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AN EFFICIENT *IN VITRO* CULTURE PROTOCOL OF STEVIA PLANTS (*STEVIA REBAUDIANA* BERTONI VAR. CHAINE.2) WITH CYTOGENETICAL STUDIES

Kasem Z. Ahmed[1], Sayed A.-M. Osman[1*], Shaban M. Saber[2] and Ahmed A. Abdel-Hamed[2]

[1] Department of Genetics, Faculty of Agriculture, Minia University, El-Minia, Eg-61519, Egypt
[2] Department of Agricultural plant, Faculty of Agriculture, Al Azhar University, Assuit Branch, Assuit, Eg-71524, Egypt

*Corresponding author: sayed.osman@mu.edu.eg

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ABSTRACT

Stevia plants **(***Stevia rebaudiana* Bertoni**)** became an economically important medicinal plant act as a sugar substitute for diabetic and obese people in many countries. In Egypt, we faced a shortage in sugar production and no more available water for irrigation of sugarcane or sugar beet crops. Stevia, a non-caloric sweetener, is being many times sweeter than sucrose also is required less water. Stevia has difficulties in propagation and improvement through seeds and vegetative methods, so *in vitro* biotechnology is the protocol has been an efficient alternative for propagation and improvement this plant. Leaf segments as the best explants were used in the present work to *in vitro* culture of stevia. For surface sterilization, the best exposure time and the combination were found that mercuric chloride (0.1%) for 2 minutes, ethanol (70%) for a one minute, and sodium hypochlorite solution (commercial Clorox®) (15%) for 15 min. Murashige and Skoog (MS) media supplemented with a wide range of concentration and combination of plant growth regulators (PGR) were tested. MS medium containing 1.0 mg/l 2, 4-D (2,4 Dichlorophenoxy acetic acid) + 1.0 mg/l NAA (Naphthalene acetic acid) was the highest and significant callus induction percentage (96%). However, the narrow range was observed for plant regeneration (1.8 – 3.6 plantlets/callus) as

well as plantlet length (1.176 – 3.370 cm). A significant variation was observed for leaves number/regenerated plantlet (3.20 to 6.14). MS medium contains 1.8 mg /l BAP (Benzyl amino purine) + 0.12 mg /l NAA resulted the highest regenerated plantlets/callus (3.600). Healthy rooted plants were obtained and transferred to pots for *ex vitro* hardiness under controlled environmental conditions. The effects of callus induction medium on the activity of mitotic cell division and their mitotic index were evaluated. Wide range and significant variation were observed among mitotic phases and mitotic indexes (MI) grown on different tested MS media. Stevia calli were grown on MS medium + 2.0 mg/l NAA exhibited the highest MI value (9.82%) with a significant difference with all other six used media. Calli cells at metaphase were showed the normal chromosome number 2n=22.

Keywords: Callus induction, Cytogenetic, *In vitro* culture, Mitotic index, Plant growth regulators, Plant regeneration, *Stevia rebaudiana*

INTRODUCTION

The genus *Stevia* (Eupatorieae, Asteraceae), which consists of approximately 150-200 species of herbaceous, shrub and sub-shrub plants, is one of the most distinctive genera within the tribe Eupatorieae (Gentry, 1996, de Oliveira *et al*. 2004, Uddin *et al*., 2006, Lavini *et al*., 2008, Sairkar *et al*., 2009, Yadav *et al.* 2011, Bawane *et al*., 2012). Among them, *Stevia rebaudiana* Bertoni originated in the Rio Monday Valley, Paraguay (Das *et al*., 2006, Bawane *et al*., 2012, Gauchan *et al*., 2014). It is a perennial shrub; the mature plant grows up to 65-180 cm esp. when cultivated or growing naturally in fertile soil (Jahan *et al*., 2014, Majumder and Rahman, 2016). It is a short-day plant and flowering from January to March in the southern hemisphere. It prefers a sandy soil and a warm sunny position with temperature 21 to 43°C, with an average 24°C (Jahan *et al*., 2014). The chronological records show that stevia leaves have been used for hundreds of years by the Guarani Indians. The main use was as a sweetener, particularly in their green tea, it was also used in medicine or as a snack (Ahmed *et al*., 2011).

After domestication and assessed agronomically of stevia in near years, *Stevia rebaudiana* Bertoni became an economically important medicinal plant act as a sugar substitute for diabetic and obese people in the early 1970s. Stevia a non-caloric sweetener being many times sweeter than sucrose (Ghallab and Saleh 2012, Hassanen and Khalil 2013, Lata *et al.,* 2013 a & b, Razak *et al.,* 2014, Namdari *et al.,* 2015) that does not ferment in the human body and their leaves are the principal source of stevioside, which has clinical

significance as they are reported to maintain glucose levels in human blood and to date there have been no reports of adverse effects from its use (Yadav *et al.* 2011 and Razak *et al.*,2014).

Propagation of stevia considered as a recalcitrant task, it's usually done by stem cuttings, which root easily, but require high labor inputs. Some plant varieties/selections produce virtually no viable seed and vegetative propagation is the only way of multiplication (Yadav *et al.* 2011.) Many different parts of the plant viz., leaves, auxiliary shoots, root-neck sprouts, shoot primordia, internodal explants etc., can be used successfully for tissue culture propagation. Seed germination of Stevia is often poor (Ahmed *et al*., 2007, Majumder and Rahman, 2016). Moreover, Shock (1982), Duke (1993), Carneiro *et al.* (1997) and Lester (1999) reported a poor and highly variable percentage of viable seeds.

The genus *Stevia* shows great variation in chromosome number. Previously, chromosome number of *Stevia rebaudiana* (2n=22) was reported by Monteiro (1980 and 1982) and Frederico *et al.* (1996) for various strains. However, strains with 2n=33 and 2n=44 (representing triploid and tetraploid cytotypes) also occur, which show a high degree of male sterility owing to the chromosomal abnormalities during gamete formation (de Oliveira *et al*. 2004). Although, most of the *S. rebaudiana* strains had 2n = 22, however, genetic variability has been observed in plant size, flowering period, and stevioside content, which may vary from 2% to 10% (Magalhães, 2000). The induction of polyploidy to improve agronomic yields is a process commonly used in plants of economic interest (Allard, 1960) and has been applied to other species. Polyploidy results in the better adaptability of individuals and increased organ and cell sizes. Tetraploids have larger leaf size, thickness and have potential use in increasing biomass and yield in comparison with diploid strains (Yadav *et al.,* 2011). Development of new varieties of *S. rebaudiana* with a higher content of rebaudioside-A and a reduced content of stevioside is the primary aim of plant breeders concerned with the improvement and utilization of this source of natural sweeteners (Lavini *et al*., 2008, Bawane *et al*., 2012, Namdari *et al.,* 2 015).

