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CALLU FORMATION AND DIFFERENTIATION OF *STEVIA REBAUDIANA* BERTONI. A METHOD OF PLANT IMPROVEMENT IN EGYPT

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ABSTRACT: **Stevia rebaudiana Bertoni, is an important non-caloric sweetening perennial herb belongs to the Asteraceae family. S rebaudiana is the single sweetener which is also used for the treatment of diabetes. S. rebaudiana explants were collected from a nursery of Genetic Engineering and Biotechnology Research Institute (GEBRI), Menofiya University, Sadat City, Egypt.. Various concentrations of growth regulators, BAP and NAA were examined for shoot proliferation and callus formation. The highest shoot proliferations resulted from 1mg/l BAP+0.5mg/l NAA. Effect of different NAA concentrations (0, 1, 2, 3, 4 and 5 mg/l) and various explants (nodes, normal leaves, vitrified leaves and roots) on calli formation and quality was examined. Vitrified leaves were the earliest callused explants when it were planted on 3mg/l NAA (21 days), followed by normal leaves (25 days) when it were planted on the same NAA concentration. Effect of different culture containers (tube, jar and cubic magenta) and various initial explants (nodes and leaves) on calli formation and quality were studied. The highest calli amount resulted from both explants when they planted in jars or cubic magenta. Shoot formation from leaves callus was observed on calli when it transplanted on MS medium supplemented with 0.2 or 0.4mg/l 2,4D.On the other hand, Calli differentiated to roots when it were planted on MS medium supplemented with 0.4mg/l IAA. Every differentiated shoot has a genetic structure may be similar or a similar to the mother plants. Further work to study the genetic fidelity and phytochemical availability is underway.**

Key words: ***Stevia rebaudiana – in vitro – callus induction – differentiation-growth regulators***

INTRODUCTION

Stevia, botanically known as *Stevia rebaudiana*, originally came from rain forests of Brazil and Paraguay. Now it is also cultivated in Japan, Korea, Thailand, China and India. About 200 species are native to South America and it is also found in Israel and Central America. Stevia has been used to sweeten tea for centuries, dating back to the Guarani Indians of South America. For hundreds of years, native Brazilians and Paraguayans have

employed the leaves as a sweetening agent. Europeans learned about the plant in 16th century, whereas North American interest began to 20th century when researchers heard of its sweetening property. Paraguayan chemist Ovidio Rebaudi documented stevia in early 1900's then in 1905 a botanist of same country Moises Bertoni gave the present name to the plant (Sapna *et al.,*2008).

***Stevia rebaudiana* Bertoni, is an important non-caloric sweetening perennial herb belongs to the Asteraceae family. It is a natural sweetener plant known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf", which is estimated to be 300 times sweeter than cane sugar (Chalapathi and Thimmegowda, 1997, Liu and Li, 1995 and Uddin *et al*., 2006). Glycoside responsible for sweetening were discovered in 1931. Stevia extracts are used today as food additives by the Japanese and Brazilians as a non calorie sweetner. beat. Stevia extract has exhibited strong bactericidal activity against a wide range of pathogenic bacteria including certain E. coli strains. Steviol, stevia's aglycone is mutagenic towards Salmonella and other bacterial strains under various conditions and towards certain cell lines. It may also be effective against Candido albicans. It may also play a role against dental plaque. The drug is neither mutagenic nor genotoxic.**

Now it's being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia and overall South America for food and pharmaceutical products. Products can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. In Japan alone, 50 tones of stevioside are used annually with sales valued in order of 220 million Canadian dollars (Brandle and Rosa, 1992). Also, it has a special importance to diabetic persons and diet conscious.

Over 100 phytochemicals have been discovered in stevia since. It is rich in terpenes and flavonoids. Stevia contains a complex mixture of diterpenes, triterpenes, stigmasterol, tannins and volatile oil. The constituents responsible for stevia's sweetness were documented in 1931, when eight novel plant chemicals called diterpenic glycosides (5-14%) were discovered and named as Stevioside, Dulcoside and Rebaudioside A, B, C, D and E. (Sapna *et al.,*2008, Taylor, 2003, Starratt *et al.,*2002 and Mitsuhashi *et al.,*1975). The leaves of stevia are the source of diterpene glycosides, Stevioside and rebaudioside, (Yoshida, 1986). Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamazaki and Flores, 1991 and Toyoda and Matsui, 1997). Length of day under which stevia is cultivated has an effect on stevioside concentration (Uddin *et al*.,2006).

