JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1289-1298

Micropropagation of Seven *Stevia rebaudiana* Bert. Genotypes Via Adult Leaf Explants

Mohamed El-Zaidy[1], Mohamed Zayed[1], Sulaiman Alharbi[1], Abdullah Doaigey[1], Abdulaziz Al Sahli[1] and Reda Ammar[2]

[1]Department of Botany and Microbiology, [2]Department of Chemical Sciences, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia.

(Received: 11 January 2014; accepted: 09 March 2014)

***Stevia rebaudiana* Bertoni is a perennial herb belongs to Compositae family used as a natural sweetener for diabetic patients, and it is native to Paraguay. Moreover, the tissue culture techniques were used to identify the best medium for callus induction and the regeneration using different concentrations of phytohormones to produce a high number of shoots and roots in each genotype. Results indicate that the Murashige and Skoog (MS) or (Linsmaier & Skoog) LS medium supplemented with 1.0 mg/l 2,4-D + 0.5 mg/l BAP + 1.0 mg/l GA3 was the favorable for all calli characters and the genotype-4 was the best one for callus induction. The green embryos character was highest in genotypes-4 with the regeneration media containing MS salts + 2.0 mg/l BAP. The high percent of multiplication and elongation shoots were obtained with genotypes-5 and 3 with a medium which containing MS salts + 0.1 mg/l BAP. Finally, the medium which contained MS salts only was the best one for differentiated roots.**

Key words: *Stevia rebaudiana,* Micropropagation, Callus, Organogenesis, Regeneration.

Stevia rebaudiana Bertoni is an herbaceous perennial plant of the Compositae family. (Singhand Rao, 2005). *Stevia* is self-incompatible (Chalapathi, 1997) and probably insect pollinated (Oddone, 1997). The dry leaves of this plant are up to 30-times sweeter than sucrose, it contains various chemicals called glycosides, that collectively give 100 to 300 times the sweetness of sucrose. Seeds of Stevia show a very low germination percentage (Orioand Toffler, 1981). Furthermore vegetative propagation through cuttings is limited by the small number of individuals (Sakaguchiand Kan, 1982). Das *et al*. (2011) reported micropropagation of *S. rebaudiana* through shoot tip culture. Uddin *et al*. (2006) noted that the inter-nodal segments initiated callus earlier than node and leaf. Callus culture and suspension culture are the basic technique used to produce the desired metabolites of plants (Vyas and Dixit, 1999). Gupta *et al*. (2010) developed a protocol for callus induction and multiplication by culturing nodal, leaf and root explants on MS medium with different concentrations of plant hormone. Alhady (2011) reported the micropropagation using stem node segment obtained from two year old plants. Sairkar *et al*. (2009) reported standardization of *in vitro* culture techniques to explore the potentials of *S. rebaudiana* for micro-propagation and callus culture. Filho *et al*. (1993) inoculated *S. rebaudiana* leaf explants with different concentrations of 2, 4-D and BA under a high concentration of sucrose (120 g/L)to induce somatic embryogenesis. Patel and Shah (2009) reported the regeneration of *S. rebaudiana* through callus culture from nodal as well as leaf segments. Also, Moktaduzzaman and Rahman (2009) reported the regeneration of *S. rebaudiana*, and they analyzed the somaclonal variation among regenerated plants. Sairkar *et al*.

* To whom all correspondence should be addressed.
Tel.: +966590699863; Fax: +966114675833;
E-mail: m_elzaidy@hotmail.com

(2009) reported standardization of *in vitro* culture technique to explore potential of *S. rebaudiana* for micropropagation and callus culture. Anbazhagan *et al*. (2010) also reported the mass propagation of *S. rebaudiana*. Shoot tip, nodal segment and leaf were used as explants.Recently, Singh *et al*. (2011) also reported the plant regeneration under *in vitro* conditions.Banerjee *et al*. (2008 & 2010) reported that the leaf explants of *Stevia rebaudiana* Bertoni induced callusing when put on a mixtureof different cyanobacterial cultures as a medium for regeneration. Taware *et al*. (2010) establishedefficient plant regeneration via shoot and callus organogenesis.Janarthanam *et al.* (2009) reported that the juvenile leaf explants of *Stevia rebaudiana* Bertoni produced maximum callus than the nodal explants. Arvind *et al.* (2012) reported thatthe *in vitro* regeneration of *Stevia rebaudiana* was performed through callogenesis and organogenesis from different explants. Jones *et al*. (2003) developed an efficient and rapid tissue culture system for *Stevia rebaudiana* via shoot tip multiplication and somatic embryogenesis from leaf explants. Sairkar *et al*. (2009) reported standardization of *in vitro* culture technique to explore potential of *S. rebaudiana* for micro-propagation and callus culture. The aim of this study istomonitor the seven Stevia (*Stevia rebaudiana*) genotypes for micropropagation using leaf explants.

