

Effect of Growth Retardants on Shoot and Root Development of Stevia(*Stevia rebaudiana* Bertoni) Plant Grown *in Vitro*

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Abstract: *Stevia rebaudiana* Bertoni is a natural sweetener herb, which is promising in food and pharmaceutical production. In Egypt, the gap between sugar production and consumption represents a serious problem, in order to close this gap; Stevia could be cultivated in the Egyptian environmental conditions. The objective of this study was to evaluate the effect of paclobutrazol (PBZ) and ethephon (ET) on shooting and rooting of stevia plant *in vitro* to improve the survival and growth of Stevia plantlets during acclimatization. The highest shoot number (48.0 shoots/explant) and the highest number of leaves (7.34) were recorded on MS medium containing 0.1 mg/l PBZ. Whereas, the highest shoot length (7.36 cm) was obtained on MS medium without supplementation with PBZ or ET. The highest fresh and dry weights of shoots was recorded on MS medium containing 0.5 mg/l PBZ, which gave 2.83 and 0.39 g, respectively. The highest number of roots (8.44 roots/shoot) was obtained with 0.1 mg/l PBZ. Also, this study indicates that PBZ or ET increased chlorophyll a, chlorophyll b, carotenoids and carbohydrates content in the plant. PBZ and ET had significant effect on the survival of plants *ex vitro*, which recorded 85 and 75%, respectively.

Keywords: Stevia, micropropagation, paclobutrazol, ethephon

I. Introduction

Stevia rebaudiana Bertoni (Stevia) is a perennial herb, belonging to family Asteraceae. The leaves of Stevia are source of diterpene glycosides, such as stevioside and rebaudiosides, which are estimated to be 100-300 times sweeter than sucrose (Tanaka, 1982). It is being commercially cultivated in China, Taiwan, Thailand, Korea, Japan, India and Malaysia (Jain *et al.*, 2009). Stevia extracts have no reported side effects and can be used as an alternative to sugar and other synthetic sweeteners (Thiyagarajan and Venkatachalam, 2012). Stevioside from Stevia is regenerated as a valuable natural sweetening agent due to its relatively good taste and chemical stability. It is of special interest to diabetic persons with hyperglycemia and the diet conscious (Toyoda *et al.*, 1997 and Arpita *et al.*, 2011). Summon *et al.* (2008) reported that Stevia also can act as an antimicrobial in pharmacological studies and according to Debnath (2008), the crude leaf chloroform extract of leaves can inhibit *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis* and *Escherichia coli*. Secondary metabolites in Stevia are used as functional component in the food industries (Savita *et al.*, 2004). The large-scale cultivation of Stevia plants is important for the human health because of the many benefits it gives. Seed germination is often poor and rates are below 10% (Miyazaki and Wantenabe, 1974). Propagation using the seed also causes great variability on stevioside level and composition (Nakamura and Tamura, 1985). The stem cutting method has limitations, such as low number of new plants and destruction of the donor plant. Micropropagation or *in vitro* culture appears to be the best method to overcome those problems and has the potential to produce large quantity of stevia plantlets in short time (Uddinet *et al.*, 2006). The tissue culture is also a powerful tool that can accelerate the genetic breeding (Alves *et al.*, 2011).

The quality of *in vitro* produced plants should be at least the same as that of traditionally propagated planting materials, the plantlets have less developed cuticle, epicuticular waxes and functional stomatal apparatus, causing high stomatal and cuticular transpiration or a transplantation shock during the first step of the acclimatization process (Hazarika, 2006; Chandra *et al.*, 2010 and Kumar and Rao, 2012). It is therefore important to use in the micropropagation procedure such treatments that increase the ability to survive the acclimatization stress and enable a quick adaptation to heterotrophy. Growth retardants are known to be effective in improving survival of plantlets after their transfer to the soil (Eliasson *et al.*, 1994). Plant growth retardants are commonly used to inhibit stem elongation of many ornamental plants, they act by inhibiting cell division in the sub-apical meristem of the shoot or by inhibiting cell elongation (Grossman, 1992). Paclobutrazol (PBZ) {2RS, 3RS)-(4-chlorophenyl)-4,4-dimethyl-2-(1H,1,2,4-triazol-1-yl)-pentan-3-ol} is one of the growth retardants, which inhibits kaurene oxidase and thus blocks the oxidative reactions from ent-kaurene to entkaurenoic acid in the pathway leading to gibberellic acid (Graebe, 1987). PBZ is among the compounds that can positively affect the acclimatization of microplants (Roberts *et al.*, 1992 and Sopher *et al.*, 1999). Ethephon (ET) {(2-chloroethyl) phosphonic acid} is a growth retardant that release ethylene (C₂H₄), chlorine (Cl) and hydrogen phosphate (H₂PO₄) on application and is known to inhibit internode elongation, induce branching (Glady *et al.*,