The main plant chemicals in stevia include: diterpene glycosides, dulcosides A-B and stevioside which is stable at high (100^0C) temperature, stable at a range of pH values(Evans, 1994), non Calorific, non Fermentable, not darkens upon cooking and approximately 250 times more sweet than cane sugar (Sakamoto *et al*., 1975). Stevia has been used for centuries as a

natural sweetner and may be helpful in treating diabetes. Plant effects on blood pressure, on fertility control. It is used as a sweetening agent, hypotensive, hypoglycemic and bactericidal (Brunet *et al*., 1998). Since *S. rebaudiana* is the single sweetener which is also used for the treatment of diabetes, it is important to standardize it for use as a drug (Sapna *et al.,* 2008)

Seeds of stevia show a very low germination percentage (Felippe and Lucas, 1971 and Toffler and Orio, 1981) and vegetative propagation is limited by lower number of individuals (Sakaguchi and Kan, 1982). Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid mass propagation of Stevia plants. Plant tissue culture technology may help to conserve rare and endangered medicine plants. Many important medicinal herbs have been successfully propagated *in vitro*, either by organogenesis or by somatic embryogenesis (Debnath *et al.,* 2006).

Tissue culture is the only rapid process for the mass propagation of stevia and there have been few reports of *in vitro* growth of stevia (Miyagawa and Fujioka, 1986), *in vitro* micropropagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species (Akita *et al,*1994 and Uddin *et al,* 2006). Clonal propagation of *S. rebaudiana* has been established by culturing stem tips with a few leaf primodia on agar medium supplemented with a high concentration (10mg/l) of kinetin.(Tamura *et al,.* 1984). A method was described for production maintenance and plant regeneration from cell suspension cultures of *S. rebaudiana* (Bert.) (Ferreira and Handro, 1988).) Somatic embryogenesis from leaf explants of *S. rebaudiana* were establish (Bespalhok *et al.,* 1993). Embryogenic callus formation and histological studies from *S. rebaudiana* (Bert.) floret explants were carried out (Bespalhok and Hattori, 1997). Inter-nodal segments initiated callus earlier than leaf and nodal explants. The highest amount of callus was found in the MS medium with 3.0 mg/l 2,4-D.

Nodal explants were incubated on MS medium fortified with different concentrations of BA (0.2, 0.5, 1.0 and 1.5 mg lG) and Kn (0.1, 0.2, 0.5 and 1.0 mg lG) alone or BA with Kn or with NAA. The response was best at 1.5 mg lG BA+ 0.5 mg lG Kn combination where highest percentage of explants (85.33%) showing shoot proliferation, highest number of total shoots (8.75±0.34) and highest average length of longest shoot (4.45±0.45 cm) were recorded. The response was poor and considerable amount of callus was formed with BA and NAA combination. The study revealed that 1.5 mg lG BA +0.5 mg lG Kn was found to be the ideal concentration for high frequency multiple shoot induction (Ahmed *et al.,* 2007).

Benzyl adenine increased multiplication rate, vitrification and somaclonal variation. However, the best results were recorded with MS nutrient medium without plant growth regulators during *in vitro* growth and development of *Stevia rebaudiana*. MS basal medium supplemented with 2 mg/l BA recorded the highest number of shoots, but these shoots were very thin and vitrified

and not suitable for multiplication through several subcultures (Ibrahim *et. al.,* 2008).

Callus induction and multiplication medium was standardized from nodal as well as leaf segments. It is possible to maintain callus on Murashige and Skoog medium supplemented with 6-benzyl amino purine and naphthalene acetic acid. Maximum callus induction was obtained on Murashige and Skoog medium incorporated with 6-benzyl amino purine (2.0-3.0 mg/l) and naphthalene acetic acid (2.0 mg/l) treatments. However, Murashige and Skoog medium containing 2.0 mg/l 6-benzyl amino purine+2.0 mg/l naphthalene acetic acid was found to be the best for callus induction. Higher regeneration frequency was noticed with Murashige and Skoog medium supplemented with 2.0 mg/l 6-benzyl amino purine+0.2 mg/l naphthalene acetic acid (Patel and Shah, 2009)

Janarthanam *et al.,* (2009) *stated that* 1.34 µM of NAA was most effective for inducing 80% of the calls to form shoots and induced greatest number of shoots (14.0 ± 1.0) per explant. Addition of higher amounts of NAA was super optimal causing a reduction in both percentage of shoot regeneration and their number per explant.