MATERIALS AND METHODS

The present study was carried out at the Botany and Microbiology Department, Faculty of Science, King Saud University, Saudi Arabia. All genotypes were brought from the Agricultural Research Center in Egyptand cultivated in the greenhouse. The basic culture mediawere MS (Murashigue & Skoog's, 1962) or LS (Linsmaier& Skoog's, 1965) macro and micro elements (1X), vitamins (Nitsch & Nitsch,1969), all media were supplied with3% sucrose as carbon source and different concentrations of the phytohormones (2, 4-D, K, GA3, BAP and NAA). All media were solidified with 0.8 % agaradded after adjusting the pH to 5.5 using 1.0 M HCL or 1.0 M NaOH(Table1), then the media were sterilized by autoclaving at 121 °C for 20 min. After cooling to 50- 65 °C, the medium was poured in glass Petri dishes using 25-30 ml per dish in the Lamin air hoods and left at room temperature to be solidified. The explantswere surface sterilized by immersing in 70% ethanol for 30 Sec, then in 0.1% mercuric chloride for 8 min. Afterwards,they were washed with three changes of sterile distilled water. Leaf discs (1 cm diameter) were cultured on the surface of solidified medium. Each genotype was cultured in 10 Petri dishes in ten replications for each the protocol treatment (A, B and C) supplemented withdifferent types of phytohormones Table (1). Afterwards,cultures were incubated in dark at 20 ±5 °C for four weeks and thin calluses induction were recordedas the percentage of explants which had calluses on the initiation media, the fresh weight (gm) of individual and initiative calli and the percentage of embryogenic calli derived from this explants.For the embryo formation, embryogenic calliwas transferred to MS mediasupplemented with different concentrations of phytohormones as indicated in Table (1) protocols D, E, F and G and then incubated for four weeks, then the percentage of calli with green embryos were recorded.For regeneration of plants, the embryos were transferred to MS media with different concentrations of benzyl amino purine (BAP) protocols H and I as shown in Table (1) then incubated for four weeks.When shooted plantlets reachedapproximately 5 cm in height, they were transferred to the rooting medium that contained auxin (NAA) or wasphytohormones-free as indicated in Table (1) protocols J and K. In all protocols for embryo formation, regeneration, and rooting the explants were incubated at the same condition in a format Scientific (USA) growth incubator at 25 ± 2°C and 3000 Lux of white cool fluorescent (16/8: light/dark cycle).

Statistical analysis

Data from these experiments were analyzed as a randomized complete block design (RCBD) with ten replicates for each genotype according to Steel and Torrie (1984). Comparisons among means were made by using the Least Significant differences test (L.S.D.).

RESULTS AND DISCUSSION

Callus induction

The production of callus of all Stevia genotypeswas recorded after incubation for four

weeks. Analysis of variance (ANOVA) Table (2), showed significant differencesdifferences in callus production between the seven Stevia genotypes, and the media protocols. However, the interaction between the media and the seven genotypes were non-significant.

The results of callus induction indicate that medium (B) induced more callus production than the other two media (A and C),(Figures 1aand 2a, Table 2 and 3). The obtained results were confirmed by those of Handro *et al*., (1977), Lee *et al*., (1982) and Swanson *et al*., (1992) who reported the optimal concentration of growth regulators (BA, NAA, IAA and 2,4-D) for callus induction of *Stevia rebaudiana*, while Bespalhok *et al.,* (1997) discussed that the somatic embryogenic callus formation occurred from leaf explants of *Stevia rebaudiana* cultured on MS medium. The same results were obtained by Bondarev *et al*., (1998).

Callus weight

The ANOVAof the seven *in vitro* traits of Stevia summarized in Table (2), showed that the sources of media, and genotypes with the interaction of media had highly significant differences in callus weight. All genotypes (Table(3) and Figure(2b)), showed that the medium B high effect on the callus weight characteristic when compared with the other two media (A and C, respectively).Therefore, the comparisons between the genotype found in Table (3), indicated that genotype-6 had the highest callus weight followed by genotypes (5 and 7), which have the same weight on media (B). The latest results of callus weight were almost matched with those of Bondarev*et al*., (1998) which showed the effects of addition of GA3 to callus and suspension cultures of Stevia .