2007). Ethephon is an ethylene-releasing compound, and it is widely used as a plant growth regulator. The effect of the application of exogenous gaseous ethylene or ET solution varies with plant species, chemical concentrations, timing and duration of application. ET regulates phases of plant growth and development by application to various growth sites (Kidd and James, 1991). The objective of this study was to assess the influence of two plant growth retardants (paclobutrazol and ethephon) on shoots and root growth to improve the *ex vitro* survival and growth of Stevia plantlets during acclimatization.

II. Materials And Methods

Plant material

Stevia plants were obtained from the Sugar Crop Institute, Agricultural Research Center, Giza, Egypt. The plants were maintained under greenhouse conditions at the Desert Research Center, Cairo, Egypt, for at least three months prior to removal of materials for culture.

Explant sterilization

Shoot tips and stem nodal segments were placed under running tap water for 30 min, then surface sterilized under complete aseptic conditions. Stem nodal segments were cut into 2-3 cm and then were soaked in a 25% (v/v) of commercial bleach solution (1.25% sodium hypochlorite) for 30 min with gentle agitation. Shoot tips were soaked in 20% (v/v) commercial bleach solution (1.0% sodium hypochlorite) for 15 min. Finally, the explants were rinsed three times in sterile distilled water.

Culture medium and conditions

All the surface sterilized explants were cultured on a full strength basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa, Haarlem, Netherlands) supplemented with 30 g/l sucrose. The pH of the medium was adjusted to 5.7-5.8 before being solidified with 3.0 g/l phytigel (Duchefa, Haarlem, Netherlands), then autoclaved at a pressure of 1.06 kg/cm² and 121°C with a 16-h photoperiod under cool white fluorescent tubes.

Effect of PBZ and ET on shoot multiplication

Explants were established *in vitro* on MS medium supplemented with 1 mg/l kinetin. After, four weeks, the *in vitro* established shoots were transferred to be multiplied on MS medium supplemented with 2 mg/l 6-benzyl adenin (BA) with four concentrations of PBZ or ET (0.1, 0.5, 1.0 and 2 mg/l). Average number of shoots per explant, average shoot length (cm), average number of leaves per shoot and fresh and dry weights of shoots (g) were recorded after six weeks for eight subcultures.

Effect of PBZ and ET on root formation

Single shoots of Stevia of length of 2 cm were excised and transferred to MS medium supplemented with 2 mg/l indole butyric acid (IBA) and the same concentrations of PBZ or ET (0.1, 0.5, 1.0, and 2.0 mg/l). Data were recorded in terms of percentage of rooting, average number of roots/shoot and average root length (cm) after six weeks from culturing.

Acclimatization

After six weeks, the plantlets were removed from the rooting media, washed in water to remove phytigel and soaked in fungicide solution (maxim) for 30 s, and then transferred to plastic pots containing equal volumes of sterile mixture of peat moss, perlite and sand. The transplanted plants were covered with plastic bags to maintain humidity, placed in greenhouse; the plastic bags were opened gradually. The survival percentage of the *in vitro* propagated plants was evaluated after eight weeks.