Plant may be regenerated directly or indirectly through callus formation. For callus induction both auxin an cytokinin are supplemented with media. Shoot apex, nodal and leaf explants of *S. rebaudiana* can regenerate shoots when be cultured on MS medium supplemented with 6-benzyladenine (BA) and indol-3-acetic acid (Sivaram and Mukundan, 2003) It was reported that the leaves of *Stevia rebaudiana* produced callus when supplemented with various concentrations and combinations of auxins (Khanam *et al.,* 2005) and also nodal segments as explants were used for Micropropagation of S. rebaudiana (Mitra and Pal, 2007). Early callus formation from internode explants of *S. rebaudiana* was reported using MS containing 13.56 µM 2, 4-D which gave maximum callus production (Uddin e*t. al.,* 2006)

Moktaduzzaman and Mahbubur Rahman (2009) stated that various degrees of callus induced from the leaf segments cultured on MS medium supplemented with the different concentrations and combinations of NAA+BA and 2,4-D +BA. Among them, 1.5 mg L-1 NAA with 1.0 mg L-1 BA was the best for callus induction (91.67%) which also produced highest fresh weight (621.7 mg) and dry weight (79.00 mg) of callus. For shoot formation, calli were transferred on to MS medium supplemented with different concentrations and combinations of BA and NAA with control. The highest number of shoots (2.17) and the highest average length of the shoot (3.22 cm) per culture was observed at 1.8 mg L-1 of BA with 0.12 mg L-1 of NAA. Data revealed that some plantlets regenerated from *in vitro* culture were 100% similar to the mother plants and some were 71, 57 or 14% similar may be due to variation in *in vitro* condition.

Shoot initiation from callus was maximum in MS+BA+2,4-D . Plantlets and calli grown on MS medium with different plant growth regulators were

screened for proximate analysis. Plant and callus regenerated from shoot explant on MS+2, 4-D+IBA was proved to be a substantial source for stevioside (Taware *et. al.,* 2010)

This study aimed to determined the optimal concentration and combination of both auxin and cytokinin for calli induction from different explants and determined the best culture vessel which lead to produce maximum calli which be differentiated in order to produce *S.rebaudiana* lines as a method of plant development.

MATERIALS AND METHODS

***S. rebaudiana* plants were harvested from a nursery of Genetic Engineering and Biotechnology Research Institute (GEBRI), Menofiya University, Sadat City, Egypt. *In vitro* work was initiated using shoot apex and axillary buds explants ranging in size from 2.5 to 3 cm, which were collected from young growing plants. The mother plants were maintained in the nursery. They were watered twice a week. After excision, stevia explants were rinsed in running tap water for one min and immersed in Tween 20 solution for another one min. After three washes with double-distilled water, further sterilization was carried out in the laminar airflow chamber using 0.2% (v/v) NaOCl for 5 min. The explants were then rinsed three times with sterile water. *Stevia* explants were cultivated on MS medium (Murashige and Skoog, 1962), supplemented with 6-benzylamino purine (0.0,0.5 and 1mg/l BAP) and Naphthalene acetic acid (0, 1 and 2 mg/l NAA) used singly and in combination. 30 g /l sucrose and 0.6% Difco Bacto-agar were added. The pH of the nutrient medium was adjusted to 5.7 with 0.1 NaOH or 0.1M HCl before autoclaving at 1.2 kg/ cm2 and 121°C for 20 min. The cultures were maintained at a temperature of 25±2°C and 16h photoperiod and light intensity 2000lux. Various concentrations of growth regulators, BAP and NAA were examined for shoot proliferation and callus formation. *S. rebaudiana* explants were prepared aseptically and were vertically planted on MS media. Shoot length, shoot number, leaf number and callus formation were recorded after four weeks.**

Examination of callus induction factors:

Shoots of *Stevia rebaudiana* Bertoni which resulted from the first stage of *in vitro* culture technique were used as initial explants in the following experiments:

Different explants of *S. rebaudiana* Bert (nodes, normal leaves, vitrified leaves and roots) were cultured on MS medium supplemented with 0.5mg/l BA, 3% sucrose, 6% Bacto agar and different NAA concentrations (0,1,2,3,4 and 5 mg/l) in order to study the effect of different NAA concentrations and various explants on calli formation and quality of *S. rebaudiana* Bert. Culture jars were containing 50 ml nutrient medium and three explants were planted in every jar.

