Plant Archives Vol. 19 No. 1, 2019 pp. 1275-1284 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF PLANT GROWTH RETADANTS ON STEVIA (*STEVIA REBAUDIANA BERTONI*) ACCLIMATIZATION PRODUCED *IN VITRO*

Rania A. Aziz[1] and Sudad K. Al-Taweel[2*]

[1]Sugar crop Res. Inst., Agricultural Res. Center. Giza. Egypt
[2*]Medical and Aromatic Plants Research Unit, College of Agriculture Engineering Sciences, University of Baghdad, Al-Jadiriya,Baghdad, Iraq

Abstract

An experiment was carried out at laboratories and greenhouses of Sugar Crops Research Institute – Agricultural researches center -Egypt to investigation, an efficient protocol for rapid *In vitro* propagation of stevia and reduction of the large number of lost plantlets during acclimatization. Murashige and Skoog (MS) medium supplemented with 2.5 mg/l N6-benzyl amino purine (BAP) recorded the maximum number of shoots of 48.1 shoots/explant, but these shoots were very thin, containing many lateral shoots, but with low survival percentage during acclimatization. Hundred % of rooted plantlets were recorded on MS medium with 0.5 and 1.0 mg/l indole 3- butyric acid (IBA). Pre-acclimatization treatments using growth retardants; Alar (B9; succinic acid 2-2-dimethylhydrazide) and Cycocel (CCC; chloroethyltrimethyl ammonium chloride) in culture medium were applied to study their effect for improving root system and post acclimatization of rooted plantlets. The highest number of roots (7.72 roots/shoot), root length (2.81 cm) and plant height (4.62 cm) were obtained on MS medium supplemented with 1.0 mg/l IBA and 0.5 mg/l Alar, respectively. It could be concluded that, it is clear that growth retardants increased the survival percentage in acclimatization under greenhouse conditions and, this protocol could be useful in producing a true-to type plant and for the use of *Stevia rebaudiana* medicinally and commercially Bertoni.

Key words:*Stevia rebaudiana*, Microprogration, acclimatization, Plant growth retardants Alar, Cycocel, Anatomy.

Introduction

Stevia rebaudiana Bertoni is a small, herbaceous, semi-bushy, tropical perennial shrub belongs to Asteraceae family. It is native to Paraguay and Brazil (Jain *et al.,* 2009). The leaves of stevia are the source of diterpenoid glycosides (stevioside and rebaudioside). These compounds are 300 times sweeter than sugar (sucrose) obtained from sugar beet, sugarcane etc. (Uddin *et al.,* 2006). Steviosides is regenerated as a valuable natural sweetening agent because of its relatively good taste, non-caloric and chemical stability (Anbazhagan *et al.,* 2010). Consequently, stevia does not have any effects on blood sugar and therefore it is friendly to human health (Taware *et al.,* 2010) Moreover, it has therapeutic values such as obesity, hypertension, heartburn, hypoglycemia, anticancer and to lower the uric acid levels (Ahmed *et al.,* 2007). Additionally, stevia is a good source of carbohydrates, protein, fibers, and antioxidant compounds. (Al Amrani, *et al.,* 2018).

The seeds of stevia show a very low germination percentage and genetic variability (Sivaram and Mukundan 2003) Vegetative propagation is slow and limited by the low number of individuals obtained from single plants (Mishra *et al.,* 2010) Hence, to overcome all these obstacles, *In vitro* propagation can play a vital role for mass propagation and production of genetically identical plants. Although, earlier, attempts have been made for propagation of stevia through tissue culture (Hossain *et al.,* 2008;Ibrahim *et al.,* 2008; Verma *et al.,* 2011 and Laribi *et al.,* 2012) but a considerable effort is still required to make it more practical.

Considerable efforts have been directed to optimize

***Author for correspondence :** E-mail : sudad.altaweel@coagri.uobaghdad.edu.iq

the conditions for *In vitro* stages of micropropagation, but the process of acclimatization of micropropagated plants remains to be not meticulously studied and continues to be a major bottleneck in the micropropagation of many plants. Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. These contribute a culture-induced phenotype that cannot survive the environmental conditions when directly placed in a greenhouse or field. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field. Acclimatization continues to be a major hurdle in the micropropagation of stevia too with lower success reported (Meera and Sathyanarayana 2010).

The terms growth retardants are used for all chemicals that slow cell division and cell elongation in shoot tissues and regulate plant height physiologically without formative effects. One of the most widely used growth retardants is alar (Succinic acid 2-2-dimethylhydrazide) was highly effective, especially in a wide range of ornamental plants. It improved the rooting system of chrysanthemum stem cutting and it also reduced the plant height of pot chrysanthemum (El-Sheibany *et al.,* 2008). Cycocel (chlormequat; 2-chloroethyltrimethyl ammonium chloride) is a synthetic plant growth retardant used on ornamentals for inducing dwarfism in plants and shorter internodes, stronger stems and green leaves (Wasfy 1995).

The present study aimed to find an efficient protocol for *In vitro* mass propagation of *Stevia rebaudiana* with an efficient pre-acclimatization treatment to improve survival of plantlets in greenhouse and to assess the physiological behaviors through some anatomical traits study to observe the effect of growth retardants on improving survival of plantlets in greenhouse.

Materials and Methods

In vitro Propagation

Plant Material

Shoot tips and stem node segments were used as explants for shoots multiplication. All the explants were collected from three months old plants of *Stevia rebaudiana* Bertoni variety Spanti. Plants grown and maintained in the greenhouse of Sugar Crops Res. Inst., Agric. Res. Center. The explants were cut into small pieces (about 1.5 cm long) and then were treated with a few drops of Dettol for 5 min. with constant shaking and washed thoroughly by adding a drop of liquid soap to remove the most external contamination, then, rinsed in running tap water for 20 minutes. Explants were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 5 min followed by rinsing for five times with double distilled water under aseptic conditions in laminar air flow cabinet. Then explants were inoculated aseptically on culture medium.