Determination of photosynthetic pigments

Chlorophyll a and b and carotenoids were extracted from shoots and estimated according to Fadeel's method (1962) and Von Wettstein (1957). The testing procedures were carried out at low light to protect the pigments from breakdown. Fresh leaf samples (0.5 g) were homogenized in mortar with 85% acetone according to Fadeel's method (1962). The optical densities were measured spectrophotometrically using Pharmacia LKB Novasspec at 622, 644 and 440 nm. The pigment concentrations were calculated using Wettstein's formula (Von Wettstein, 1957) as following:

$$\text{Chlorophyll a (mg/l)} = 9.784 \times E_{662} - 0.99 \times E_{644}$$

$$\text{Chlorophyll b (mg/l)} = 21.426 \times E_{644} - 4.65 \times E_{662}$$

$$\text{Carotenoids (mg/l)} = 4.695 \times E_{440} - 0.268 (\text{Chlorophyll a+b})$$

Where, E = Optical density at the wavelength indicated.

The concentration of pigments was then expressed in mg/g fresh weight of leaves according to the following formula:

$$\text{mg/g} = (\text{mg/l} \times \text{dilution}) / (\text{sample weight} \times 1000)$$

Determination of total carbohydrates

For determination of total carbohydrates content, a known weight of powdered samples were extracted from the dried terminal shoots in 1 N HCl solution for six hours at 100°C and filtered. The filtrate was raised to known volume. Total carbohydrate content was determined photometrically at 485 nm according to the phenol

sulphuric acid method described by Dubois *et al.* (1956). Phenol reagent was prepared by adding 10 ml of H₂O to 90 ml of the 5% phenol solution. The resultant solution should be colorless, but may develop a pale yellow color with time. Standard glucose solution was prepared by adding 50 mg of glucose dissolved in water and raised to a final volume of 100 ml. A standard curve was prepared with a range of 20-150 µl of glucose standard solution. A blank of distilled water was used. Five ml of phenol reagent were added to each tube and mixed thoroughly, then rapidly 5 ml of H₂SO₄ were added to each tube. Samples were allowed to stand at room temperature for 30 min and the absorbance at 485 nm was recorded. The concentrations of glucose were plotted against the corresponding absorbance to generate a standard curve. The concentration of the unknown carbohydrate is determined graphically.

Experimental design and statistical analysis of data

The experiments were subjected to a completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan,1955), as modified by Snedecor and Cochran (1998) were used to analyze the obtained data. Each treatment of PBZ and ET had three replicated and each replicate consisted of 9 jars. The experiments were repeated twice. The differences between the averages of the recorded parameters for all treatments were tested for significance at the 5% level. The averages followed by the same letter are not significantly different at $p < 0.05$.

III. Results And Discussion

Growth retardants are known to affect of morphological and physiological characters quality of the *invitro* produced plants. There are problems at acclimatization stage, in which the plantlets are very thin with low rate of survival when transfer to soil. After transferring multiple shoot buds into MS basal medium containing 2.0 mg/l BA and various concentrations of PBZ and ET separately (0.1, 0.5, 1.0, and 2.0 mg/l), the amount of PBZ and ET affected of multiple shoot formation, shoot length, leaves number, fresh and dry weights as presented in Table 1 and Figure 1.

Table 1. Effect of different concentrations of PBZ and ET in MS medium supplemented with BA (2.0 mg/l) on shoot multiplication, shoot length, leaves number and fresh and dry weights of Stevia after six weeks from culturing.

Treatment	No. of shoots/explant	Length of shoots (cm)	No. of leaves/shoot	Fresh weight of shoot (g)	Dry weight of shoot (g)
Control	37.33b	7.37a	7.00a	2.03bc	0.20de
0.1 mg/l PBZ	48.00a	6.5ab	7.43a	2.40ab	0.27bcd
0.5 mg/l PBZ	30.67b	5.63bc	5.63b	2.83a	0.39a
1.0 mg/l PBZ	27.67bc	4.93cd	4.30c	1.87bcd	0.29abcd
2.0 mg/l PBZ	21.00d	4.00de	3.87cd	1.43cd	0.26bcd
0.1 mg/l ET	38.67b	6.13b	6.33ab	2.20ab	0.32abd
0.5 mg/l ET	24.34cd	4.50d	4.33c	2.83a	0.36ab
1.0 mg/l ET	20.33de	3.13ef	3.67cd	1.30de	0.22cde
2.0 mg/l ET	15.67e	2.13f	3.00d	0.70e	0.14e

Means followed by the same letter within a column are not significantly different at $p > 0.05$.