Optimum explants and NAA concentrations were chosen according to the results of the results of the latest trail to begin a study that aimed to know responses of various explants (nodes and leaves) when cultured in different culture containers (tube, jar and cubic magenta) on calli formation and quality of *S. rebaudiana* Bert. Each culture container (jar and cubic magenta) was containing 50 ml nutrient medium and three explants were planted in every container, while, tubs culture were containing 15ml medium and one explant was planted in each tub.

Calli resulted from leaves were planted on MS medium supplemented with different IAA and NAA concentrations (0.0, 0.2, 0.4 and 0.6mg/l) in order to determine the optimum auxin and concentration lead to the best calli differentiation. After four weeks observed responses were recorded. The resulted shoots were proliferated according to Ibrahim *et al.,* (2010)

Statistical analysis: Data of results were statistically analyzed by one or two factorial randomized complete design using SAS(1988) package. The least significant difference among levels of each treatment were compared using LSD. test at 5%level according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Results in Table (1) and Fig (1) indicated that BAP concentrations negatively affected shoot length; the tallest shoot length was obtained from free hormone MS medium. Also, shoot length of *S rebaudiana* was affected negatively by increasing NAA concentrations. Inter action between BAP and NAA concentrations individual or in combinations showed the tallest shoots on free MS medium (6.50cm) followed by 0.5mg/l BAP +1.0mg/l NAA (6.16cm). Similarly, data revealed that both BAP and NAA concentrations positively affected shoot proliferation of *S. rebaudiana* Bert. Anyway, the highest shoot number resulted from 0.5mg/lBAP+1.0mg/l NAA followed by 1.0mg/lBA+1.0mg/l NAA as well as 1.0mg/l BAP+ 2mg/l NAA (9, 7 and 7, respectively). Callus formation was observed on the physiological base of the shoots at 0.5 mg/l BAP+2mg/l NAA as presented in fig (1, J and k).on the other hand, number of nodes and leaves were significantly ascended with increasing BAP concentrations, regarding effects of NAA concentrations, the same results were observed. Concerning, the effect of inter action between BAP and NAA concentrations on node and leaf number,1.0mg/l BAP+1.0mg/l NAA possessed the highest node and leaves number (52.0 and 103.33 node and leaves, respectively).

Shoots of *S. rebaudiana* Bert which resulted from the first stage of *in vitro* culture technique were used as initial explants in order to study the effect of different NAA concentrations (0,1,2,3,4 and 5 mg/l) and various explants (nodes, normal leaves, vitrified leaves and roots) on calli formation and quality of *S. rebaudiana* Bert. Results in Table (2) and Fig (2) cleared that callused explants number was the best when normal and vitrified leaves were cultured on MS basal medium supplemented with 2mg/l NAA (100% callused

Table (1): Effect of different concentrations of BAP and NAA on shoot proliferation and callus induction of *S. rebaudiana* Bert