Culture Medium and Growth Conditions

The culture medium consisted of Murashige and Skoog medium (MS) salts and vitamins. The medium containing 3% (w/v) sucrose was solidified with 0.7% (w/v) agar. The pH of medium was adjusted to 5.8 with, 0.1 KOH or HCl. Media were autoclaved under 1.1 kg/ cm2 and 121°C for 20 minutes. The cultures were incubated in growth room at 27°C±2°C and 16 h photoperiod provided by white fluorescent lamps.

Shoot Initiation

For shoot initiation shoot tips and stem node explants were cultured on MS medium supplemented with (BAP) at different concentrations (0.0, 1.0, 1.5, 2.0 and 2.5mg/ l). The medium was dispensed in the culture tube (150 × 25 mm) containing 15 ml of MS medium and capped with polypropylene closure caps. After six weeks of culture, data on survival percentage, growth to survival percentage and shoot length (cm.) were recorded.

Shoot Multiplication

In this experiment, explants were cultured on MS medium supplemented with different concentrations of BAP and Kin (1.0, 1.5, 2.0 and 2.5 mg/l), MS medium without growth retardants was used for control (0.0 mg/ l). The explants were cultured in 5 jars (350 mm) containing 40 ml of culture medium, Average shoot number of shoots, average shoot length (cm) and average number of leaves were recorded every four weeks for three subcultures.

In vitro Roots Formation

Auxins (IBA, IAA and NAA) were used at different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) of each auxin type for *In vitro* developed shoots. MS medium without growth retardants was served as control. Data were recorded in terms of percentage of rooting, average number of roots/ shoot and average roots length (cm) after three weeks of culture *In vitro* (Pre-Acclimatization) of Micropropagated Plantlets. Mainly this experiment was conducted to study the effect of growth retardants Alar and Cycocel at different concentrations (0.5, 1.0 and 1.5 mg/l) plus IBA at 1.0 mg/l (was used as control and the best result from the previous treatment) on shoot higher,

number and length of roots per shoot.

Ex vitro Acclimatization of Micropropagated Plantlets

In vitro rooted plantlets were removed gently from medium, washed in running tap water and soaked in 2g/l fungicide solution (Benlate 50% WP) for 3 minutes. Plantlets were transported to 6 cm diameter plastic pots filled with sterile mixture of sand, peat moss and vermiculite at equal volume. Plantlets were covered with plastic bags and maintained in greenhouse at 30°C 2±°C humidity was reduced gradually by making a hole on the plastic bag increasing its size every 3 days over 2-3 weeks. Data were collected for survival percentage, plant height and leaves number of the acclimatized plantlets after four weeks.

Anatomy Study

A laboratory experiment was carried out at the laboratory of Anatomy, College of Agriculture Engineering Sciences, Baghdad, Iraq in 2018, Fresh materials of seedling stem and root were fixed in formalin acetic acid (FAA) at 24-48 h and changed the solution to put samples in the ethanol (70%). Fresh plant samples of seedling stems and roots were sectioned using hand sectioning method of Hutchinson(1954) and Hasan (2018) as stems and roots of a selected seedlings were cut at the middle into small pieces of a length ranged 5-7 cm. Segments were sectioned into thin pieces by a razor blade and treated with 1% sodium hypochlorite for 10 min to remove the chlorophyll pigments. The section samples were socked in distilled water for 5 Min. Finally, the samples were placed on the slides and mounted the cover slides by D.P.X. and fixed by Olympus KRÜSS light microscope then photographed using Olympus Am scope camera (Hassan *et al*. 2018). Traits were studied on seedling to measure stem epidermis thickness, stem cortex thickness, stem vascular bundle thickness and stem diameter. The number of stomata plastid's in the upper and lower epidermis, morphological changes of stomata for leaf number10 from different treatments were measured through a Joel Scanning Electron Microscope (T.33A) linked with the software program in the Central Laboratory of the Faculty of Agriculture, Cairo University. The statistical analysis of the data was done according to the variance analysis of the CRD design with three replicates. The means of treatments were compared with a test of least significant difference (LSD) at 5% probability (Steel and Torrie, 1981).

Statistically Analysis

Data were statistically analyzed and subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan's multiple range test (Duncan1955).

Results and discusson

In vitro Propagation

Shoot Initiation

Concerning the establishment of *Stevia rebaudiana*, data in table 1 and fig 1A showed that percentage of survival ranged between 48 to 89% for both shoot tips and stem segments, respectively. Percentage of growing explants ranged from 58.6% to 92.1% and 41.6% to 82.7 % for shoot tips and stem node segments, respectively.

Data obtained after six weeks of culture revealed that shoot tips gave the highest growth percentage (92.1%) and highest average length (3.92 cm) on MS medium supplemented with 1.0 mg/l BAP, followed by MS medium containing 1.5 mg/l BAP which gave 83.4% growth percentage and average shoot length of 3.5 cm. While, the same medium gave the highest growth percentage 82.7% for stem segments, but the average length was 2.11cm. However, MS medium containing 1.0 mg/l BAP gave average length of 2.77cm. On the other hand, the lowest growth percentage and the least average shoot length for both explant types were recorded on MS medium without plant growth regulators (control). The average length of shoots decreased gradually with an increase in BAP (1.0 mg/l) concentration. From the previous results, it is clear that MS medium containing 1.0mg/l BAP was rise to best result for shoot tips. These results are in agreement with that obtained by (Ibrahim *et al.,* 2008) on stevia. Similar report regarding the efficiency of shoot tips explant on initial culture establishment was also obtained from the study of (Anbazhagan *et al.,* 2010) and (Das *et al*. 2011) on stevia. Whereas, in contrary to the present account,