In Egypt, the promising stevia plant was introduced at the Agriculture Research Centre, Giza, Egypt during the late 2000s, and studies on its adaptability were initiated (Alaam, 2007). However, at the beginning of adaptation of this plant, research focused on cultivation rather than crop improvement and very recently, few steps for genetic improvement were published (Ghallab and Saleh, 2012, Hassanen and Khalil, 2013, Ali *et al.,* 2014).

Use of biotechnological approaches, such as tissue culture for the mass propagation of elite genotypes, anther culture for

development of pure homozygous doubled haploid and molecular marker technology for identification of marker loci linked to rebaudioside-A trait, can create new opportunities for plant breeders (Yadav *et al.*, 2011).

In the present study, we try to establish *in vitro* cultural protocol to improve stevia under Egyptian condition. Induction of calli and derived somaclones may prove easy and efficient tools for raped and overcome problems of traditional plant breeding methods with this new commercial crop. Stevia *rebaudiana* Bertoni Chaine.2 cultivar used as donor explant and tested most effective steps in *in vitro* culture and establishment *in vitro* regeneration protocol with cytogenetically test.

MATERIAL AND METHODS

In vitro callus induction:

Plant material: Stevia transplants (*Stevia rebaudiana* Bertoni var. Chaine.2) were obtained from Sugar Crops Research Institute, Agriculture Research Center, Giza, Egypt and replanted in suitable pots with clay soil and all agriculture practices were applied correctly under greenhouse condition of Department of Genetics, Faculty of Agriculture, Minia University, El-Minia, Egypt. The growth of those plants was followed weekly and best healthy growing plants were selected as explant source for our experiments.

Explant preparation: Based on published previous studies of many laboratories worldwide on stevia *in vitro* tissue culture and successfully used leaves as optimum explant, we were followed. At optimum physiological conditions of stevia plants (June, July, and August), the four youngest tip leaves (~ 3-4 cm) of each plant were collected and directly transfer to Biotechnology Lab, Department of Genetics, Faculty of Agriculture, Minia University for further works.

Surface sterilization: Hence, surface sterilization is a very critical step in tissue culture process. We tested six different exposure times for three different detergents and we tested the viability of leaf tissues to produce callus. In the beginning, collected leaves were washed under running tap water for 20 minutes to remove the dust, and minimize the contamination. Explants were moved under aseptic conditions in laminar air flow cabinet. Leaves were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride), then treated in ethanol solution 70 %, then surface sterilized with 15 % sodium hypochlorite solution; different exposure times of these three detergents were tested (Table 1). Finally, sterilized explants were washed 3-4 times with sterile double distilled water and cut in small parts (1-2 cm). Then explants were inoculated on culture medium.

Table (1) Different treatment (in minutes) and concentration used in surface sterilization of stevia (*Stevia rebaudiana* Bertoni var. Chaine.2) young leaves for *in vitro* culture studies.

Treatment. No.	$HgCl_2$ (0.1%) Exposure Time	Ethanol (70%) Exposure Time	Clorox (15%) Exposure Time
1	1	1	5
2	1	1	10
3	2	1	15
4	2	1	5
5	3	1	10
6	3	2	15

Culture medium and plant growth regulators used for callus induction: The culture medium consisted of (MS) salts with vitamins (Murashige and Skoog, 1962). The medium supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. In this experiment, explants were cut into small parts cultured on MS medium supplemented with different (PGR) and combination to check the best induction/growth medium (Table 2). MS medium (without plant growth regulators) was used as a control. Five explants were cultured per jar as one replicate.

Table (2) Different concentrations (mg/l) and the combination of four plant growth regulators (two auxins: NAA, 2,4-D) and (two cytokinines: Kin and BAP) used for callus induction in stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2).

Treatment	NAA	Kin	BAP	2,4-D
Control	0.0	0.0	0.0	0.0
T_1	0.0	0.0	0.0	3.0
T_2	2.0	0.0	0.0	0.0
T_3	1.0	0.0	0.0	1.0
T_4	1.5	0.0	0.1	0.0
T_5	1.0	0.2	0.0	0.0
T_6	0.0	0.0	0.5	1.5
T7	0.0	0.5	0.0	1.0

All media pH was adjusted to 5.7±0.1 with 0.1 KOH and HCl before autoclaving. Specific MS medium was distributed into Baby Food Jars (200 ml) with 25 ml each. Media were autoclaved under 1.1 kg/cm² and 121°C for 20 minutes and were ready for culture.

Culture condition: The cultures were incubated in incubators at 27°C±2°C at the darkness for 3-5 weeks with weekly observation and following up with any changing in growth habit. At the end of the growth period, the percentage of obtained calli were calculated based upon a number of cultured

explants and statistical analysis using MSTAT program (Version 4) to get the best medium.

Plant regeneration from obtained calli: For plant regeneration, healthy, well-grown calli were selected to obtain plants. Firstly, calli were separated into small pieces (~3-4 mm) then were transplanted to regeneration medium. To regenerate plants, four different MS media with different concentration and combination of (PGR) were used, as well as hormones-free MS media was used as control (Table 3).

Table (3) Different concentration (mg/l) and the combination of plant growth regulators (BAP, Kin: Kinetin, NAA, and GA3: Gibberellic acid) which added to MS media for plant regeneration of stevia plant (*Stevia rebaudiana* Bertoni var. Chaine.2).

Treatment	BAP	Kin	NAA	GA_3
Control	0.0	0.0	0.0	0.0
T_1	2.0	0.0	0.0	0.0
T_2	1.5	0.5	0.0	0.0
T_3	1.5	0.0	0.0	1.0
T_4	1.8	0.0	0.12	0.0

Culture condition: The growth room conditions maintained for *in vitro* cultures were 26 ± 2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16h day light and 8h dark. Cultures were maintained for 4-6 weeks and a number of regenerated plantlets/plants per callus, plant length and number of leaves per plantlet were recorded and also, statistically analyzed using MSTAT program (Version 4).

Root formation: To obtain healthy roots, regenerated plantlet/plant about (1.5-2.0 cm length) were subcultured into Baby Food Jars (200-ml) with 25 ml of PGR-free MS medium containing 0.8% agar (w/v) and 3% sucrose (w/v), with pH 5.7±0.1. Media were autoclaved under 1.1 kg/cm² and 121°C for 20 minutes.