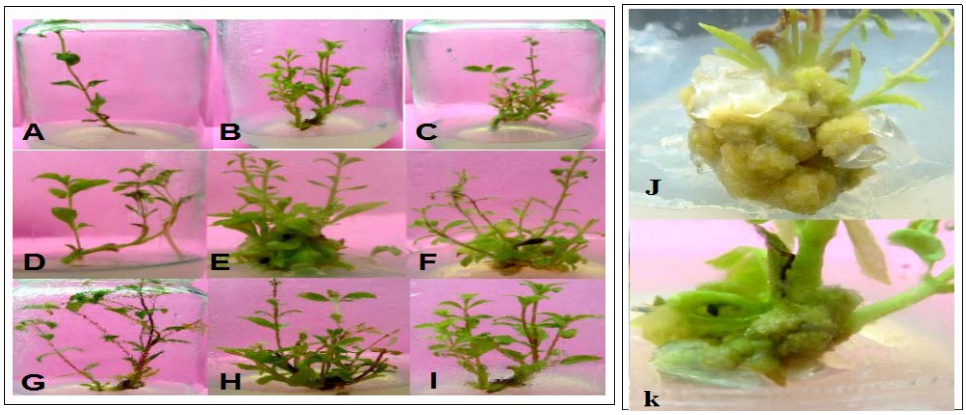
BA	0.0	0.5	١.٠	Mean
NAA	Shoot length			
٠.٠	٦.٥٠	٥.٨٣	٤.٣٣	٥.٥٥
1.0	٥.٣٣	٦.١٦	٥.١٦	٥.٥٥
2.0	٤.٨٣	٤.١٦	٣.٨٣	٤.٤٧
Mean	٥.٥٥	٥.٣٨	٤.٤٤	
L.S.D	A	B	A*B	
	٠.٦٠	٠.٦٠	١.٠٣	
NAA	Shoot Number			
٠.٠	١.٣٣	٦.٣٣	٦.٦٦	٤.٧٧
1.0	٣.٣٣	٥.٠٠	٧.٠٠	٥.١١
2.0	٦.٠٠	٩.٠٠	*٧.٠٠	٧.٣٣
Mean	٣.٥٥	٦.٧٧	٦.٨٨	
L.S.D	A	B	AxB	
	٠.٣٩	٠.٣٩	٠.٦٧	
NAA	Node Number			
٠.٠	٧.٦٦	٢٢.٣٣	٣١.٣٣	٢٠.٤٤
1.0	٢٢.٦٦	٤١.٣٣	٥٢.٠٠	٣٨.٦٦
2.0	٤١.٦٦	٤٥.٦٦	٣٤.٣٣	٤٠.٥٥
Mean	٢٣.٩	٣٦.٤٤	٣٩.١١	
L.S.D	A	B	AxB	
	٤.٥٧	٤.٥٧	٧.٩١	
NAA	Leaf Number			
٠.٠	١٥.٣٣	٤٤.٦٦	٦٢.٦٦	٤٠.٨٨
1.0	٤٥.٣٣	٨٢.٦٦	١٠٣.٣٣	٧٧.١٠
2.0	٨٣.٣٣	٩١.٣٣	٦٨.٦٦	٨١.١٠
Mean	٤٧.٩	٧٢.٨٨	٧٨.٢١	
L.S.D	A	B	AxB	
	٩.١٤	٩.١٤	١٥.٨٣	

*** Induced callus was observed**

Table (2): Effect of different NAA concentrations and various explants on calli formation and quality of *S. rebaudiana* Bert

NAA (mg/l)	Explant	Explant number	Callused explant no.	Callused explant %	Days to calli initiation	callus colour	callus type	callus amount
0	Nodes	12	1	8.3	35	light green	fraiable	poor
	Leaves	12	2	16.7	38	light green	compact	poor
	*Vit.leaves	12	4	33.3	30	light green	fraiable	poor
	Roots	12	0	0	∞	--	--	--
1	Nodes	12	3	25.0	35	light green	fraiable	medium
	Leaves	12	1	8.3	38	yellow	compact	poor
	*Vit.leaves	12	6	50.0	28	light green	fraiable	medium
	Roots	12	0	0	∞	--	--	--
2	Nodes	12	8	66.7	30	light green	fraiable	medium
	Leaves	12	12	100.0	32	light green	compact	medium
	*Vit.leaves	12	12	100.0	25	light green	fraiable	high
	Roots	12	1	8.3	35	yellow	compact	poor
3	Nodes	12	12	100.0	30	light green	fraiable	high
	Leaves	12	8	66.7	25	light green	compact	medium
	*Vit.leaves	12	10	83.3	21	light green	fraiable	high
	Roots	12	5	41.6	30	yellow	compact	poor
4	Nodes	12	12	100.0	28	light green	fraiable	high
	Leaves	12	12	100.0	25	light green	compact	medium
	*Vit.leaves	12	7	58.3	21	light green	fraiable	high
	Roots	12	12	100.0	30	yellow	compact	poor
5	Nodes	12	6	50.0	35	light green	fraiable	medium
	Leaves	12	6	50.0	30	light green	compact	medium
	*Vitr.leaves	12	4	33.3	30	light green	fraiable	medium
	Roots	12	8	66.0	28	yellow	compact	medium

***Vit. Leaves: vitrified leaves*Vit. Leaves: vitrified leaves**



Where: A: 0mg/l BAP+ 0mg/l NAA, B: 0.5mg/l BAP+ 0mg/l NAA,C: 1mg/l BAP+ 0mg/l NAA
J and K: calli initiated on basal nodes
D: 0mg/l BAP+1mg/l NAA, E: 0.5mg/l BAP+1mg/l NAA, F: 1mg/l BAP+1mg/l NAA,
G: 0mg/l BAP+1mg/l NAA, H: 0.5mg/l BAP+1mg/l NAA and I: 1mg/l BAP+1mg/l NAA.