Table 1: Effect of BAP concentrations and explants type on shoot initiation of *Stevia rebaudiana* Bertoni *In vitro*

BAP Conc. (mg/l)	Explant type: Shoot tip			Stem node segment		
	Survival %	Growth to survival %	Shoot length (cm)	Survival %	Growth to survival %	Shoot length (cm)
0.0	48c	58.6c	1.23c	51.5e	41.6d	0.85c
1.0	89a	92.1a	3.92a	78.8b	77.3b	2.77a
1.5	83b	83.4b	3.05b	83.2a	82.7a	2.11a
2.0	79b	78.9b	2.86b	71.1d	73.5b	1.81b
2.5	77b	66.2c	1.74c	74.9c	63.4c	1.67b

Means followed by the same letter are not significantly different at 0.05 level

(Laribi *et al.,* 2012) reported that the nodal segments from adult *Stevia rebaudiana* Bertoni plants cultured for shoot proliferation, produced better results than the shoot tip explants.

Shoot Multiplication

In order to increase the number of shoots/explants, *In vitro* regenerated shoots were cultured on MS medium supplemented with BAP or Kin at 1.0, 1.5, 2.0 and 2.5 mg/l for both cytokinins, in addition to the control treatment. From data in table 2 and fig 1B, it is clear that shoot number was significantly affected by BAP concentrations. Increasing BAP concentration increased the number of shoots. Shoot multiplication rate was significantly high and ranged from 8.06 to 48.1 shoots/ plant due to BAP application, which increased multiplication rate. The maximum number of shoots (48.1 shoots/explant) was recorded on MS medium supplemented with 2.5 mg/l BAP compared to the other BAP treatments. Whereas, the same concentration of kinetin (2.5 mg/l) gave 17.13 shoots/explant. On the other hand, shoot length decreased by increasing BAP concentration, average shoot length ranged from 0.80 to 4.06 cm. Also, increasing Kin concentrations up to 2.5 mg/l decreased the average shoot length; it was ranged from 5.01 to 6.02 cm. MS basal medium without plant growth regulators was found the best medium to improve the length of *Stevia rebaudiana* Bertoni, which reached 6.02 cm. Average number of leaves ranged from 2.62 to 7.31 and it was significantly affected among the different treatments. The highest number of leaves (8.32) was achieved on the control medium (table 2). Increasing BAP or Kin concentrations reduced leaves number significantly per shoot. So, MS medium without growth regulators is better than MS medium containing BAP or Kin concentrations with respect to shoot length and number of leaves. These results are in agreement with that obtained by (Ibrahim *et al.,* 2008) who reported that multiplication of stevia without BAP is better to produce normal plants. Our finding suggested the BAP increased the shoot number and decreased of shoot and leaf growth as compared to control medium with significant differences amongst the treatments. MS medium supplemented with 2.5 mg/l BAP recorded the maximum number of shoots, but these shoots were very thin, vitrified and irrelevant to subculture completion (results not presented). On the other hand, (Das *et al.,* 2011) found that Kin is much more effective than BAP. They reported that MS medium supplemented with 2.0 mg/l Kin performed best in multiple shoot proliferation and resulting more than 11 multiple shoots in stevia within 35 days of culture.

Table 2: Effect of different concentrations of cytokines (BAP or Kin) on shoot multiplication of *Stevia rebudiana* Bertoni *In vitro*

Conc. (mg/l)	Average no. of shoots/explants	Average shoot length (cm)	Average no. of leaves/shoots
0.0	8.06h	7.2a	8.32a
BAP			
1	30.1d	4.06e	5.2f
1.5	38.8c	2.81f	4.1g
2	42.5b	2.01g	3.46h
2.5	48.1a	0.80h	2.62i
Kin			
1	10.3g	6.02c	7.31b
1.5	14.11f	5.81c	6.82c
2	16.82e	5.63b	6.11d
2.5	17.13e	5.01d	5.44e

Means followed by the same letter are not significantly different at 0.05 level

Roots Formation

Data presented in table 3 and fig. 1C showed that the effect of IBA on root formation was significantly higher than IAA and NAA, where roots percentage was 100% at low concentrations of IBA (0.5 and 1.0 mg/l). The highest average number of roots (4.71) and average root length (3.49 cm) were obtained on MS medium supplemented with 1.0 mg/l IBA. Regarding the effect of IAA and NAA on root formation, data indicated that the percentage of shoots formed roots ranged from 81.8

Table 3: Effect of different concentrations of auxins (IBA, IAA and NAA) on root formation of *Stevia rebudiana* Bertoni *In vitro*

Conc. (mg/l)	Rooted shoots %	Average no. of roots/shoots	Average root length (cm)
0.0	66.94c	3.52b	2.84c
IBA			
0.5	100a	3.89b	3.05b
1	100a	4.71a	3.49a
1.5	66.7c	3.46b	2.66c
2	61.5d	3.26b	2.13d
IAA			
0.5	83.2b	1.77f	1.31g
1	82.9b	2.28d	1.7f
1.5	51.6e	1.43f	0.98h
2	33.2f	1.03g	0.90i
NAA			
0.5	82.1b	2.02e	1.63f
1	81.8b	2.61d	1.92e
1.5	71.6c	1.81f	1.41g
2	52.0e	1.27g	0.96h

Means followed by the same letter are not significantly different at 0.05 level

to 83.2% on MS medium supplemented with IAA or NAA at 0.5 or 1.0 mg/l. For both auxins; IAA and NAA concentrations (0.5 to 2.0 mg/l), number of roots ranged from 1.03 to 4.71 roots per explant. However, the average of roots length was ranged from 0.90 to 1.92 cm. On the other hand, MS basal medium without plant growth regulators recorded 66.94% rooting percentage, 3.52 roots number and 2.84 cm root length. Several researchers showed that addition of auxins into the nutrient medium was successful in initiating of roots from *In vitro* shoots for *Stevia rebaudiana* Bertoni (Anbazhagan *et al.,* 2010; Rafiq *et al.,* 2007 and Thiyagarajan and Venkatachalam 2012).