Figure 1. Multiple shoots formation of Stevia on MS medium supplemented with 2.0 mg/l BA in addition to a) 0.1, b) 0.5, c) 1.0 and d) 2.0 mg/l PBZ.

Shoot proliferation was observed after six weeks from culturing. PBZ at the lower concentration of 0.1 mg/l was significantly superior than the other concentrations; it gave the highest shoot number of 48.0 shoots/explant. These results are in agreement with that obtained by Anchalee and Bodhipadma (2012), who reported that low concentration of PBZ seems to have a power to enhance multiple shoot and plantlet formation in some species of *Curcuma*. Kongbangkerd and Yanaphan (2005) found that MS medium consisted of 0.1 mg/l PBZ was able to induce shoots of *Curcuma longa* *in vitro* better than 0.5 mg/l PBZ. Also, some authors reported that PBZ enhanced cytokinin activities and induced adventitious shoot proliferation in *Araceae* (Werbrouck and Debergh, 1996). In addition, there are synergistic effects of PBZ and cytokinin, especially BA that enhancing

shoot proliferation (Tefera and Wannakrairoj, 2006). PBZ induced suppression of gibberelin biosynthesis should have resulted in changes in hormonal constellation favorable for axillary bud initiation and branching as observed by Woodward and Marshall (1988). Such effect of intensification of cytokinin activity can result from the fact that growth retardants increase the contents of endogenous cytokinin (Rademacher, 2000). PBZ, like other growth retardants combined with cytokinin induced strong shoot proliferation (Ziv, 1990). Also the concentration of 0.1 mg/l ET gave 38.67 shoots/explant, which is the highest number of shoots comparing to the other concentrations of ET. On the other hand, the highest concentration of 2.0 mg/l of PBZ and ET gave the lowest shoot number of 21.0 and 15.87 shoots/explant, respectively. On the other hand, the influence of PBZ and ET on shoot length in *Stevia rebaudiana* was clearly observed, where a gradual decrease in shoot length for all the treatments was noticed as compared to that of control, where the tallest shoots (7.36 cm) were recorded. Treatments with 1.0-2.0 mg/l PBZ gave significant reductions in stem length and fresh to dry weight ratios of shoots of *Chrysanthemum* (Elaine *et al.*, 1990). Foster *et al.* (1991) reported that plant height was reduced by ET application. Alternatively, some of the applied ET may be translocated in the plant, as been demonstrated by Edgerton and Hatch (1972). Such translocated ET could then release ethylene in subapical elongation regions, leading to inhibited stem growth.

PBZ is a useful PGR that has been applied in agricultural systems for decades. PGRs are used on crops, such as rice and wheat to inhibit plant height as well as increase the number of tillers (Assuero *et al.*, 2012). Average number of leaves ranged from 7.43 to 3.0. The highest number of leaves (7.43 and 7.0) was recorded with 0.1 mg/l PBZ and the control. On the other hand, the lowest number of leaves was achieved on 2.0 mg/l ET, which gave 3.0 leaves /shoot. Regarding the mean shoot fresh and dry weights, the addition of PBZ and ET at 0.5 mg/l positively affected the obtained shoot weights, comparing to the different tested concentrations of PBZ and ET. It could be noticed that the fresh and dry weights were gradually increased with increasing the concentration from 0.1 to 0.5 mg/l. Moreover, fresh weight rate was not varies significantly in the concentration of 0.1 to 0.5 mg/l. While 0.5 mg/l PBZ gave the maximum dry weight (0.39 g) of shoots, the highest concentration of PBZ (2.0 mg/l) led to a considerable decrease in shoot fresh weight (1.43 g). The highest fresh and dry weights of shoot (2.83 and 0.35 g), regarding different concentrations of ET, was obtained with 0.5 mg/l ET, while the lowest fresh and dry weights of shoot (0.7 and 0.14 g) were recorded at 2.0 mg/l ET. Inhibited gibberellin biosynthesis and increased cytokinin and abscisic acid content induced by triazoles might be the cause of increased root growth and corresponding dry or fresh weight in plants (Rajalekshmi *et al.*, 2009). The obtained results indicate that increasing PBZ and ET concentrations resulted in a significant decrease in shoot number, shoot length, leave number and fresh and dry weights.