Rooting condition: for rooting, the growth room conditions were adjusted to 26±2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16h day light and 8h dark (as mentioned above).

Acclimatization Stage: The *in vitro* healthy rooted plantlets/plants were transferred to acclimatize pots contained the sterile composition of soil:sand (1:1; V: V) and irrigated periodically with sterile water. These pots were kept in controlled environmental conditions suitable for hardiness of *ex vitro* stevia plants. We considered one independent plant derived from independent callus as one somaclonal.

Cytological study:

Obtained healthy, vigorous growth calli from different MS callus indication/growth media were selected for the mitotic study.

Calli after refresh growth for 2-3 weeks in callus growth MS medium were collected and some of them were soaked in colchicine solution (0.05%; an antimitotic agent) for 3 hours then all samples were fixed in fresh Farmer's solution (ethanol:acetic acid; 3:1, v:v) overnight. The calli were stored in 70 % ethyl alcohol and kept in the refrigerator (5°C) tell microscopic examination. Percentage of cells with prophase, metaphase, and Ana-telophase were recorded and (MI) was calculated for colchicine-non-treated calli for each different callus induction media to explore the effect of MS medium on callus growth and (MI).

Stevia calli were prepared as mentioned above and stained with aceto-carmine (Ahmed *et al*. 1999). Temporary squash preparations of calli cells were made in one percent aceto-carmine. Slides were prepared by smearing the callus pieces and macerated on a glass slide in a drop of aceto-carmine stain. Around 300 divided mitotic cells were examined for estimating and calculating mitotic index per each MS medium. Data were statically analyzed (in RCBD) using MSTAT program (Version 4). Obtained calli cells were examined and photographed using an Olympus BX 51 microscope at an initial magnification of X 2000. Photomicrographs of suitable mitotic cells were taken for illustration using C-4040 digital camera through the microscope eyepiece.

RESULTS AND DISCUSSION

Successful plant tissue culture process depends upon many factors e.g. before and through *in vitro* culture as well as *ex vitro* adaptation. Starting with explant-donor plants and its environmental conditions (field grown or greenhouse conditions), washing collected materials and outmost important are surface stabilization and healthy of prepared explants. Through sterilization process, many different microorganisms-toxic chemical components have been tested through several decades. The detergent type, concentration, and exposure time strongly affect target plant materials. For more efficient surface sterilization, many research groups were used more than one chemical component to ensure a high degree of disposal of many contaminated microorganisms with maintain the vitality of plant cells and increase the efficiency of sterilization.

Surface sterilization protocol:

Therefore, surface sterilization is essential step to obtain successful tissue culture protocol and *in vitro* systems. The efficiency of the sterilization varies according to the chemical concentration used and time of exposure etc... Our first aim was the establishment of efficient surface sterilization protocol for stevia plant grown under greenhouse conditions. Freshly harvested stevia leaves explants were treated with (0.1%) mercuric chloride ($HgCl_2$), then (70%) ethanol and finally with (15%) sodium hypochlorite (commercial Clorox®, Na ClO) with different

times, to explore the best time combination for surface sterilization, with careful observation of the sterilized leaves on *in vitro* culture (leaves color and what if there is microbial contamination appear in the new culture).

Among the six different combinations of sterilization treatments, three of them were contamination-free cultures, but only one of them their explants were still alive with green color and further produced healthy calli (Table 4). The other three combinations were completely covered with microbial contamination, although the explants were green in the begging of its growing, but with the passage of time, these explants were completely disappeared under strongly growing of contaminating microorganisms. The best exposure time and the combination were found that mercuric chloride (0.1%) for 2 minutes, ethanol (70%) for a one minute, and finally Clorox® (15%) for 15 min. However, careful washing many times with double distilled serialized water was very critical to deposable of residual effects and complete removal of the toxic chemical agents. Therefore, our all next experiments were followed this efficient protocol and we get hundreds of very healthy calli from leaves explants.

Our finding was cordoning with other research laboratories e.g. Preethi *et al*. (2011a) reported that the cultured explants showed more than 80% contamination-free cultures when treated with 0.1% $HgCl_2$ for 2 min for surface sterilization. On the other hands, Guruchandran and Sasikumar (2013) observed that treatment of explants with 0.3% mercuric chloride for five minutes duration was the best for obtaining contamination-free cultures and higher survival percentage**.** This concentration of mercuric chloride prevented the growth of the microbial contaminants. They also reported that the cultured explants showed more than 80% contamination-free cultures when treated with only 0.1% mercuric chloride for two minutes. As a general observation from many experiments, a higher concentration of mercuric chloride and longer time of exposure produced cultures with less contamination, but the percentage of survival of explants decreased. Therefore, a moderate concentration of mercuric chloride (0.1%) with suitable exposure time (~2 minutes) may produce a higher percentage of survival sterilized explants which can produce healthy *in vitro* culture plant organs.

Using two detergent chemicals for surface serialization was efficient for this goal, where, Gauchan *et al*. (2014) found that the dipping of explants in 70% ethanol for 1 minute followed by sterilization with $HgCl_2$ (0.1%) for 3 minutes as it prevented browning of tissues and microbial contamination. Based on our results and available literature, we can recommend use three sterilization steps (as described above) which we believe that it is more efficient

for leaves surface sterilization and subsequent *in vitro* culturing.

Table (4) Effect of different exposure time of different detergents solutions and combinations on biological and plant contamination status of surface sterilization of stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2).

Treatment. No	$HgCl_2$ (0.1%) Exposure Time (minute)	Ethanol (70%) Exposure Time (minute)	Clorox® (15%) Exposure Time (minute)	Reaction of explants	
				Response of Explants	Contamination Status
1	1	1	5	Green (live)	Contaminated
2	1	1	10	Green (live)	Contaminated
3	2	1	15	Green (live)	clean
4	2	1	5	Green (live)	Contaminated
5	3	1	10	Brown (dead)	clean
6	3	2	15	Brown (dead)	clean

Callus induction from leaves segments:

Surface sterilized leaf segments (1-2 cm) of stevia plants were used as a primary explant to callus induction on MS media supplement with various combination and concentration of PGR (Table 2). This experiment was repeated many times, each with five replicates, all of the replicates contain five explants, and callus induction data were recorded every week, and after three weeks of culture, initiation recorded the final data.

Inoculated leaf segments on MS medium fortified with different concentrations of auxins (NAA and/or 2, 4-D) singly and/or in combination with cytokinins (BAP and/or Kinetin; Table 2) gave a varied callusing response (Table 5, Fig. 1). Leaf segments were also cultured on MS medium without PGR as (control). There is no response for callus induction even after five weeks of incubation or more on control MS.