IBA treatments proved to be better than NAA and IAA. These results are in agreement with those obtained by (Ibrahim *et al.,* 2008 and Stapathy and Das 2010) who stated that IBA was better than NAA and IAA for shoot and root formation of stevia. Also, (Jitendra *et al.,* 2012) found that MS medium with 1.0 mg/l IBA proved to be the best for *In vitro* rooting of *Stevia rebaudiana* Bertoni, which gave the highest rooting percentage, roots number and length. Whereas, it has been reported that the best rooting response was observed on MS medium supplemented with 2.0 mg/l IBA (Verma *et al.,* 2011 and Preethi 2011). On the contrary, (Laribi *et al.,* 2012) reported that the highest percent (97%) of rooting, maximum number of roots and rooting length were observed on MS medium supplemented with 0.5 mg/l IAA for rooting.

Effect of growth retardants on rooting improvement *In vitro*

Data in table 4 and fig. 1 D presented the effect of alar (B9) and Cycocel (CCC) separately at different concentrations (0.0, 0.5, 1.0 and 1.5 mg/l) for each, in MS medium supplemented with 1.0 mg/l IBA, on rooting improvement of stevia shoots before acclimatization. It was cleared that both B9 and CCC gave rise to a large number of roots. The highest roots number was induced at 1.0 and 1.5 mg/l B9 compared to the control and other levels of CCC. MS medium supplemented with 1.0 mg/l IBA alone (control) andwithout any growth retardants, gave the highest mean length of roots and plant height (3.42 and 5.33 cm, respectively). However, the average of roots length was ranged from 1.72 to 3.07 cm for both retardants at concentrations from 0. 5 to 1.5 mg/l. Moreover, data also showed that root length and plant height were decreased by increasing B9 or CCC concentrations. Results in Table 4 illustrated that B9 and CCC treatments at different concentrations reduced root length, height of plants and increased number of roots as compared with plants produced in culture medium without any growth retardants. These results are in agreement with those obtained by (Wasfy 1995 and EL-Mokadem and Heikal 2008). Growth retardants application at 0.5 mg/l for B9 and CCC increased the percentage of plantlets survival to about 76-80% over their control in greenhouse. In this regard, (Ziv 1986) reported that about 50 to 90 % of *In vitro* propagated plantlets of many species have been lost at the time of transfer to soil. At least 7-10% of rooted plantlets that were not treated with retardants were successfully acclimatized in greenhouse after three months. A large portion of the plantlets were loss during hardening. There are various problems met at other levels have their origin at the multiplication, rooting and acclimatization stages. These problems could be summarized as follows:

- The shoots were very thin, very long internodes and a few shoots were vitrified.
- Low efficient rooting and low rate of survival upon transfer to soil.

Table 4: Effect of growth retardants (Alar & CCC) in MS medium enriched with IBA (1.0 mg/l) on rooting improvement of *Stevia rebaudiana* Bertoni *In vitro*

Growth retardant conc. (mg/l)	Average no. of root/ hoots	Average root length (cm)	Average plant height (cm)
0.0	5.02e	3.42a	5.33a
Alar (B9)			
0.5	5.93c	3.07b	4.62c
1	7.72a	2.81c	4.33d
1.5	6.44b	2.21d	4.26e
Cycocel (CCC)			
0.5	5.06e	2.14d	5.14b
1	6.12c	1.78e	3.96e
1.5	5.36d	1.72e	4.01e

Means followed by the same letter are not significantly different at 0.05 level

So, this experiment revealed that growth retardants especially alar helped in better rooting and acclimatization of *Stevia rebaudiana* Bertoni, plantlets. Whereas, growth retardants suppress growth because they block the terpenoid pathway that is responsible for the production of gibberellins and compress internodes into a shorter length. The roots of treated stivia were shorter and thicker (fig.1D). Plant growth retardants are a group of synthetic compounds that modify plant structure, mainly by inhibition of gibberellins biosynthesis. Also, growth retardants have a more general inhibitory action on isoprenoid biosynthesis in plants (Weber and Baker 2010) Alar still has many uses for ornamental plants, where it is used to control vegetative growth, reduce plant height (induce dwarfism) and add to the general vigor of plants by stimulating

resistance to stress conditions. It also stimulates strengthens stems and root growth. Its mode of action is inhibition of gibberellins transport, as opposed to gibberellins biosynthesis and this accounts for the internode inhibitions observed in treated ornamentals.

Alar and Cycocel prevent cell elongation and inhibit cell division due to their effect as anti-gibberellins (Hammer *et al.,* 1975) Moreover, retarded stem elongation by preventing the formation of kaurence, a precursor of gibberellins biosynthesis, subsequently inhibiting or reducing only elongation of stem cells (Wasfy1995) These results are in agreement with those reported by (Porwal *et al.,* 2002) on Rosa damascene. Also, alar treated chrysanthemum has a more branched root system (Barras 2002) In general, to encourage root formation (high number of roots), low plant high and strong stem, the alar (B9) treatment proved to be better than Cycocel (CCC).