Fig (1): Effect of different NAA concentrations on proliferation and callus induction of *S. rebaudiana* Bert.

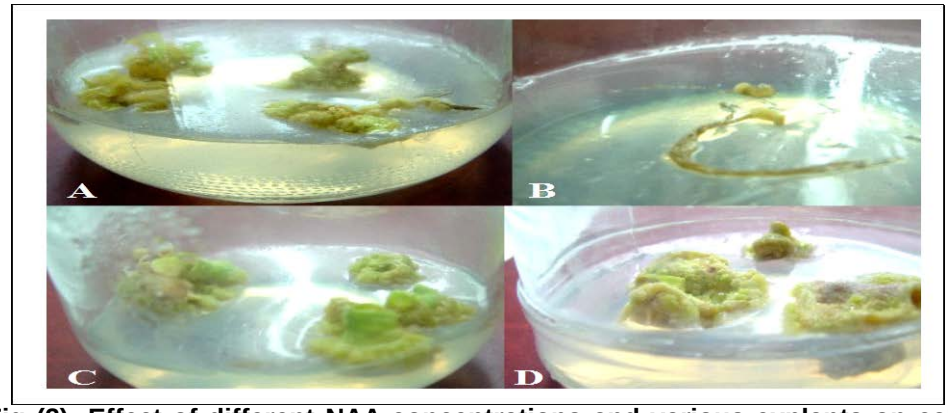


Fig (2): Effect of different NAA concentrations and various explants on calli formation and quality of *S. rebaudiana* Bert

explants), while node segments achieved the best results for callus formation with MS basal medium containing 3 mg/l NAA. Root explant response was different than leaves and nodes explants for callus formation. It may be due to the endogenous hormones containing explant. Days to initial calli were various according to both NAA concentrations and different explants Vitrified leaves were the earliest explant formed callus when these vitrified leaves were planted on MS basal medium supplemented with 3mg/l NAA (21 days), followed by normal leaves (25 days). Calli types differed due to explants, node and vitrified leaves explants results in friable calli while

normal leaves and roots explants gave compact calli. Callus amount varied according to NAA concentrations and various explant types. Low concentration of NAA produced the poorest calli, high calli results from 2 and 4mg/l NAA when vitrified leaves, nodes were the explants, respectively.

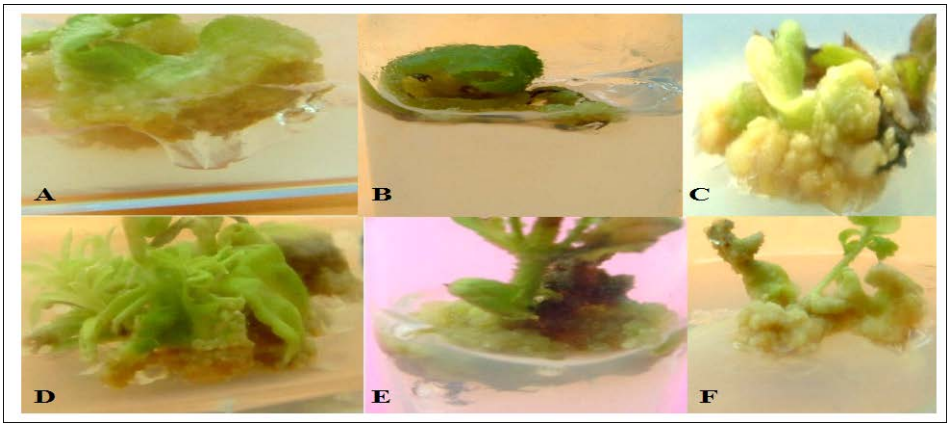
Results in Table (3) and Fig (3) clearly show the response of various explants (leaves and nodes) when they were planted in different culture containers on calli formation and quality of *S. rebaudiana* Bert. Both explants (nodes and leaves) initiated friable calli earlier when they were planted in tubs (25 and 21 days, respectively). The highest calli amount resulted from both explants when they planted in jars (250ml) or cubic magenta (250ml).