Root quality is an important index of *in vitro* plantlets quality and a critical factor for transplanting. adventitious rooting. Shoots formed roots on MS medium containing 2.0 mg/l IBA and different concentrations of each of PBZ and ET separately (Table 2). It was observed that there is no significant difference in rooting percent among the different concentrations of PBZ and the control treatment, giving 88.8–100% of rooting. The higher dosage of ET (1.0 and 2.0 mg/l) suppressed rooting percentage and gave 44.44% rooting, while 0.1 and 0.5mg/l ET were insignificantly different comparing to the other treatments. The highest roots number was achieved on 0.1 and 0.5 mg/l PBZ, which gave 8.44 and 8.33 roots/shoot, respectively (Figure 2). Therefore, cell division may have been promoted, which in turn increased number of roots.

The increase in number of roots is necessary to keep shoot–root ratio in balance (Mukhtar *et al.*, 1998). PBZ applied in combination with IBA increased the rooting capacity of cutting, by increasing root formation (Wiesman and Lavee, 1995). Depending on the plant species and the concentration applied, PBZ induce root anatomical and morphological modifications. It increased root diameter of rows and diameter of cortical cells (Burrows *et al.*, 1992). PBZ increased root diameter in soybean by increasing the size of cortical parenchyma cells (Barnes *et al.*, 1989). The increase in fine root development implies a more favorable root/crown balance and less stress in treated trees. Improved water status has been reported after treatment with PBZ (Swietlik and Miller, 1983 and Warren *et al.*, 1991). Followed by 0.5 mg/l ET that gave 7.44 roots/shoot. These results are similar to that of Rossi *et al.* (2008) who found that pulverization of ET in the aboveground tissues of *Pinus taeda* seedlings caused thickening of tap roots, and an increase in the number of secondary roots in the middle and lower portion of the root system. ET affected adventitious rooting with IBA. Li *et al.* (2006) showed that ethylene treatment activated transcription of ARF19 in roots of *Arabidopsis* seedlings, which might provide an explanation for the effects of ET IBA combination. Krishnamoorthy (1972) and Roy *et al.* (1972) reported that ET promoted rooting, whereas Mullins (1972) showed that rooting was inhibited by ethylene. ET treatment promotes adventitious rooting of some herbaceous plants (Pan *et al.*, 2002). Stepanova *et al.* (2005) reported that IAA was accumulated by ethylene treatment. On the other hand, the highest average root length (7.74 cm) was obtained on MS medium without growth retardants (control). However, the average of root length was ranged from 6.14 to 2.86 cm for PBZ and ET concentrations. Data also revealed that root length was gradually decreased with increasing PBZ and ET concentrations. So, it is suggested that high concentrations of PBZ and ET cease reduction of root length and in contrast increased root thickness and number of roots, except the higher

concentrations. In agreement with these results, Grazia (1988) reported that PBZ caused reduction of root length, especially at the end of the rooting phase when added to IBA.

Table 2. Effect of different concentrations of PBZ and ET in MS medium supplemented with IBA (2.0 mg/l) on rooting formation of Stevia after four weeks from culturing.

Treatment	Rooting %	No. of roots/shoot	Length of root (cm)
Control	100.0a	6.00c	7.74a
0.1 mg/l PBZ	100.0a	8.44a	6.14b
0.5 mg/l PBZ	100.0a	8.33a	6.08b
1.0 mg/l PBZ	88.8a	6.33bc	4.93cd
2.0 mg/l PBZ	88.8a	5.00cd	2.91e
0.1 mg/l ET	88.8a	5.22cd	5.66bc
0.5 mg/l ET	77.78ab	7.44ab	5.48bc
1.0 mg/l ET	44.44c	5.11cd	4.37d
2.0 mg/l ET	44.44c	4.33d	2.86e

Means followed by the same letter within a column are not significantly different at $p>0.05$.