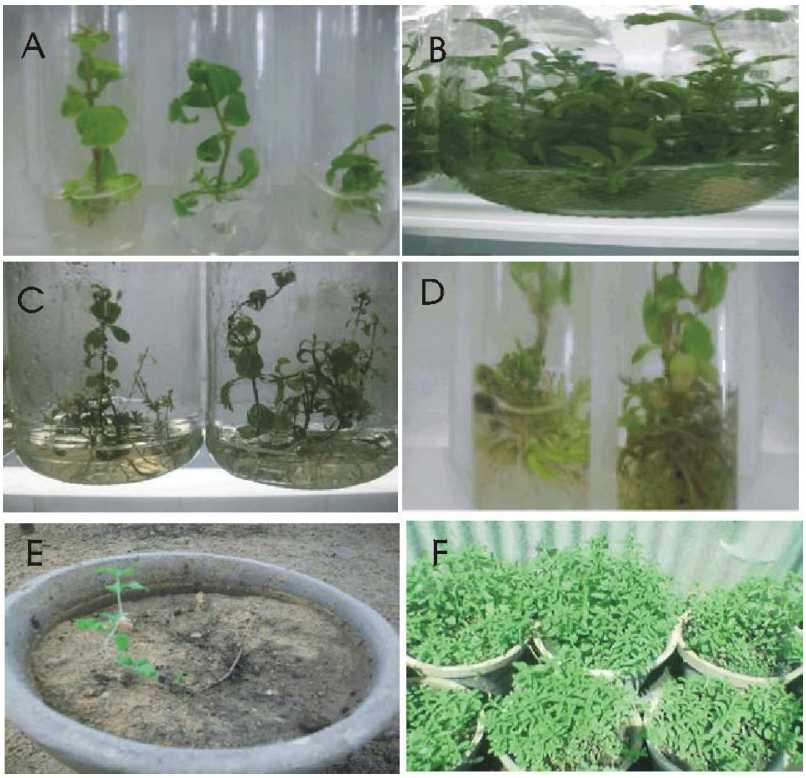

Fig. 1: Micropropagation of stevia (*Stevia rebaudiana*): (A) Starting stage (B) Multiplication of shoots, (C) Rooting without growth retardant (D) Rooting with growth retardant, (E) Acclimatization plantlets which non-treated with growth retardant. (F) Acclimatization plantlets treated with growth retardant after one month.

***Ex vitro* Establishment**

After attaining the rapid *In vitro* acclimatization rate, successful acclimatization or establishment of tissue culture raised propagates in the greenhouse, is the key parameter of a micropropagation protocol) Ahmed*et al.,* 2007) Ultimate triumph of any *in vitro* propagation venture depends on the accomplishment of ex vitro adaptation of tissue cultured plantlets. Two types of rooted plantlets were derived from *in vitro* pre-acclimatization, non- treated plantlets (control) and treated plantlets with Alar at 1.0 mg/l. As seen in table 5, with respect of treated plantlets, 94.5% survival percentage was assured. While, it's reduced for non-treated plantlets to 21%, because plantlets through tissue culture are heterotrophic, lack cuticle on their epidermis (Laemmli 1970) as well as having non- functional stomata (Murashige and Skoog 1962) In addition, plantlets were very thin, higher and have non-functional roots and they could not survive and eventually died in the greenhouse. Thus, it was needed to acclimatize the plants *in vitro*, where they receive a special treatment before they can be transferred to greenhouse (fig.1E).

In present study, pre-acclimatization treatments of micropropagated plantlets with growth retardants that inhibit gibberellins biosynthesis (alar) in culture were used to study their effect on post acclimatization survival. Alar, the best result (1.0 mg/l), reduced the plant height and number of leaves considerably (Rademacher 1991) (table 5 fig. 1F). Alar indirectly inhibits cell elongation and division through blockage of ent-kaurene synthetase activity in the gibberellins biosynthetic pathway (Sponsel 1987) This result is in agreement with those reported by (Meera and Sathyanarayana 2010) who found that growth retardant (Ancymidol) reduced the length of the shoot, number of leaves and internode length considerably. Also, they reported that the use of alar resulted in a higher number of multiple shoots, average growth parameters and a better survival percentage. Finally, growth retardants have other biological effects besides retarding stem elongation. Also, leaves of treated plants are frequently darker green than in untreated plants.

Effect of plant retardants on leaf anatomical traits

A: The effect of plant hormone retardants on stem anatomical traits

Fig. 2 shows that the quadrilateral stem consists of a single layer of epidermis consisting, the cells oval, covered by the cuticle layer. The cortex consists of two layers of cells, the first layer is near the epidermis known

Table 5: Effect of growth retardant (Alar at 1.0 mg/l) on *ex-vitro* acclimatization of *Stevia rebaudiana* Bertoni

Plantlets after *ex-vitro* transfer	Plantlets survival %	Plant height (cm)	Number of leaves	Plant health remark
Non-treated (control)	21b	7.72a	12.24a	+
Treated	94.5a	6.68a	10.88b	+++
+++ very good, ++ good, + fair				

Means followed by the same letter are not significantly different at 0.05 level

collenchyma cells consist of three rows, so several rows of them are grouped in the corner of stem, after the collenchyma layer can show several layers of the parenchyma cells separate between as the ordinary intercellular space, followed the cortex the vascular tissue that consists from vascular bundles, each bundle is connected to the other by a group of cells called the intervascicular cambium. The vascular bundle consists as the follows; vascular bundle cup, beneath of it the phloem elements, followed by xylem elements and between the xylem and phloem there is the fascicular cambium layer, so it appears as four small vascular bundles in the corner of the stem. From fig 2, pith is in the center of the stem and has a cavity in the center of pith due to the dissolution of many pith cells. All the results above described in corresponding with Evert (2006).