Different types of MS media differ in concentrations and combination of PGR were used to callus induction. The MS medium containing (1.0 mg/l 2,4-D + 1.0 mg/l NAA) gave the best result with highest mean of callus induction/5 explants (4.8 with 96% percentage). However, wide range and significant effects were detected for callus induction (2 to 4.8 callus/replicate which contain 5 leaf segments), the percentage also ranged from 40% to 96% between the 7 different MS media used in this study (Table 5, Fig. 1). This explains the critical role of plant growth regulators can play in *in vitro* culture of stevia plant. This conclusion supports the well-known phenomena in plant tissue culture (Gupta *et al*. 2010**)**.

Back to our results, where two MS media also produced relatively high callus indication rate, the first

one was containing 2.0 mg/l NAA (Preethi *et al*. 2011a), which give 3.8 calli/5 explants i.e. 76%, and the second MS medium supported by 3.0 mg/l 2,4-D (Uddin *et al*. 2006), give 3.4 calli/5 explants, i.e.72%. However, the moderated success media were MS medium containing 1.0 mg/l NAA + 0.2 mg/l Kin give 2.4 calli/5 explants i.e. 48% and MS + 1.5 mg/l NAA + 0.1 mg/l BAP or MS with 1.0 mg/l 2,4-D + 0.5 mg/l Kin which both produced 2.2 calli/5 explants i.e. 44%. Preethi *et al*. (2011a) used also the same media and get similar results. However, the least productive MS medium in our experiment was induced only two calli (40%) which contains 1.5 mg/l 2, 4-D + 0.5 mg/l BAP, this can be considered as the poorest callus induction medium in our experiments (Table 5, Fig. 1); as reported also earlier by Guruchandran and Sasikumar (2013).

Generally, the present results were agreeing with the findings of many research group worldwide e.g. Uddin *et al*. (2006), Gupta *et al*. (2010) and Preethi *et al*. (2011a).

Using a combination of two synthetic auxins, 2,4-D at 1 mg/l and NAA at 1 mg/l represent auxin-cytokinin interaction to induce stevia calli was perfect than any other combination or using each PGR alone. Maybe using auxin:cytokinin in 1:1 ratio very suitable for stimulating stevia leaves to induce calli. Also, Gupta *et al*. (2010) used the same ratio and has got the same results.

Table (5) Effect of plant growth regulators and its combination in MS media on callus induction from leaf segments explant of stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2).

Treatment		Means of Callus induction/5 explants*	Callus Induction (%)
1	Control	0.000	0.0
2	3.0 mg/l 2,4-D	3.400^{bc}	72%
3	2.0 mg/l NAA	3.800^{ab}	76%
4	1.0 mg/l 2,4-D+1.0 mg/l NAA	4.800^{a}	96%
5	1.5 mg/l NAA+0.1 mg/l BAP	2.200^{d}	44%
6	1.0 mg/l NAA+ 0.2 mg/l Kin	2.400^{cd}	48%
7	1.5 mg/l 2,4-D+ 0.5 mg/l BAP	2.000^{d}	40%
8	1.0 mg/l 2,4-D+0.5 mg/l Kin	2.200^{d}	44%

*****The different letters following the means of callus indication were indicated significantly different at LSD 5%, while the means followed by the same letter were not significant at LSD 5%.

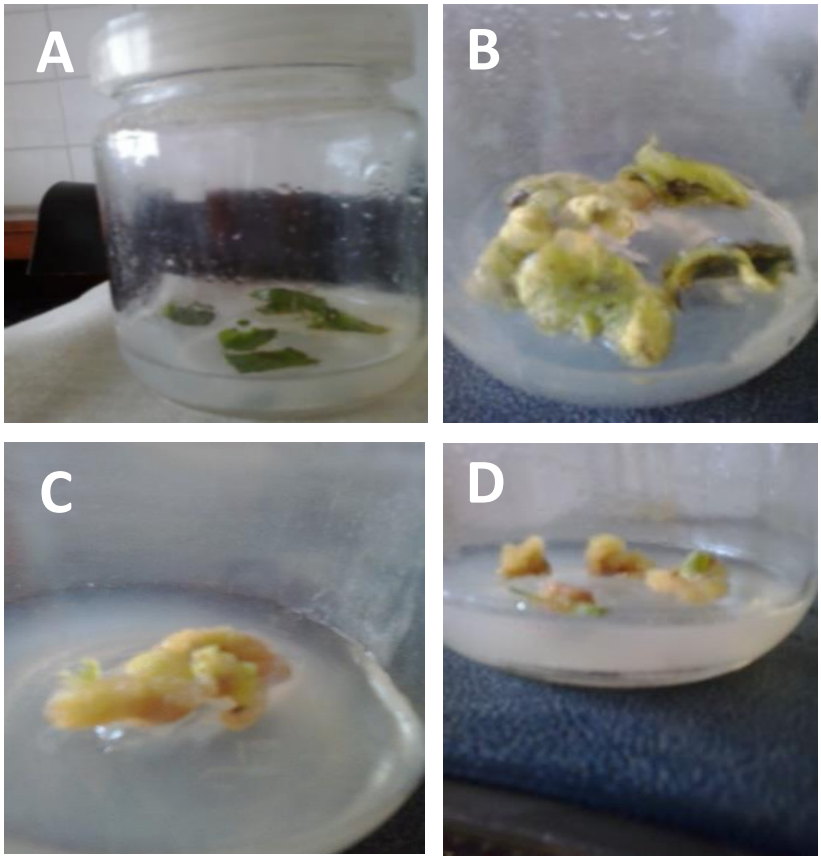

Figure (1): A-D, Steps for callus induction from leaf segments explant of stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2) using MS medium supplemented with different plant growth regulators and its combination; A, leaf explant incubated on MS callus induction medium; B, Callus initiation on leaf segments, C & D; induced calli.

Plant regeneration from obtained stevia calli:

The obtained calli have been divided into small pieces (3-4 mm) and subcultured to five different MS regeneration media which supplemented with a different combination of PGR. Plantlets were regenerated on all types of media except the control MS medium with the PGR-free medium. However, narrow range was observed (1.8 – 3.6 plantlets/callus) as well as means of plantlet length (1.176 – 3.370 cm), therefore, no significant between these MS media were detected. But for means of leaves number/regenerated plantlet, significant variation was observed between the MS media used to regenerate those plantlets and wide range was notice (3.20 to 6.14 leaves/plantlets; Table 6; Fig 2). The obtained results are going to the same manner of several published results e.g.