Total calluses weight

The ANOVA of the total callus weight for the seven Stevia genotypes showed in Table (2) indicated that the mean square of the media and the media in combination with that of the genotypes was highly significant, while that of genotypes alone was significant. The mean values of *in vitro* traits for all characters, showed in Table (3) and Figure 2c, which indicate the medium A is favored. The comparisons of genotypes (Table, 3) showed that the genotype-4-6 gave the highest value of total calli weight, while the lowest value found with genotypes (1). All genotypes gave high total calli weight on medium (A) when compared with other mediums (B and C). Whereas the response of total calli weight on medium (A) was found to be greater with the genotypes (7, 4 and 1), respectively. On the other hand, the genotypes (4 and 7) gave the higher total weight of calli on medium (A and B), respectively, when compared with medium (C), while the genotype-2 and 3 gave the lowest weight on medium C.

Embryogenic calluses

In this part of study the cultures were incubated in complete darkness, after that a small and slow-growing calli appeared. The growing calli of all genotypes cultured in the first medium (A) was found to be hard, white and appeared on the explants. The calli that has sprung up on the second medium (B) showed a color of calli ranged from white to yellow and were friable, easy to separate from the explants and appeared under or a above of the explants.Finally the calli of the genotypes after growing on the third medium (C), showed that color ranged from white to brown and were friable, easy to separate from the explants and appeared on all the explants and this agree with the result of Swanson *et al.,* (1992) when cultured the leaf explants of *Stevia rebaudiana* in MS medium which yielded friable callus cultures. The ANOVA showed that the genotypes and the interaction between media and genotypes was non-significant differences for embryogenic callus, whereas the protocol of media were significantly affected on embryogenic calluses (Table 2). The result in Table (3) showed that the medium (B) gave the highest percentage of embryogenic calli while the medium (C) gave the lowest percentage of it and the medium (A) showed intermediate.

The comparison between the genotypes means results which represented in Table (3) and Figure(2d) indicated that all genotypes were statistically in almost similar to this character of embryogenic calli percentages, either illustrates the result of the interaction between media and genotypes on the produced embryogenic callus. From these results we indicated that the genotypes varied in their responses to the media protocols. The result in Table (4)showed that the genotype-5 gave the lowest embryogenic callus percent on medium (C), while genotype-3 gave the highest embryogenic callus percent on the same media. On medium (B) genotype-1 gave the highest

Table 1. Components of medium protocols (A, B,… and K) which used for the calli induction, Green embryos, Shoot differentiation, multiplication and elongation and Root differentiation of the seven Stevia genotypes.

Protocol components	Protocol A Conc. mg /L	Protocol B Conc. mg /L	Protocol C Conc. mg /L`	Protocol D Conc. mg /L	Protocol E Conc. mg /L	Protocol F Conc. mg /L	Protocol G Conc. mg /L	Protocol H Conc. mg /L	Protocol I Conc. mg /L	Protocol J Conc. mg/L	Protocol K Conc. mg /L
LS or MS salts	1 X	1 X	1 X	1 X	1 X	1 X	1 X	1 X	1 X	1 X	1 X
Inositol	100	100	100	100	100	100	100	100	100	100	100
Folic acid	0.5	-	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nicotinic acid	5	0.5	1.0	5	5	5	5	5	5	5	5
Thiamine HCL	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCL	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycine	2.0	-	-	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Biotin	0.5	-	-	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Coconut milk (ml/L)	100 ml/L	100 ml/L	100 ml/L	-	-	-	-	-	-	-	-
2 , 4- D	1.0	1.0	2.0	-	-	-	0.1	-	-	-	-
Kinetin	-	-	0.4	2.0	-	-	3.0	-	-	-	-
GA3	-	1.0	1.0	-	-	-	-	-	-	-	-
BAP	0.5	0.5	1.0	0.02	2.0	6.0	-	0.1	1.0	-	-
NAA	-	-	-	-	-	2.0	-	-	-	0.1	-
Casine hydrolysate	400	400	400	-	400	-	-	-	-	-	-
Sucrose	30000	30000	30000	30000	30000	30000	30000	30000	30000	30000	30000
Agar	8000	8000	8000	8000	8000	10000	8000	7000	7000	7000	7000

Table 2. Analysis of variance of *in vitro* traits for the seven Stevia genotypes (*Stevia rebaudiana* Bertoni)

S.O.V	D.F	Mean Square			
		Callus induction %	Callus weight (gm)	Total weight of calli (gm)	Embryogenic calli %
Media	2	52726.190**	0.161**	28.758**	44683.075**
Genotypes	6	1169.683**	0.002 *	00.217*	433.342
M×G	12	227.302	0.005**	00.294**	982.097
Error	209	251.481	0.0014	00.094	889.940

, * significant and highly significant at the 0.05 and 0.01 level of probability, respectively.