Results came in agreement with Janarthanam *et al*., (2009) who stated that juvenile leaf explants of *Stevia rebaudiana* Bertoni produced maximum callus than the nodal explants cultured on MS containing 11.31 µM 2, 4 -D and 2.22 µM BAP. Callus transferred to MS supplemented with 4.44 µM BA and 1.34 µM NAA showed better growth response and produced 14.0 ± 1.0 shoots with an average length of 5.6 ± 0.1 cm after 28 days. Callus response varied between nodal and leaf explants with 60 and 80%, respectively. Leaf explants produced maximum callus, the formation of early callus from leaf explant of *Stevia rebaudiana*.

Results in Table (4) and Fig (4) showed the effect of IAA and 2, 4-D concentrations on calli differentiation of *Stevia rebaudiana*. Calli differentiated in to organogenesis or embryogenesis. Calli differentiation of *Stevia rebaudiana* was affected by both IAA and 2, 4-D concentrations. Stevia calli differentiated to adventitious buds and subsequently shoots on MS medium supplemented with 0.2 or 0.4mg/l 2,4-D. On the other side, Stevia calli differentiated to roots on MS basal medium supplemented with 0.4mg/l IAA. Adventitious shoots produced from leaves callus were transferred to MS medium containing 0.25mg/l IBA (according to Ibrahim *et al*., (2010) . The survival plantlets were transferred to planting medium containing peatmoss, sand and perlite at equal volume and left in greenhouse for adaptation and subsequently for more growth and development (Fig, 5). Each produced plant has a genetic structure may be similar or a similar to the mother plants (this point under studies).

Table (3): Effect of different culture containers and various explants on calli formation and quality of *S. rebaudiana* Bert.

Container	Explant	Days to calli initiation	Callus amount	Callus type
Tube	Nodes	25	medium	friable
	Leaves	21	medium	friable
Jar	Nodes	28	high	compact
	Leaves	24	high	compact
Cubic majenta	Nodes	24	high	friable
	Leaves	20	high	friable

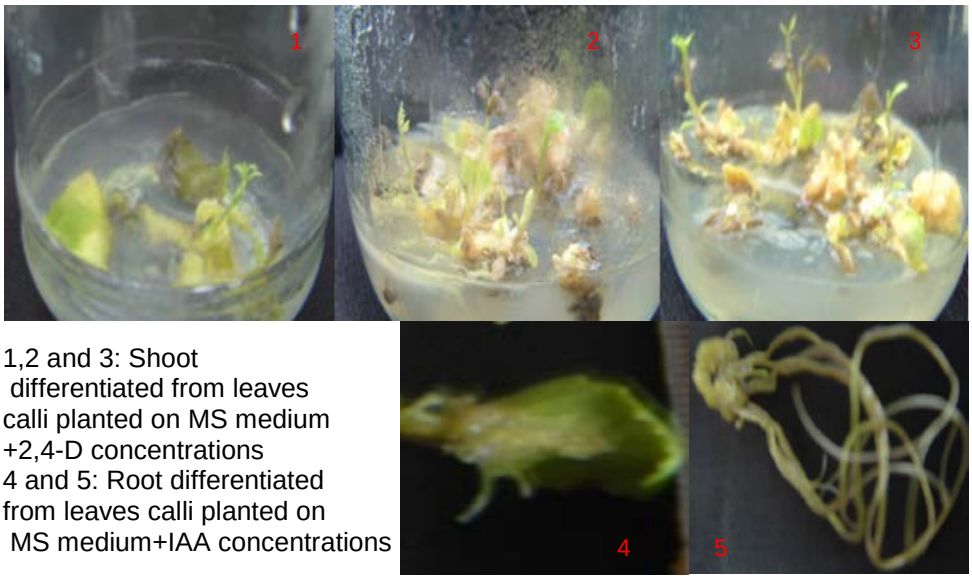


A: Leaf callus planted in cubic magenta. B: Leaf callus planted in tube
C: Leaf callus planted in jar
D: Node callus planted in cubic magenta E: Node callus planted in tube
F: Node callus planted in jar

Fig (3): Effect of different culture containers and various explants on calli formation and quality of *S. rebaudiana* Bert

Table (4): Effect of different concentrations of IAA and 2.4-D on calli differentiation of *S. rebaudiana* Bert.

Auxin type	Concentration	Type of response	No of differentiated
IAA	0.0	-	-
	٠.٢	-	-
	٠.٤	Root	+ +
	٠.٦	-	-
2,4-D	٠.٠	-	-
	٠.٢	Shoot	+ +
	٠.٤	Shoot	+
	٠.٦	-	-



1,2 and 3: Shoot differentiated from leaves calli planted on MS medium +2,4-D concentrations
4 and 5: Root differentiated from leaves calli planted on MS medium+IAA concentrations

Fig (4): Effect of different concentrations of IAA and 2.4-D on calli differentiation of *S. rebaudiana* bert.