Moktaduzzaman and Rahman (2009), Preethi *et al*. (2011a), Guruchandran and Sasikumar (2013) and Ali *et al.* (2014).

Although, no significant variation between MS regeneration media for number of regenerated plantlets, but its real visible that MS medium contains 1.8 mg /l BAP + 0.12 mg /l NAA resulted the highest obtained plantlets (3.600) from one inoculated callus, as reported also by other research groups **(**e.g. Moktaduzzaman and Rahman, 2009 and Ali *et al.*, 2014). In contrast, MS medium with 1.5 mg/l BAP + 1.0 mg /l GA_3, reported by Guruchandran and Sasikumar (2013), produced the least number (1.800 plantlets). Very interesting observation appeared when we compare the above results with the plantlet length results, hence the MS medium which produced the highest number of plantlets, those plantlets were very short relative to the plantlets derived from MS produced the least number of plantlets, maybe so kind of negative correlation between the number of regenerated plantlets and their length.

Respect to a number of leaves/regenerated plantlet, MS medium contains 1.8 mg /l BAP + 0.12 mg /l NAA, produced plantlets with the highest number of leaves (6.14/plantlet), and significantly different from the other three regeneration media (Table 6, Fig. 2).

From the present results, the best medium for regenerate plantlets from calli of stevia leaf segment was MS medium supplemented with 1.8 mg/l BAP + 0.12 mg/l NAA, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, pH 5.7±0.1 before autoclaving and medium packed into 200-ml Baby Food Jars with 25 ml each. The cultures were grown under 26 ± 2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16h daylight and 8h dark.

***In vitro* rooting and *ex vitro* acclimatization:**

Several root-less plantlets were regenerated in vitro (as reported above). Therefore, those plantlets (~1.5-2.0 cm length) were subcultured into 25 ml of PGR-free MS rooting medium solidified with 0.8% agar (w/v) and 3% sucrose (w/v) with pH 5.7±0.1 (in 200-ml of Baby Food Jars). These cultures were kept under 26±20C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16 h daylight and 8 h dark. Tens of healthy rooted plants were obtained.

To acclimatize the *in vitro* healthy rooted plants, we transferred them to pots contained the sterile composition of soil: sand (1:1; v:v) covered for few days with transparent plastic bags and irrigated periodically with sterile water (Fig. 2). These pots were kept in controlled environmental conditions suitable for *ex vitro* hardiness of stevia plants.

Table (6) Effects of using MS regeneration medium with different plant growth regulators and its combination on plant regeneration from calli induced from leaf segments explants of stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2).

	MS medium with different plant growth regulators combinations & concentrations	Means of No. plantlets/callus	Means of Plantlet length (cm)	Means of No. of leaves/plantlets**
1	control	-	-	-
2	1.8 mg/l BAP+ 0.12 mg/l NAA	3.600 a	1.724 a	6.140a
3	2.0 mg/l BAP	2.600 a	1.176 a	3.880b
4	1.5 mg/l BAP+0.5 mg/l Kin	2.600 a	1.210 a	3.860b
5	1.5 mg/l BAP+ 1.0 mg/l GA	1.800 a	3.370 a	3.200b
	LSD.5%	N.S*	N.S*	1.220

***** N.S, non-significant LSD 5%.

******The different letter following the means of a number of leaves/plantlets indicate significantly different at LSD 5%.

Cytological study:

The growth rate of calli may be affected strongly by the composition of callus induction/growth medium. The above present results show that wide range and significant differences were detected between used callus induction media. Therefore, in this section will present and discuss the results of effects of MS medium type on the percentage of mitotic stages as well as a mitotic index (MI).

As discussed above, seven different callus induction/growth media were used for callus indication and growth, those media were varied according to their contents of PGR (Table 2 & 5; Fig. 1). Also, we checked the effects of these media on the activity of mitotic cell division and their mitotic index (Table 7). Wide range and significant variation were observed among mitotic phases and indexes of calli grown on different tested MS media. Prophase was the most common recorded phase in all calli grown on different growth media. Calli obtained from 1.0 mg/l 2,4-D + 1.0 mg/l NAA (was the best medium) and 1.0 mg/l 2,4-D + 0.5 mg/l Kin MS media gave the highest values of prophase index (92.59% and 91.78%, respectively), while, calli obtained from MS+1.0 mg/l NAA + 0.2 mg/l Kin medium showed the lowest value (81.42%) of prophase index when compared with the other media. Metaphase and ana- & telophase index (2.46% -12.85% and 2.38% - 7.31%, respectively) were observed with less repeat in calli grown in all different checked media (Table 7).