Table 3. Mean values of *in vitro* traits (for four characters understudies) as influenced by Genotype, and media protocols

Factor		Callus induction %	Callus weight(gm)	Total weight of calli (gm)	Embryogenic calli %
Media	A	34.000 b	0.08964 c	0.3076 b	52.798 b
	B	82.571a	0.17975 a	1.4594 a	83.787 a
	C	36.143 b	0.10660 b	0.3962 b	33.728 c
Genotypes	1	56.333 ab	0.118 b	0.7327abc	51.444 a
	2	49.667 bc	0.119 b	0.6334 c	57.845 a
	3	49.000 bc	0.121 b	0.6512 c	60.403 a
	4	59.000 a	0.130 ab	0.8608 a	56.694 a
	5	45.000 c	0.124 ab	0.6829 bc	51.611 a
	6	42.000 c	0.141 a	0.6773 bc	59.991 a
	7	55.333 ab	0.124 ab	0.8090 ab	59.407 a

Factor means followed by a common letter are not significantly different according to LSD 0.05

Table 4. Mean values as interactions between the *in vitro* traits for four character studies of the seven stevia genotypes with the protocols (A, B and C) of media

Media × Genotypes		Callus induction(%)	Callus weight(gm)	Total weight of calli (gm)	Embryogenic calli (%)
A	Genot.1	33.00	0.079	0.267	33.63
	Genot.2	29.00	0.091	0.254	60.00
	Genot.3	31.00	0.105	0.330	45.97
	Genot.4	45.00	0.095	0.434	41.70
	Genot.5	33.00	0.074	0.244	50.09
	Genot.6	31.00	0.102	0.340	51.45
	Genot.7	36.00	0.080	0.285	48.33
B	Genot.1	90.00	0.151	1.347	74.78
	Genot.2	82.00	0.139	1.122	66.62
	Genot.3	79.00	0.159	1.260	70.31
	Genot.4	93.00	0.188	1.742	69.08
	Genot.5	75.00	0.212	1.567	71.56
	Genot.6	69.00	0.213	1.395	73.52
	Genot.7	91.00	0.196	1.783	68.95
C	Genot.1	46.00	0.123	0.584	24.14
	Genot.2	38.00	0.128	0.525	27.33
	Genot.3	38.00	0.098	0.364	42.25
	Genot.4	39.00	0.106	0.407	39.58
	Genot.5	27.00	0.087	0.239	14.95
	Genot.6	26.00	0.109	0.296	32.95
	Genot.7	39.00	0.096	0.359	38.41
	L.S.D	13.971	0.032	0.269	26.28

Table 5. Analysis of variance for green embryos formation of the seven Stevia genotypes (*Stevia rebaudiana* Bertoni)

S. O. V	D. F	Mean square (Callus regeneration)
Media	3	7277.500**
Genotypes	6	2040.595**
Media × Genotypes	18	3069.167**
Error	279	384.087

** highly significant at the 0.01 level of probability

Table 6. Percentages of green embryos formation as influenced by genotypes and media protocols tested by LSD

		Green embryos (%)
Media protocols	D	74.000 [b]
	E	91.570 [a]
	F	70.430 [b]
	G	87.290 [a]
Genotypes	Genot. 1	92.500 [a]
	Genot. 2	82.500 [bc]
	Genot. 3	73.000 [d]
	Genot. 4	76.500 [d]
	Genot. 5	76.250 [cd]
	Genot. 6	88.000 [ab]
	Genot. 7	77.000 [cd]

Factor means followed by a common letter are not significantly different according LSD 0.05

embryogenic callus percent, but the genotype-2 gave the low percent. On the contrary, genotype-2 gave the highest embryogenic callus percent on medium (A), while the genotype-1 gave the lowest one. Therefore, the high percent of embryogenic calli was found with the protocol of medium (B) especially with genotypes (1, 6, 5 and 3), respectively,Table (4). The same results found by Wada *et al*., (1981), Bespalhok *et al*., (1993 & 1997) and Jones *et al*., (2003) which reported that the somatic embryos of *Stevia rebaudiana* were induced from leaf explants. Also they observed the various developmental stages of embryos after subculturing of calli on the same composition medium.

Green embryos Formation

In another sets of experimentsfour protocols of media (D, E, F and G) were used to study embryo formation of the seven Stevia genotypes as shown in Table (1). Cultures were incubated under the circumstances of 300Lux of light for 16 hours.'s and in darkness for 8 hours. The AVOVA for green embryos formation presented in Table (5). The results indicated that all the media, genotypes and the interaction between media and genotypes had highly significant effects on the green embryos formation. Figures (1 b) showed that the development stages of green embryogenic calli of the Stevia genotype - 1 under the regenerated media protocol G.On the other hand,

Table 7. The percentages of differentiated shoots and the averages of shoot number (multiplication) and of shoots height (elongation) of seven Stevia genotypes cultured on the tow media (H&I).