The results in (table 7) showed significant differences between the studied treatments in the anatomical characteristics of the plant stem. The C2 treatment was the mostly significant. Table 6 shows that the highest stem diameter was 519.3 mm in C2 treatment compared to the control treatment, then the lowest diameter of the plant stem was 376.2 mm. in treatment of C0 but this treatment showed a significant increase of epidermis thickness 3.93µm compared to C2 and C1 (Alar), which was 3.67 and 3.43 µm respectively. Treatment C2 was characterized by the largest thickness of the cortex of 20.97 µm while C1 was the least thickness of the cortex of 15.13 µm. The Alar C1treatment was recording the largest thickness of the vascular bundle in the leg was 60.30 micrometers while C2 treated the lowest thickness of the vascular bundles in the leg at 52.83 micrometers (Table 6).

Many studies have concluded that the use of the Alar growth inhibitor to increases the tolerance of plantlets to environmental conditions in the adaptation period (Armitage, 1994 and Basra, 2000). It is recommended to treat the weak vegetative growth plants which resulting from tissue culture (Biza, 1995). The summarized results obtained in table 7 was agreed with table 6 results which showed that the Alar treatments will be effect indirectly to inhibit cell division and cell elongation, reduce plant length, number of leaves and internodes of treated plants which reflecting on increased the stem diameter (Meera And Sathyanarayana, 2010). It also led to an increase in the thickness of vascular bundles, which led to the balance between the vegetative growth and root growth of acclamized plantlets.

The control treatment was significantly higher in the

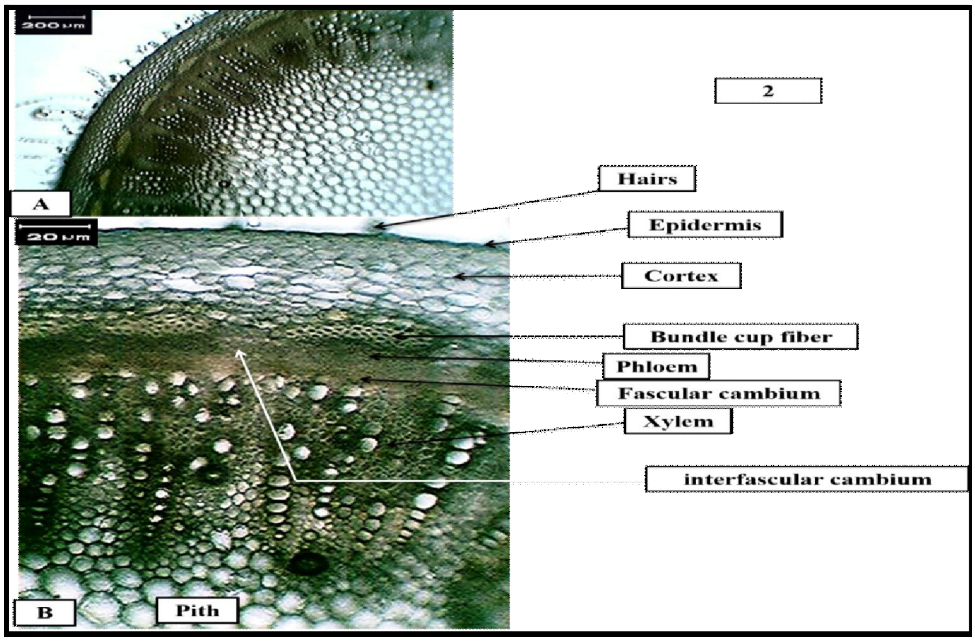

Fig 2: A cross section in stem of *Stevia rebaudiana* seedling treated with Alar after accumilization period

Table 6: Effect of plant growth retardants on stem anatomical traits of *Stevia rebaudiana* afteracclimization period

Treatment	Stem Diameter (mm)	Epidermis thickness (μm)	Cortex thickness (mm)	Vascular bindles thickness (μm)
C_0	376.2	3.93	18.70	43.97
$C_{1(1mg/l)}$	481.5	3.43	15.13	60.30
$C_{2(1mg/l)}$	519.3	3.67	20.97	52.83
L.S.D	2.043	0.346	0.845	4.84

C0: control; C1: Alar; C2 Cycocel

width and length of the gap in the lower epidermis of the plant leaf in question. It was 28.9 and 30.5 μm compared to C2, which had the lowest width and length of the lower lip of the plant at 9.2, 22 micrometers sequentially (Table 6).

Some studies found that the use of growth retardants has a clear effect on the density of stomata and the process of fixation of CO_2 and the rate of transpiration in the leaves of the plantlets which growing from the tissue culture during the acclimization period. This result will be depending on the type of growth inhibitor, time of treatment. The strongest effect of the growth inhibitor in general is after 24 days of seedling treatment and almost disappear after 43 days of treatment (Harmath *et al.,* 2014) attributed to the reason that each growth retardant has limited effected period on plantlet as a result of its partially chemical components degradation as well as due to its used effected concentration of the other hand (Harmath *et al.,*2014).

B. Stomata characteristics:

Table 7 and fig. 3 show the C1 treatment as a superior to the other treatments in the stomata size of the leaves upper epidermis, with 26.2, 33.8 micrometers respectively, compared to the C2 treatment, which showed a significant reduction in the width of the upper epidermis of 15, 22.7 micrometers of treatment C2 and C0respectively, which shows the lowest length of the upper epidermis was 31.3 and 30.9 micrometers, respectively (table 6). This table also showed that is no significant differences between C1 and C0 in the lowest stomata length and numbers at the leaf upper epidermis. The results in Table 6 show significant differences between the treatments in all the stripping characteristics of *Stevia rebaudiana* Bertoni leaf, which was culturally propagated during the transduction phase. The C1 treatment was superior to both C0 (control) and C2 (Cycocel) by recording the largest number of plastids in the epidermal cells of the epidermis, the highest and lowest plastids was 5.7, 6.7 respectively compared to the control and Cycocel treatments, which was 3.7, 4.7.3.7 and 4.7 plastids respectively.