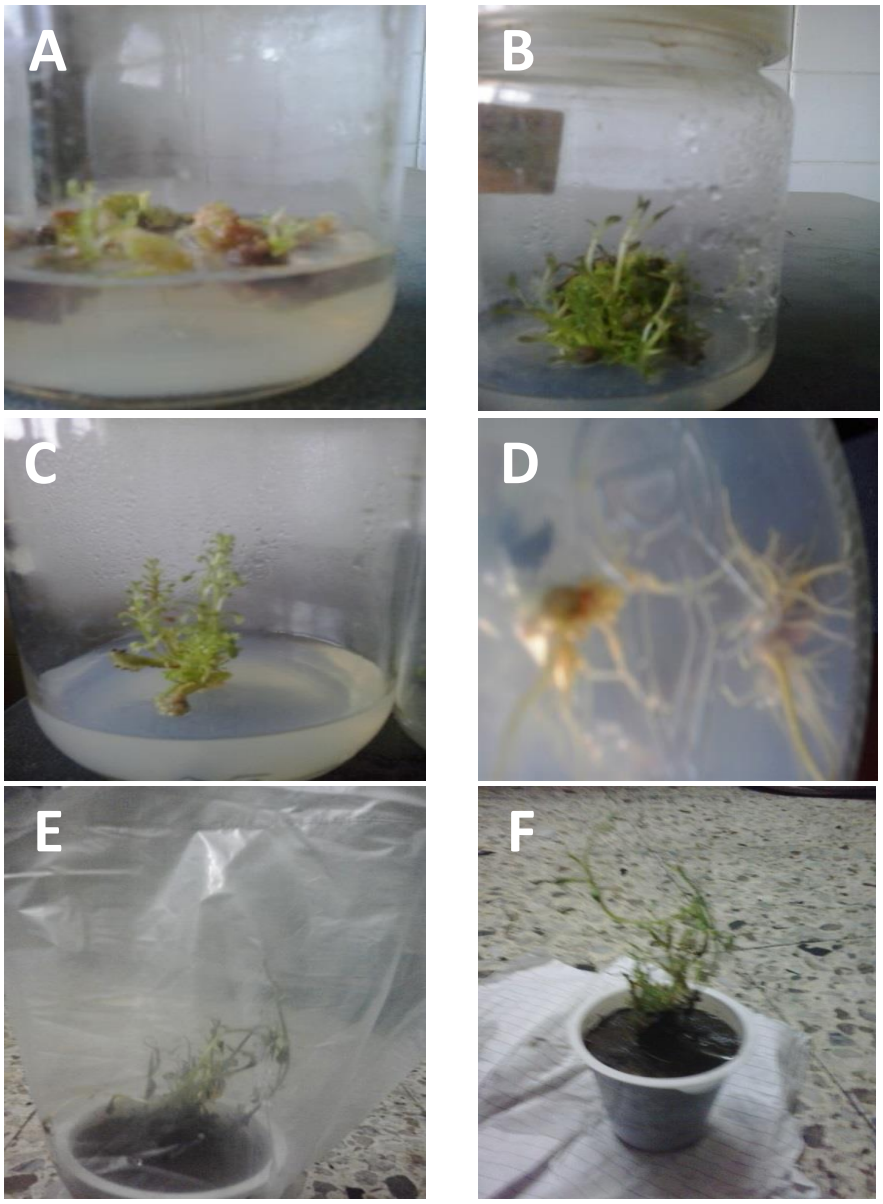

Figure (2) A-F: *In vitro* plantlets regeneration and *ex Virto* plants adaptation derived from calli induced from leaf segments explants of stevia plants (*Stevia rebaudiana* Bertoni var. Chaine.2). A, *in vitro* obtained calli starting plantlet regeneration; B, many shoots were regenerated from cultured calli; C, Complete rootless-plantlet already grown in *in vitro* culture. D, healthy roots were induced *in vitro* (note: inverted vail for photo); E, intact plant grown *ex Virto* in a pot covered with a plastic bag for hardness. F, adapted stevia plants grown normally in the pot for maturity.

The effect of different callus induction MS media on the mitotic index (MI) are shown also in Table (7). Stevia calli which grown on 2.0 mg/l NAA MS medium exhibited the highest MI value (9.82%) with a significant difference with all other used media. On the other hand, callus grown in 1.5 mg/l NAA + 0.1mg/l BAP medium gave the lowest MI value (4.36%) when compared with the other used media. These results indicate that PGRs have a clear effect on the rate of mitosis, which consequently affects the growth rate of the calli. From previously published papers, many other research teams found the same results (e.g. Frederico *et al.*, (1996) and Raina *et al*., 2013). Therefore, it is very important to select the best PGRs concentration and combination to get maximum *in vitro* culture growth.

Table (7): The percentages of different mitotic stages, as well as a general mitotic index (MI), obtained from stevia (*Stevia rebaudiana* Bertoni var. Chaine.2) calli cells grown on different MS callus induction media supplemented with different PGRs.

MS medium with PGR	Total no. of the tested cell	Prophase %	Metaphase %	Ana&telophase %	Mitotic index (MI)
NAA	271	88.75	6.25	5.00	9.82
2,4-D	391	88.09	9.52	2.38	7.16
2,4-D+NAA	371	92.59	2.46	4.93	7.27
NAA+Kin	322	81.42	12.85	5.71	7.23
2,4-D+Kin	358	91.78	6.84	1.36	6.79
NAA+BAP	313	85.36	7.31	7.31	4.36
2,4-D+BAP	307	88.46	7.69	3.84	5.65
$LSD_{0.5}$		3.54	4.98	1.13	1.74

Our investigation of mitotic calli cells at metaphase of stevia plant treated with colchicine solution (0.05%; an antimitotic agent) could observe the chromosome number was 2n=22 (Fig. 3).

These findings are in agreement with many research groups e.g. Monteiro (1980, 1982) and Frederico *et al.* (1996). However, the genus *stevia* (~ 200 species) shows great variation in chromosome number. de Oliveira *et al*. (2004) reported that strains with 2n=33 and 2n=44 (representing triploid and tetraploid cytotypes) were also observed, although most reports indicate that n = 11 (2n = 22).

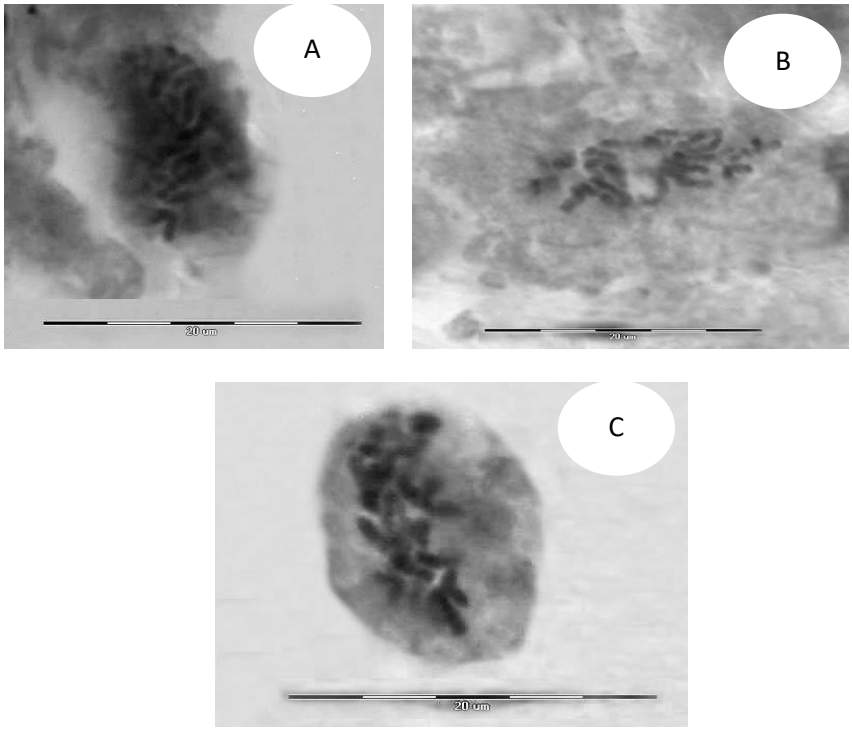

Figure (3) A-C: Mitotic calli cells at metaphase of stevia plant (*Stevia rebaudiana* Bertoni var. Chaine. 2) treated with colchicine solution (0.05%) showing chromosome number 2n=22.