Genotype	media	Differentiated shoots %	(Multiplication) average number of shoots	(Elongation) average height of shoots cm
Genot. 1	H	39.20	2	2.0
	I	32.13	7	3.3
Genot. 2	H	42.00	4	2.8
	I	40.86	2	3.2
Genot. 3	H	69.43	10	2.3
	I	71.32	3	5.1
Genot. 4	H	51.23	3	2.2
	I	32.16	8	2.6
Genot. 5	H	82.11	5	2.4
	I	74.26	2	3.2
Genot. 6	H	60.11	4	2.5
	I	47.20	2	3.5
Genot. 7	H	33.15	5	2.6
	I	41.19	3	3.5

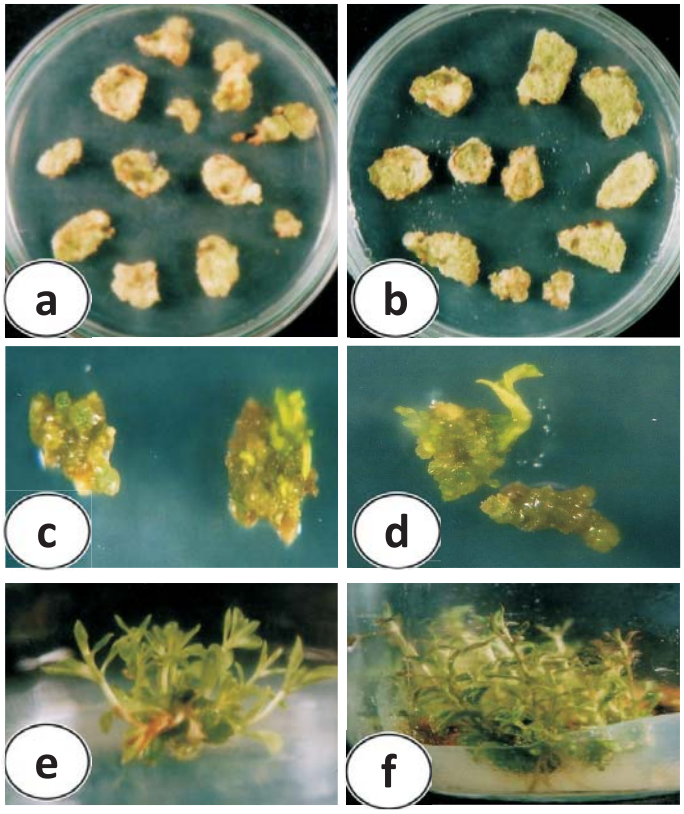

Fig. 1. (a) Embryogenic callusesof stevia genotype- 4 on protocol B, (b) Green embryos formation of stevia genotype-1 on protocol G, (c & d)Shootsdifferentiation of stevia genotype-5 on protocol H, (e) Multiplicationin genotype-3 on protocol H (f) Root differentiation of Stevia genotypes -3, on the protocol k (without hormone).

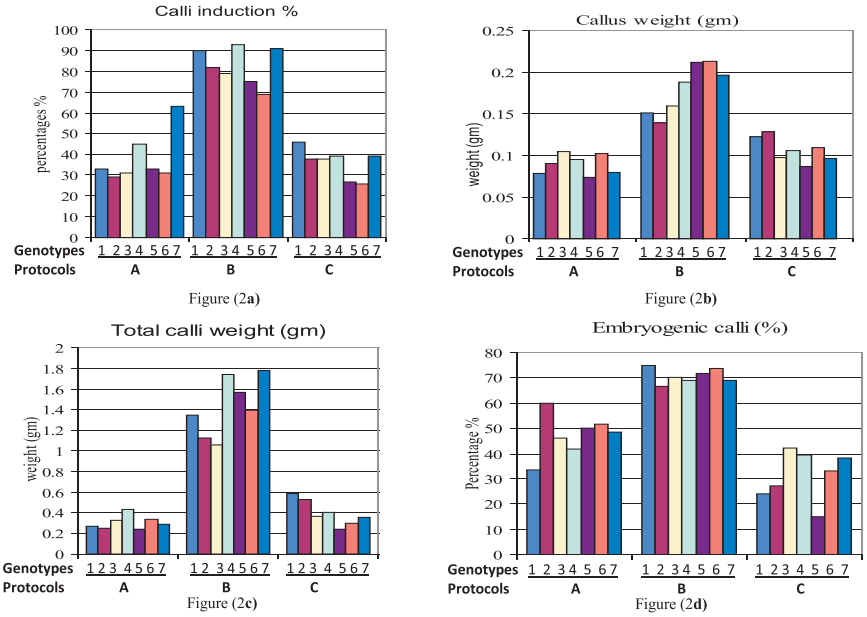

Fig. 2. Calli induction in percentages (%) (2a), calli weight in grams (2b) total weight of calli (2c) and embryogenic calli in percentages (%) (2d) of the seven stevia genotypes with the three media protocols (A, B and C)

