochemical analysis of fi eld established *Stevia* rebaudiana Bertoni.Pakistan J. Bot., 39, 2467–2474.

Nakamura S, Tamura Y. Variation in the main glycosides of *Stevia* (*Stevia* rebaudiana) (1985). Jpn J Trop Agric.; 29: 109-116.

Nishiyama, P., Alvares, M. and Vieira,L.G.E (1992) Quantitative analysis of stevioside in the leaves of *Stevia* rebaudiana by near infrared reflectance spectroscopy, J sci food Agric, 59:277- 281.

Nishiyama, P., Kusumoto, I. T., Costa, S. C., Alvarez, M. and Vieira, L. G. E. (1991). Correlation between the contents of total carbohydrates and steviosides in leaves of *Stevia* rebaudiana Arquivos De Biologia E Tecnologia. 34: 425-434.

Rahman M; and O. Rajora 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). Plant Cell Reports 20:531-536.

Sharuti V, Kuldeep Y, Narender S. (2011) Optimization of the protocols for surface sterilization, regeneration and acclimatization of *Stevia* rebaudiana Bertoni. Am Eurasian J Agric Environ Sci. ; 11(2): 221-227.

Silva ALL, Franco ETH, Dornelles EB, Reichert Bortoli CL, Quoirin M. (2009) *In vitro* multiplication of *Vriesia scalaris* E. Morrem (*Bromeliaceae*). *Iheringia Sér Bot*.; 64: 151-156.

Singh, S.D., Rao, G.P. (2005). *Stevia*: The herbal sugar of the 21st century. *Sugar Technol.* 7:17-24.

Sneath,P.H.A and Sokal,R.B.(1973).*Numerical taxonomy*. The principles and practice of numerical classification. San Francisco: W. H. Freeman.

Soliman, H. I. A., Metwali, E. M. R., Almaghrabi, O.A.(2014). Micropropagation of *Stevia* rebaudiana Betroni and assessment of genetic stability of *in vitro* regenerated plants using inter simple sequence repeat (ISSR) marker. *Plant Biotechnology* 31: 249-256.

Steven,M.S.,Gail,B.M. and Christopher,W.W.B.I(1992). Stevioside biosynthesis by callus root,shoot and rooted shoot culture invitro plant,cell tissue.org., 28:151-158.

Summon MH, Mostofa M, Jahan MS, Kayesh MEH, Haque MA.(2008) Comparative efficacy of powdered form of *Stevia* (*Stevia* rebaudiana Bertoni) leaves and glimepiride in induced diabetic rats. Bangl J Vet Med. 6(2): 211-215.

Tanaka O. (1988). Isolation of sweetener from *Stevia* rebaudiana. Japanese Kokai 63:177-764.

Tateo, F.,Mariotti,M.,Bononi,M.,Lubian,E.,Martello,S.and Cornara,L. 1998. Stevioside content and morphological variability in a population of *Stevia* Rebaudiana (Bertoni)Bertoni from Paraguay. Italian J. Food Sci. 10: 261_267.

Uddin MS, Chowdhury MSH, Khan MMMH, Uddin MB, Ahmed R, Baten MA. (2006) *In vitro* propagation of *Stevia* rebaudiana Bert in Bangladesh. *Afr J Biotechnol*.; 5(13): 1238-1240.

Ummi Nur Ain Abdul Razak, Chong Boon Ong, Tiew Sing Yu and Li Kiaw Lau (2014). *In vitro* Micropropagation of *Stevia* rebaudiana Bertoni in .Malaysia.,Braz. Arch. Biol. Technol. v.57 n.1: p p. 23-28.

Yadav, A. K., Singh, S., Dhyani, D. Ahuja, P. S. (2011). A review on the improvement of *Stevia* [*Stevia* rebaudiana (Bertoni)]. Can. J. Plant Sci., 91, 1–27.

الإكثار الدقيق السريع لنباتات الاستيفيا، ومدى تراكم الستيفيوسيد والثبات الوراثي باستخدام معلمات ISSR

رانيا محمد عبد العزيز[1] و خالد عدلي محمد خالد[2]
[1] معهد بحوث المحاصيل السكرية – مركز البحوث الزراعية
[2] قسم الوراثة – كلية الزراعة – جامعة بني سويف

يزرع الاستيفيا كمحلاً طبيعياً في مناطق مختلفة من العالم. ولها استخدامات طبية متنوعة دون أي آثار جانبية. ويمكن أن تزرع عن طريق البذور أو عن طريق العقل الخضرية وطريقة الزراعة بالبذور ليست فعالة بسبب انخفاض الخصوبة بجانب أن إنبات البذور ضعيف، وأيضا معدلاته أقل من 10٪ وأيضاً وجود بها ظاهرة عدم التوافق الاتي. أما طريقة العقل الخضرية فمن عيوبها انخفاض عدد النباتات الجديدة. في هذه الدراسة، تم إكثار الاستيفيا من خلال زراعة العقد الخضرية على بيئة MS تحتوي على تركيزات مختلفة من 6-benzyladenine (BA), kinetin (Kin) وقد أعطى التركيز (2 مجم/لتر BA + 0.5 مجم/لتر Kin) أعلى قييم لكل من لنسبة النباتات الناتجة، وأقصى عدد من المجموع الخضري و أعلى متوسط طول للمجموع الخضري (0.04 و 9.07 و 4.61) على التوالي. تم تسجيل الحد الأقصى N. of root formation/shoot ، ومتوسط طول الجذر/المجموع الخضري وأعلى نسبة من الجذر/ المجموع الخضري في بيئة تحتوي على IAA 1مجم / لتر (12.93و 6.89 و 98٪). وكانت نسبة الفرق بين T1 و T2 في (٪) الرطوبة والدهون والرماد والألياف الخام وSteveoside و Reboside-A نسبة طفيفة وبالتالي فإن كل من البيئات T1, T2 هي مناسبة. ومن الواضح أن البيانات التي تم الحصول عليها عن الرطوبة والبروتين والكربوهيدرات الكلية كانت الفروق ملحوظة في T2 أكثر من T1، ويعتبر هذا كدليل على استخدام البيئة المناسبة للحصول على نسبة عالية من البروتين والمحليات ستيفيوسيد والتي تعكس القيم الغذائية. تم أقلمة النباتات السابقة في المعمل بنجاح مع معدل بقاء على قيد الحياة من 95٪ في مرحلة التقسية. تم استخدام معلمات ISSR لتحليل الثبات الوراثي للنباتات الأم والنباتات الناتجة من الإكثار الدقيق التي تنتجها استيفيا. لم يتم الكشف عن أي اختلافات بين النباتات المزروعة والنباتات الأم.