REFERENCES

Ahmed, B., Hossain, M., Islam, R., Kumar, S.A, and Mand, A. (2011). A review on natural sweetener plant - Stevia having medicinal and commercial importance. Prethodno priopcenje. 2: 75-92.

Ahmed, K. Z., Allam, H. Z., Mousa, A. M. and Ali, M. S. A. (1999). Spontaneous versus colchicine-treated dihaploid plants in wheat (*Triticum aestivum* L.) anther culture. Acta Agronomica Hung., 47: 137-146.

Ahmed, M. B., Salahin, M., Karim, R.; Razvy, M. A., Hannan, M. M., Sultana R., Hossain, M., and Islam, R. (2007). An efficient 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.

Alaam, A. I. (2007). Sugar crops council: Future view. The Proceeding of Thirty-Eight Annual Conference, Egyptian Sugar Expertise Society, Hawamdia, Egypt.

Ali, A. E. L., Abo-Shosha, A. A., Kassem, M. K. M., and El-Dabaawy, E. E. M. (2014). Biotechnological studies on

gamma irradiated stevia (*Stevia Rebaudiana*) plant under abiotic stresses. 4th Int. Conf. Rad. Res. Appl. Sci., Taba, Egypt; 95 – 109.

Allard, R. W. (1960). Principles of Plant Breeding. John Wiley & Sons, Inc., New York, 485 pp.

Bawane, A. A., Gopalakrishna, B., Akki, K. S, and Tiwari, O. (2012). An Overview on Stevia: A Natural Calorie Free Sweetener. IJAPBC, 1: 362-368.

Carneiro, J.W.P., Muniz, A. S., and Guedes, T. A. (1997). Greenhouse bedding plant production of *Stevia rebaudiana* (Bert) bertoni. Can. J. Plant Sci., 77: 473–474.

Das, K., Dang, R., and Rajasekharan, E. P. (2006). Establishment and maintenance of callus of *stevia rebaudiana* Bertoni under aseptic environment. Natural product Radiance., 5: 373 – 376.

de Oliveira, M. V., Forni-Martins, R. E., Magalhães, M. P., and Alves, N. M. (2004). Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (*Eupatorieae, Asteraceae*). Genetics and Molecular Biology, 27: 215-222.

Duke, J. (1993). *Stevia rebaudiana*. p. 422–424. In: J. Duke, CRC handbook of alternative cash crops. CRC Press, Boca Raton, FL.

Frederico, A. P., Ruas, P. M., Marin-Morales, M. A, Ruas, C. F., and Nakajima, J. N. (1996). Chromosome studies in some *Stevia* Cav. (Compositae) species from southern Brazil. Rev. Bras. Genet., 19: 605-609.

Gauchan, D. P., Ashna, D., Sharma, N., Bhandari, S., Maskey, E., Shrestha, N.; Gautam, R.; Giri, S., and Gurung, S. (2014). Regenerative callus induction and biochemical analysis of *Stevia rebaudiana* Bertoni . J. Adv. Lab. Res. Biol., 4; 41-45.

Gentry, A. H. (1996). A Field Guide of the Families and Genera of Woody Plants of Northwest South America (Colombia, Ecuador, Peru) with Supplementary Notes on Herbaceus Taxa. The University of Chicago Press, Chicago, 895 pp.

Ghallab, M. M., and Saleh, M. S. (2012). An efficient method for regeneration of *Stevia rebaudiana* (Bertoni) in Egypt. Alexandria science exchange journal, 33: 285-291.

Gupta, P., Sharma, S., and Saxena, S. (2010). Callusing in *Stevia rebaudiana* (Natural Sweetener) for Steviol Glycoside Production. Int. J. Agric. Biol. Sci., 1: 30-34.

Guruchandran, V., and Sasikumar, C. (2013). Organogenic plant regeneration via callus induction in *Stevia rebaudiana* Bert . Int. J. Curr.

Microbiol. App. Sci., 2: 56-61.

Hassanen, A. S., and Khalil, M. A. R. (2013). Biotechnological studies for improving of stevia (*Stevia rebaudiana* Bertoni) *in vitro* plantlets. Middle-East Journal of Scientific Research, 14 : 93-106.

Jahan, M. T., Islam, M. R., Roy, P. K., Mamun, A. N. K., and Islam, M. A. (2014). *In vitro* clonal propagation of *Stevia Rebaudiana* Bertoni through node and shoot tip culture. Nuclear Science and Applications, 23: 61-65.

Lata, H., Chandra, S., Techen, N., Wang, Y, and Khan, I. A. (2013b). Molecular analysis of genetic fidelity in micropropagated plants of *Stevia rebaudiana* Bert. using ISSR marker. American Journal of Plant Sciences, 4: 964-971.

Lata, H., Chandra, S., Wang, Y. H., Raman, V., and Khan, A. I. (2013a). TDZ-Induced high frequency plant regeneration through direct shoot organogenesis in *Stevia rebaudiana* Bertoni: An important medicinal plant and a natural sweetener. American Journal of Plant Sciences, 4: 117-128.

Lavini, A., Riccardi, M., Pulvento, C., De-Luca, S., Scamosci, M., and Andria, R. (2008). Yield, quality and water consumption of *Stevia rebaudiana* Bertoni grown under different irrigation regimes in Southern Italy. Ital. J. Agron. / Riv. Agron., 2: 135-143.

Lester, T. (1999). *Stevia rebaudiana* sweet leaf. The Australian New Crops Newsletter, 11, 1.

Maamoun, M. K. M., El-Mahrouk, M. E., Dewir, Y. H., and Omran, S. A. (2014). Effect of radiation and chemical mutagens on seeds germination of black cumin (*Nigella sativa* L.). Journal of Agricultural Technology, 10: 1183-1199.

Magalhães, P. M. (2000). Agrotecnología para el cultivo de estévia o hierba dulce. In: Fundamentos de Agrotecnología de Cultivo de Plantas Medicinales Iberoamericanas. CYTED-CAB, Bogotá, pp 441-450.

Majumder, S., and Rahman, M. M. (2016). Micropropagation of *Stevia rebaudiana* Bertoni. through direct and indirect organogenesis. Journal of Innovations in Pharmaceuticals and Biological Sciences, 3: 47-56.

Moktaduzzaman, M., and Rahman, S. M. M. (2009). Regeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. Biotechnology, 8: 449-455.

Monteiro, R. (1980). Taxonomia e biologia da reprodução de *Stevia rebaudiana* Bert. Master Thesis, Universidade Estadual de Campinas, Campinas, Brasil.