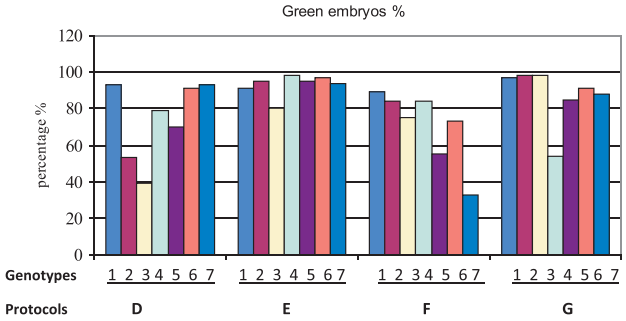

Fig. 3. Percentages (%) of the green (calli) embroys of the seven stevia genotypes with the four media protocols (D, E, F and G)

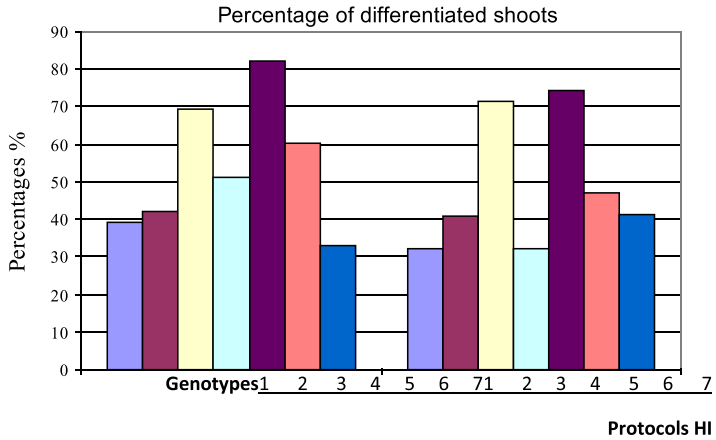

Fig. 4. a) Diagrams of the differentiated shooting percentages (%) with the two media protocols (H and I) of the seven stevia genotypes (b) Diagrams differentiated root percentages (%) with the two media protocols (J and K) of the seven stevia genotypes

Table (6) shows thePercentages of green embryos formation with protocol E and G that gave the high mean of green embryos respectively, whereas the medium F and D gave the low percentage of it respectively. The analysis of data showed that the genotypes 1and 6 gave the high percent of green embryos formation, andgenotype-3 gaves lowest percent of green it. An interaction between genotypes and media protocols illustrated in (Table,6 and Figure, 3). The previous data showed that the protocols E and G were the favorable than the other protocols (D and F) (Table, 6 and Figure, 3). The results obtained from the present study regarding the establishment of green embryos are in agreement with those of Jones *et al*., (2003) who found the maximum number (94%) of embryogenic calluses from leaf explants on MS medium.In the same composition medium, they observed the embryos at different stages of development after subculturing of the calli.

Shoot differentiation, multiplication and elongation

Two different concentrations of (BAP) were added to the protocols of culture media H and I (Table 7 and Figure 4a) for shoot differentiation, elongation and multiplication (Figure 1 c, d and e) of the present seven selected Stevia genotypes. From the previous data it can be noted that the high percent of differentiated shoots were obtained with genotype-5 on medium H, (Table 7 and Figures 1 c and 4a), also was the same genotype on mediumI then genotype-3.The opposite effects of the low differentiated percent were Stevia genotype-7 with medium H while genotypes (1 and 4) were given the lowest percent with medium I, respectively. The average number of shoots as indicator to the multiplication cells was high in genotype-3 with medium H, (Table 7 and Figures 1e and 4a), while was also high in

genotype-4, then genotype-1 with medium I more than the other genotypes. The low number of shoots as multiplication found in general with medium I more than with medium H in almost genotypes. Again, the average height of shoots as elongation was almost similar in all genotypes with medium H, but with medium I the genotype-3 had the highest average number (5.1cm) more than the other genotypes which were low in almost.The results obtained from the present study regarding the differentiation of the callus tissue and that induce shoot cultures to grow roots thereby differentiating into rooting-shoot cultures are in agreement with those of Swanson *et al.,* (1992) and Bondarev *et al.,* (1998).