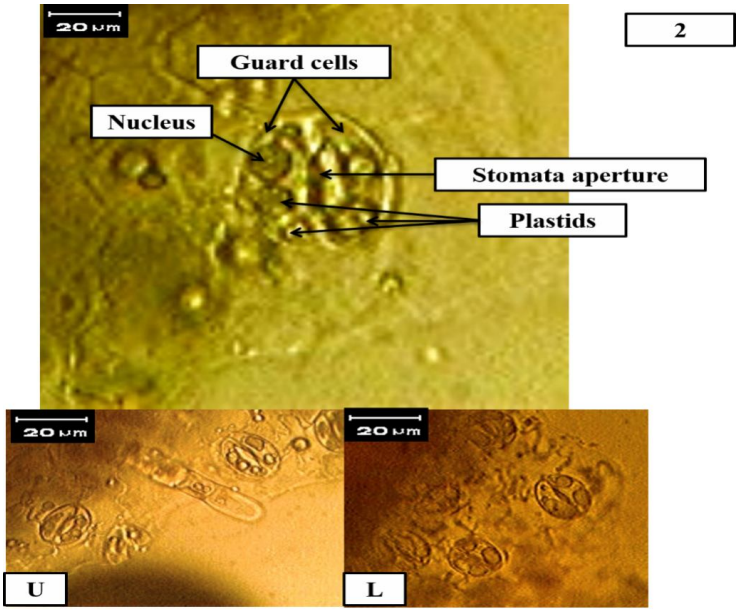

Fig 3: A cross section in upper and lower leaf epidermis of *Stevia rebaudiana* seedling treated with Alar after accumilization period

Table 7: Effect of Plant hormone retardants on leaf anatomical traits in *Stevia rebaudiana* Bertoni after accumilization period.

Treatments	Upper Epidermis			Lower Epidermis		
	Plastids number	Stomata length	Stomata dimeter	Plastids number	Stomata length	Stomata diameter
C_0	3.7	30.9	22.7	4.7	30.5	28.9
$C_{1(1mg/l)}$	5.7	33.8	26.2	6.7	28.9	24.0
$C_{2(1mg/l)}$	3.7	31.3	15.0	4.7	22.0	9.2
L.S. D	1.153	0.765	1.063	1.153	1.193	0.951

C_0: control; C_1: Alar; C_2 Cycocel

Conclusion

The present study, with no prior studies, details was performed on an efficient protocol for micropropagation of stevia plant and solving the problem of weak and low percent of survived stevia plantlets during acclimatization period in greenhouse. Growth retardant treatments proved to be optimum method for improving rooting and survival of plantlets in acclimatization. Also, It could be concluded that the obtained leave anatomical results could be concluded to explain the physical effect of some Plant growth retardant which their results will be agreed with the anatomical results of the table 4 and table 5 which shows that the treatment of Alar (C1) is better one for roots formation and increase the plastids number in the upper and lower leaves epidermis which correlated with increasing the chlorophyll content of the

leaves (Table 6), this means to be increasing the CO_2 fixation rate due to the length and width of the stomata or optimum stomata size in the upper of the leaf, which may have been to reduce the transpiration process as well as that the Alar may be has a longer effect due to its long stability on the leaves than the Cycocel.

References

Acclimatization and Establishment of Micro propagated Plants.*(ISHS) Acta Hort.,* 171-178.

Ahmed, A.T., S.A.Al-samaraee, Z.Hanan and Elmeer (2012). Inter-simple Sequence Repeat (ISSR) Analysis of Somaclonal variation in date palm plantlets regenerated from callus. *2nd International Conference on Environment and Industrial Innovation,* pp: 35.

Ahmed, A., M.N. Huda, C. Mandal, K.A. Alam, M.S. H. Reza and A.Wadud (2007). *In vitro* morphogenic response of different explant of stevia (*Stevia rebaudiana*). *Int. J. Agric. Res.,* **2**: 1006-1013.

Al Amrani, H.A., Sudad K.AlTaweel and Z.M. Abdul-Qader (2018). Steviosides composition in *Stevia rebaudiana* bertoni: Effect of drying methods*Plant Archives,***18(2)**: 2025-2030.

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

Armitage, A.M. (1994). Ornamental bedding plants. *Crop Production Science in Horticulture 4. Wallingford: CAB International,* p. 62–81

Barras, A. (2002). Effect of Plant Growth Retardant ALAR on Growth and Flowering of Chrysanthemum MSc.thesis, *Garyounis University,* Benghazi, Libya.

Basra, A.S.(2000). Plant growth regulators in agriculture and horticulture, their role and commercial uses. Binghamton, *New York:The Haworth Press, Inc.,* 74–81.

Biza, K. (1995). Törpésítés vegys zerekkel. Kertészetés Szõlészet. Growth regulation with chemicals. *Horticulture and Viticulture,* **44(42)**: 15.

Das, A., S.Gantait and N.Mandal(2011). Micropropagation of an Elite-Medicinal plant: *Stevia rebaudiana* Bertoni. *International J. Agric. Research,* 40-48.

Duncan, D.B. (1955). Multiple Range and multiple F Test. *Biometric,***11**: 1-42.

El-Sheibany, O.M., N.A. El-Malki and A. Barras-Ali (2008). Effect of growth retardant Alar on some anatomical and chemical changes in local cultivar of *Chrysanthemum morifolium*. *Journal of Sci. and Its Appl.,* **2(1)**: 1-5.

Evert, R.S. (2006). Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development, *Third Edition, John Wiley & Sons, Inc.*

Hammer, P.L., D.C. Kiplinger and H.K. Tayama (1975). Greenhouse studies on the effect of chemical growth retardants on shoot growth of chrysanthemum and Poinsettias. *Ohio Florists Association Bulletin,* **549**: 2-5.