Monteiro, R. (1982). Estudos cromossômicos em *Stevia*

rebaudiana-Série *Multiaristatae* no Brasil. Rev. Bras. Bot., 5: 5-15.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473-497.

Nadmdari, N., Shooshtari, L., and Qaderi, A. (2015). *In vitro* Mmicropropagation of *Stevia rebaudiana* Bertoni . Biological Forum – An International Journal, 7: 1750-1754.

Preethi, D., Sridhar, T. M., and Naidu, C. V. (2011). Efficient protocol for indirect Shoot regeneration from leaf explants of *Stevia rebaudiana* (Bert.) An important calorie free biosweetner. Journal of Phytology, 3: 56-60.

Raina, R., Bhandari, S. K., Chand, R, and Sharma, Y. (2013). Strategies to improve poor seed germination in *Stevia rebaudiana*, a low calorie sweetener. Journal of Medicinal Plants Research, 7: 1793-1799.

Razak, U. N. A.A., Ong, C. B., Yu, T. S., Lau, L.K. (2014). *In vitro* micropropagation of Stevia rebaudiana Bertoni in Malaysia. Brazilian Archives of Biology and Technology, 57: 23-28.

Sairkar, P., Chandravanshi, M. K., Shukla, N. P., and Mehrotra, N. N. (2009). Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. Journal of Medicinal Plants Research, 3: 266-270.

Shock, C. C. (1982). Rebaudi's Stevia: natural noncaloric sweeteners. California Agric., 36: 4-5.

Uddin, S. M., Chowdhury, S. H. M., Khan, H. M. M., Uddin, B. M., Ahmed, R., and Baten, A. M. (2006). *In vitro* propagation of *Stevia rebaudiana* Bert in Bangladesh. African Journal of Biotechnology, 5: 1238-1240.

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

بروتوكول فعال لزراعة انسجه نباتات الاستيفيا (*Stevia* var. Chaine.2) *rebaudiana* Bertoni مع دراسات خلوية وراثية

قاسم زكي احمد[1]– سيد عبد المقصود عثمان [1]– شعبان محمد صابر[2] – احمد علي عبد الحميد [2]

[1]قسم الوراثة، كلية الزراعة، جامعة المنيا، المنيا، جمهورية مصر العربية؛ رمز بريدى 61519

[2]قسم النبات الزراعي ،كلية الزراعة ،جامعة الازهر –فرع اسيوط؛ جمهورية مصر العربية؛رمز بريدي 71524

أصبحت نباتات الاستيفيا من النباتات الهامة من الناحية الاقتصادية والطبية كبديل آمن لمرضي السكر والسمنة فى كثير من الدول. ونظرا لان المواد المحلاة المستخلصة من نباتات الاستيفيا أكثر حلاوة من السكروز المستخلص من قصب السكر وبنجر السكر، وكذلك احتياجات الاستيفيا من الماء قليلة بالمقارنة بقصب السكر وبنجر السكر. لذا يسعى الباحثون في مصر، والتي تعاني من نقص في انتاج السكر، لجعل محصول الاستيفيا مساعدا في انتاج السكر وسد الفجوة الغذائية والمحافظة على الصحة. و لكن تواجه زراعة الاستيفيا والتحسين الوراثي لها صعوبات فى استخدام البذور للتكاثر وكذا بطئ وصعوبة عملية التكاثر الخضري. لذلك فان التكنولوجيا الحيوية ربما تساعد في إيجاد حلول لتلك المشاكل، فعن طريق الزراعة فى القوارير (زراعة الانسجة) يمكن اكثار الاستيفيا بسهولة واجراء التحسين الوراثي.

في هذه الدراسة تم استخدام اجزاء ورقية كمنفصل نباتي (Explant) بهدف التوصل الي بروتوكول مناسب لزراعة الأنسجة النباتية، يتضمن التوصل الي أفضل معامله للتعقيم السطحي والحصول علي اجزاء نباتيه خاليه من التلوث بالكائنات الحيه الدقيقة وكذلك علي درجه عالية من الحيوية للاستمرار في مزارع الأنسجة واستيلاد نباتات.

فتم التوصل الي ان أفضل معامله للتعقيم السطحي بوضع الأجزاء النباتية المستخدمة تحت الماء الجاري لمده 20 دقيقة وذلك للتخلص من اي اتربة وتقليل الملوثات، ثم التعقيم السطحي بمحلول من كلوريد الزئبق 0.1% لمده دقيقتان ثم كحول الايثانول 70% لمده دقيقه واحد، ثم محلول الكلوروكس بتركيز 15% (من الكلوروكس التجارى 5.25% هيبوكلوريد الصوديوم) لمده 15 دقيقه. وفي النهاية يتم الغسيل بالماء المقطر المعقم 3–4 مرات وذلك للتخلص من الاثر السام للمواد المستخدمة. ولاستحداث نمو الكالوس (Calli) تم اختبار سبعة بيئات مختلفة مشتقة من بيئة موراشيج وسكووج (MS) مدعومة بمجموعة واسعة من التركيزات والمخاليط من منظمات النمو النباتية، حيث أعطت البيئة المحتوية على 1ملجم / لتر 2,4–D +1ملجم / لتر NAA النسبة العالية لاستحداث الكالس، حيث نتج من هذه المعاملة كالوسات بنسبة (96%). ولاستيلاد النباتات

(Plant Regeneration) لوحظ ان أفضل بيئة لتكشف الكالوسات الي نباتات هي بيئة MS و المدعومة 1.8 ملجم/لتر من البنزيل امينو بيورين + 0.12 ملجم /لتر من النفثالين استيك اسيد (NAA) وانتجت اعلي عدد للنباتات المستولدة من الكالوس الواحد (3.600). وتم نقل النباتات المتكشفة الي بيئة MS خاليه من اي منظمات نمو نباتيه، ونمت عليها عشرات النباتات ذات جذور قوية تؤهلها للأقلمة خارج القوارير. وبالفعل تم اقلمتها في اصص بها تربة زراعية تحت ظروف بيئية محكمة.

ولقد تم اخذ عينات من الكالوسات النامية على مختلف البيئات المغذية وتم دراسة الثبات الخلوي من خلال المراحل المختلفة للانقسامات الميتوزية لخلاياها، فكانت البيئة المحتوية علي 2 ملجم/لتر من النفثالين استيك اسيد هي الأكثر في عدد خلايا الكالوسات المنقسمة حيث اعطت أعلى معامل انقسام MI (9.82%) مقارنة بالست معاملات الأخرى. وثبت ان تلك الخلايا تحتوي على عدد زوجي من الكروموسومات مقداره 22 كروسوما في الخلية الواحدة.