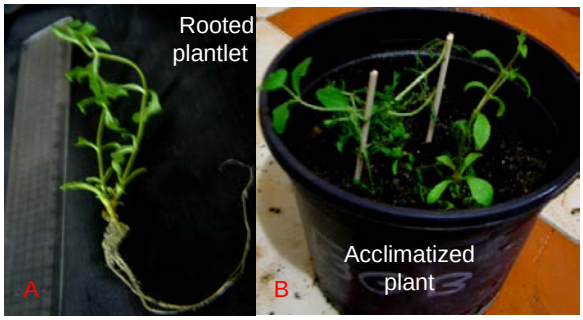


Fig (5): A: Rooted plantlet derived from differentiated leaf callus of *S. rebaudiana*.
B: Acclimatized plantlets derived from differentiated leaf callus *S. rebaudiana*

Results under discussion are in line with Janarthanam *et al.,* (2009) who demonstrated the ability to regenerate whole plants of *Stevia rebaudiana* from leaf explants. Significant numbers of shoots were produced in this study through an intervening callus phase with all regenerates showing normal phenotypes. This reseach described callus mediated regeneration of *Stevia rebaudiana* from leaf explants and describes a rapid and simple protocol to

obtain large numbers of plantlets within a short period. Also same the results obtained by Taware *et. al*., (2010) who demonstrated that Shoot initiation from callus was maximum in MS+BA+2,4-D.

Results are a step in tissue culture technique improvement of *Stevia rebaudiana* Bert. in Egypt. Other studies should be carried out to obtain the optimum conditions for producing stevia and its active components *in vitro*. Anyway, production of *Stevia rebaudiana* through in direct method may be a very effective method in plant breeding programs which may be selected to high metabolites production.

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تكوين الكالوس وتكشفه للستيفيا ريبديانا بيرتونى .طريقة لتحسين النبات في مصر

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الملخص العربي

يعد نبات ستيفيا ريبديانا من الأعشاب المستخدمة في التحلية والغير مسببه لارتفاع في السعرات الحرارية والتي تنتمي إلى العائلة المركبة. تعد نباتات الاستيفيا المحلى الوحيد الذى يستخدم في علاج مرضى السكر. جمعت الأجزاء النباتية من مشتل معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية–مدينة السادات–جامعة المنوفية–مصر.اختبر تاثير التركيزات المختلفة للبنزيل ادينين ونفثالين حمض الخليك على تضاعف الفروع وإنتاج الكالوس. نتج اكبر عدد للفروع من التركيز ١ملليجرام/لتر بنزيل ادينين+٠.٥ملليجرام/لتر نفثالين حمض الخليك.كما اختبر تأثير التركيزات المختلفة لنفثالين حمض الخليك (٠و ١و ٢ و٣ و٤ و٥ ملليجرام/لتر) والأجزاء النباتية المختلفة (العقل –والأوراق– والأوراق التي بها الظاهرة الزجاجية) على إنتاج الكالوس وجودتة. تكون الكالوس على الأوراق التي بها الظاهرة الزجاجية مبكرا (٢١يوم) تبعته الأوراق الطبيعية (٢٥يوم) عندا لزراعة على نفس التركيز من نفثالين حمض الخليك. كما تم دراسة نوع أوعية الزراعة (أنابيب– برطمانات– ماجينتات مكعبة) على تكوين الكالوس وجودته.نتجت أعلى كمية للكالوس عن كلا المنفصلين النباتين عند الزراعة في برطمانات أو ماجينتات مكعبة.لوحظ

تكوين أفرع على الكالوس الناتج من الورقة عند نقله على بيئة موراشيجى وسكوج المزودة ب ٠.٢ ملليجرام/اللتر كلوروفينوكسى حمض الخليك بينما تكشف الكالوس الناتج من الورقة الى جذور عندمانقل على بيئة موراشيجى وسكوج المزودة ب ٠.٤ملليجرام/لتر اندول حمض الخليك. كل فرع متكشف لهتركيب وراثي قد يكون مماثل أو مخالف للنبات الأم. التماثل الوراثى والمركبات الطبيعية النباتية للنبيتات الناتجة من كالوس الورقة تحت الدراسة.