Harmath, J., G. Schmidt, M. Forrai and V. Szabó (2014). Influence of some growth retardants on growth, transpiration rate and CO_2 fixation of Caryopterisincana 'Heavenly Blue'. *Foliaoecologica*, **41(1)**:24-33.

Hasan, M.A., S.K. AL-Taweel, J.H. Hamza, and W.M. Jewad (2018). Effect of seed weight on stem anatomical characters in white lupine (*Lupinusalbus* L.) cultivars. *Indian J. Agric. Res.,* **52(6)**: 666-670.

Hossain, M.A., A.M. Shamim Kabir, T.A. Jahan and M.N. Hassan (2008). Micropropagation of stevia. *Inte. J. Sustainable Crop Production,* **3(4)**: 1-9.

Hutchinson, E. P. (1954). Sectioning methods for moss leaves. *Bryologist.* **57**: 175-176.

Ibrahim, A.I., M.I. Nasar, B.R. Mohammed and M.E. Zefzafi (2008). Plant growth regulators affecting *In vitro* cultivation of *Stevia rebaudiana*. *Sugar Technol. M.,***10(3)**: 254- 259.

Jain, P., S. Kachhwahe and S.L. Kothari (2009). Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* Bertoni by using high copper levels in the culture medium. *Scientia Horticulturae,* **119**: 315- 319.

Jitendra, M., S. Monika, S.D. Ratan, G. Priyanka, S. Priyanka and D.J. Kiran (2012). Micropropagation of an anti-diabetic plant-*Stevia rebaudiana* Bertoni (Natural Sweetener) in Hadoti Region of South-East Rajasthan, *India*. *ISCA Journal of Biological Sci.,* **3**: 37-42.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of head bacteriophage T4. *Nature,* **227**: 680-685.

Laribi, B., N. Rouatbi, K. Kouki and T. Bettaieb (2012). *In vitro* propagation of *Stevia rebaudiana* Bertoni. A non-caloric sweetener and antidiabetic medicinal plant. *Int. J. Med. Arom. Plant,* **2(2)**: 333-339.

Meera, M.A.V. and B.N. Sathyanarayana (2010). Studies on pre-acclimatization treatments to improve post acclimatization survival in stevia (*Stevia rebaudiana* Bertoni). IV International Symposium.

Mishra, M., R. Singh, U. Kumar and V. Prakash (2010). *Stevia rebaudiana* a magical sweetener. *Global J. Biotechnol. & Biochem.,* **5(1)**: 62-74.

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

Porwal, R., C.L. Nagda and J.P.S. Pundir (2002). Influence of plant growth regulators on vegetative growth and flower earliness of damask rose. *South Indian Horticulture,* **50(1/3)**: 119-123.

Preethi, D. (2011). Direct shoot organogenesis from leaf explants of *Stevia rebaudiana*. *J. Phytol.,* **3(5)**:69-73.

Rademacher, W. (1991). Biochemical Effects of Plant Growth Retardants, Plant Biochemical Regulators, *Marcel Dekker,* New York.

Rafiq, M., M.O. Dahot, S.M.Mangrio, H.A.Naqfi and I.A. Qarshi (2007). *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni. *Pak. J. Bot.,* **39(7)**: 2467-2474.

Sivaram, L. and U. Mukundan(2003). *In vitro* culture studies on *Stevia rebaudiana*. *In vitro* Cell. Dev. *Biol. Plant,* **39(5)**: 520- 523.

Sponsel, V.M. (1987). Gibberellin Biosynthesis and Metabolism. In: Plant Hormones and their Role in Plant Growth and Development. *P.J. Davies (Ed.), MartinusNijhoff Publishers,* Boston.

Stapathy, S. and M. Das (2010). *In vitro* shoot multiplication in *Stevia rebaudiana* Bertoni, a medicinally important plant. *General and Applied Plant Physiol.,* **36(3-4)**: 167-175.

Steel, R.G.D. and Torrie J.H. (1981). Principles and Procedures of Statistic. Mcgraw. *Hill Book Co., Inc.* New York, pp. 485.

Taware, A.S., D.S. Mukadam, A.M. Chavan and S.D. Tawar(2010). Comparative studies of *In vitro* and in vivo grown plants and callus of *Stevia rebaudiana* (Bertoni). *International J. Integrative Biol.,* **9(1)**: 10-15.

Thiyagarajan, M. and P. Venkatachalam (2012). Large scale *In vitro* propagation of *Stevia rebaudiana* (Bert.) for commercial application: Pharmaceutically important and antidiabetic medicinal herb. *Ind. Crop. Prod.,* **37**: 111-117.

Uddin, M.S., M.S.H. Chowdhury, M.M.M.H. Khan, M.B. Uddin, R. Ahmed and M.A. Baten, (2006). *In vitro* propagation of *Stevia rebaudiana* Bertoni in Bangladesh. *African J. Biotech.,* **5(13)**: 1238-1240.

Verma, S., K. Yadav and N. Singh (2011). Optimization of the protocols for surface sterilization, Regeneration and Acclimatization of *Stevia rebaudiana* Bertoni. *American-Eurasian J. Agric. & Environ. Sci.,* **11(2)**: 221- 227.

Wasfy, E. (1995). Growth Regulators and Flowering. *Academic Book shop, Modern Egyptian Press,* pp. 503-510.

Weber, S.M. and S.R. Baker (2010). Alar cartilage grafts. *Clin. Plast Surg.,* **37(2)**: 253-264.

Ziv, M. (1986). *In vitro* Hardening and Acclimatization of Tissue Culture Plants. *In: Plant Tissue Culture and its Agriculture Application, (Withers, L.A.; Alderson, P.G. Eds.).* Butter Worths, London. pp. 187-196