Root differentiation

Data of the root differentiation for the seven Stevia genotypes on the two mediums J and K(Table 8 and Figures 1f and 4b), indicated that the medium K was the best one for root differentiation more than the other medium J. The high differentiated root percentages were the Stevia genotypes (3, 2, 4 and 7), respectively on the medium k (without hormone),Table (8). While the low root differentiation percentages found with genotypes (4 and 1), respectively on the media J which containing NAA phytohormones Table (8). The results of root differentiation are in agreement with the results of Bondarev (2001), Sivaram *et al.,* (2003), Jones *et al.,* (2003) and Dhir *et al.,* (2005). Tamura *et al.,* (1984a) found from anatomical examination, the roots were differentiation when the shooting subculture transferred to a medium containing NAA phytohormone with 0.1 mg/L concentration.

Table 8. The percentages of differentiated roots of the seven Stevia genotypes cultured on the two mediums (J & K)

Genotype	media	differentiated roots %
Genot. 1	J	77.49
	K	86.10
Genot. 2	J	88.38
	K	98.2
Genot. 3	J	88.38
	K	100
Genot. 4	J	71.24
	K	97.02
Genot. 5	J	82.00
	K	91.02
Genot. 6	J	80.42
	K	89.35
Genot. 7	J	86.72
	K	96.36

ACKNOWLEDGMENTS

The Authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project RGP-VPP-062.

REFERENCES

1. Alhady M. R. A. A. Micropropagation of *Stevia rebaudiana* Bertoni: A New Sweetening Crop in Egypt. *Glob. J. Biotechnol. Biochem.* 2011; **6**(4): 178-182.
2. Anbazhagan, M., Kalpana, Rajendran R., Natarajan V. and Dhanave D., *In vitro* production of *Stevia rebaudiana* Bertoni. *Emirates J. Food and Agric.,* 2010; **22** (3): 216-222.
3. Arvind A., Kumar S., and Kasana M.S. *In vitro* regeneration of *Stevia* and evaluation of antimicrobial and antiprotozoal properties of regenerated calli and plants. *Elect J Plant Breed,* 2012; **3**(3): 916-924
4. Banerjee M. Sarkar P. In vitro callusing in stevia rebaudiana Bertoni using cyanobacterial media – a novel approach to tissue culture. *Inter J Integ Biol.* 2008; **3**: 163-168.
5. Banerjee M. Sarkar P.Somatic embryogenesis in *Stevia rebaudiana* Bertoni using different concentrations of growth hormones. *Inter J Plant Sci,* 2010; **5**(1): 284-289
6. Bespalhok, J. C., Filho, and Hattori K. Embryogenic Callus formation and histological studies from *Stevia rebaudiana* Bertoni floret explants. Revista Brasileira de Fisiología *Vegetal* 1997; **9**: 3, 185- 188.
7. Bespalhok, J. C., Filho, Hashimoto J. M., and Vieira L. G. E. Induction of Somatic embryo genesis from leaf explants of *Stevia rebaudiana*. *Revista Brasileira de Fisiología vegetal* 1993; **5**(1): 51- 53.
8. BondarevN. I. Pecularities of propagation and development of *Stevia rebaudiana* Bertoni plants *in vitro*. Sals: Proceedings of 9th International Conference of Horticulture, September 3th - 6th 2001 Lednice, Czech Republic, ISBN 80-7157-524-0, 2001; **2**, 431-734.
9. Bondarev, N. I., NosovA. M., and Kornieko A.V. Effect of exogenous growth of regulators on callusogenesis and growth of cultured cells of *Stevia rebaudiana* Bertoni. Timiryazey Institute of Plant Physiology, Russian Academy

of Science botoniheskayaul. 35, Moscow, 127276 Russia.1997.
10. Chalapathi M. K. Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni): Future crop for India. *Crop Res.* 1997; **14**: 347-350.
11. Das A, Gantait S, Mandal N. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. *Int. J. Agric. Res.* 2011; **6**: 40-48.
12. Dhir S., Knowles K., Dhir S. K. Regeneration and cloning of Stevia plant: A low calorie sweetener. Feagin mill middle school, Hous County, Warner Robins, GA 31088 and Center for Biotechnology, Fort Valley State University, Fort Valley, GA 31030. 2005.
13. Filho J. C. B., Hashimoto J. M., Vieira L. G. Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. *Braz. J. Plant Physiol.* 1993; **5**: 51-53.
14. Gupta P., Sharma S., Saxena S. Callusing in *Stevia rebaudiana* (Natural Sweetener) for Steviol Glycoside Production. *Int. J. Agric. Biol. Sci.* 2010; **1**(1): 30-34.
15. Handro W., Hell., K. G., Kerbauy G. B. Tissue culture of *Stevia rebaudiana*, a sweetening plant. *Planta medica* 1977; **32**: 115-117.
16. Janarthanam B., Gopalakrishnan M., Lakshmi S. G., Sekar T. Plant Regeneration from Leaf Derived Callus of *S. rebaudiana* Bertoni. *Plant Tissue Cult. & Biotech.* 2009; **19**(2): 133-141.
17. Jones, Joi A., Moore., D., Knowles K.M. and Sarwan. Factors affecting plant regeneration via somatic embryogenesis in *Stevia rebaudiana*. Association of Research Directors, Inc. 13th Biennial Research Symposium March 29-April 2, 2003.
18. Lee, K. R., Park., J. R., Choi., B. S., Han., J. S., OH., S. L., and Yamada Y. Studies on the callus culture of stevia as a new sweeting source and the formation of stevioside. *Kor J Food Sci and Techn.* 1982; **14**(2): 179 -183.
19. Linsmaier, E. M., Skoog F. Organic growth factor requirements of tobacco tissue culture. *Physiol. Plant.* 1965; **18**: 100-127.
20. Moktaduzzaman M., Rahman S. M. MRegeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. *Biotechnol.* 2009; **8** (4) :449-455
21. Murashigue, T. Skoog F. A revised medium for rapid growth and possess with tobacco tissue cultures. *Physiologia Plantarum,* 1962; **15**: 473-497.
22. Nitsch, J. P. Experimental androgenesis in Nicotiana. *Phytomorphology* .1969; **19**: 389-404.
23. Oddone B. How to grow Stevia. Technical manual. Guarani Botanicals, Pawtucket, CT.1997.
24. OrioO. A., Toffler F. Acceni sulla pin ata tropicale 'Kaa-he-e' ou 'erba dolce'. *Rev. Soc. Sci. Aliment* 1981; : 225-230 [English summary].
25. Patel R. M., and Shah R. R. Regeneration of Steviaplants through callus culture. *Indian J. Pharm. Sci.* 2009; **71**: 46-50.
26. Sairkar P., Chandravanshi M. K., Shukla N. P., Mehrotra N. N. Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. *J. Med. Plant Res.* 2009; **3**: 266-270.
27. Sakaguchi M., Kan T. Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. *CiCult* 1982; **34**: 235-248.
28. Singh N., Yadav K., Kumari S. and Renu.metabolic changes during differentiation in callus cultures of *Stevia rebaudiana* (Bertoni). *Journal of Phytology.* 2011; **3**(3): 63-67
29. Singh S. D. and Rao G. P. Stevia: The herbal sugar of the 21st century. *Sugar Technol.* 2005; **7**: 17-24.
30. Sivaram, L., MukundanU. *In vitro* culture studies on *Stevia rebaudiana* . *In vitro Cellular and Development Biology Plants* 2003; **39** (5) 520-523.
31. Steel, J. D., Torrie J. H. Principles and procedures of statistics: A Biometrical Approach. 4th edition MC Graw Hill. New York. 1984.
32. Swanson, S. M., Mahady, G. B., and Beecher C. W. W. Stevioside biosynthesis by callus , root , shoot and rooted shoot cultures *in vitro*. *Plant Cell Tissue Organ Cultures.* 1992; **28**(2): 151-157.
33. Tamura, Y., Nakamura., S., Fukui. H. and Tabata., M.Clonal propagation of *Stevia rebaudiana* Bertoni by stem tip culture. *Plant Cell Reports* 1984a; **3**(5), 183-185.
34. Taware, A. S., Mukadam D. S., Chavan A. M. andTawar, S.D. Comparative studies of *in vitro* and*in vivo* grown plants and callus of *Stevia rebaudiana*(Bertoni). *International J. Integrative Biol.,* 2010; **9** (1): 10-15.
35. Uddin, M. S., Chowdhury M.S.H., Khan M. M. M. H., Uddin M.B., Ahmed R. and Baten M. A. *In vitro* propagation of *Stevia rebaudiana* Bertoni in Bangladesh. *African J. Biotech.,* 2006; **5** (13): 1238-1240.
36. Vyas S.P., Dixit V. K. *Pharma Biotechnol.* 1999; 298-299.
37. Wada, Y., Tamura., T., Kodama., T., Yamaki., T., Uchida Y. Callus Cultures and Morphogenesis of *Stevia rebaudiana* Bertoni. *Yukagawa*, 1981; **36**(4): 215-219.