Figure 2. Root development of Stevia in MS medium supplemented with 2.0 mg/l IBA in addition to 0.1 mg/l PBZ.

Chlorophyll content is one of the indirect markers of the efficiency of the photosynthetic apparatus, for all chlorophyll and not only that takes part in photosynthesis. Photosynthesis is a physiological marker related to *in vitro* plant quality (Borkowska, 2003). The effect of PBZ and ET treatments on chlorophyll a, chlorophyll b, carotenoids and carbohydrates content were evaluated (Table 3). It was clear that leaf chlorophyll a and b, carotenoids and carbohydrates content were increased in treated plantlets at the different treatments compared with the control. The highest amount of chlorophyll a and b and carbohydrates were recorded on MS medium supplemented with 2.0 mg/l PBZ; they reached 0.083, 0.179 and 1.715 mg/g, respectively. On the other hand, the control had the lowest amount of chlorophyll a and b, carotenoids and carbohydrates content were found on MS medium without supplementations (control), which recorded 0.010, 0.030, 0.061 and 0.024 mg/g, respectively. Fletcher *et al.* (2000) proposed that PBZ as one in triazol group stimulates cytokinin synthesis that enhances chloroplast differentiation, chloroplast differentiation, chlorophyll biosynthesis and prevents chlorophyll degradation. Nouriyani *et al.* (2012) reported that an increase in PBZ concentrations increased chlorophyll content significantly in two wheat cultivars. Also, Pinhero and Fletcher (1994) observed an increase in chlorophyll and carotenoid pigments after treatments with the triazole compound PBZ in maize seedlings. Aboelfazl *et al.* (2013) reported that treating plants of banana with PBZ increases the total leaf chlorophyll content when compared with the control treatment. Samani (2104) results showed that PBZ concentration had significant effect on leaf chlorophyll content. Whereas, the concentration of 0.5mg/l PBZ gave the highest carotenoids content (0.122 mg/g). Youssef and Abd El-Aal (2013) reported that PBZ significantly increased total carbohydrates and total chlorophylls content in *Tabernaemontana coronaria* as compared with untreated plants. Regarding the effect of ET, the results indicated that the ET levels had significant effect on chlorophyll a and b, carotenoids and carbohydrates. Leaf chlorophyll a and b were all significantly higher than control. The highest chlorophyll a and b contents was recorded on MS medium supplemented with 2.0mg/l ET (0.062 and 0.171 mg/g, respectively), followed by 1.0 mg/l ET, which gave 0.42 and 0.129 mg/g, respectively. Decreasing the concentration of ET caused decreasing in the level of chlorophyll a and b. Wei *et al.* (2006) reported that ET causes photosynthesis improvement. On the other hand, the lowest chlorophyll a and b content was achieved on the control medium. Carotenoids and carbohydrates were affected by ET concentrations, as 0.5 mg/l ET recorded the highest carotenoids and carbohydrates content (0.179 and 1.814 mg/g, respectively), while MS

medium (control) gave 0.061 and 0.024 mg/g, respectively. The results cleared that raising the levels of ET used increased chlorophyll a and b and carotenoids content in the leaves.

Table 3.Effect of different concentrations of PBZ and ET on the contents of chlorophyll a and b, carotenoids and carbohydrates of Stevia.

Treatments	Chl. a (mg/g)	Chl. b (mg/g)	Carotenoids (mg/g)	Carbohydrates (mg/g)
Control	0.010 ^e	0.030 ^e	0.061 ^c	0.024 ⁱ
0.1 mg/l PBZ	0.020 ^d	0.040 ^f	0.082 ^d	0.046 ^h
0.5 mg/l PBZ	0.043 ^b	0.130 ^b	0.112 ^c	1.082 ^b
1.0 mg/l PBZ	0.038 ^b	0.166 ^a	0.122 ^b	1.073 ^b
2.0 mg/l PBZ	0.083 ^a	0.179 ^a	0.073 ^d	1.715 ^a
0.1 mg/l ET	0.031 ^c	0.068 ^d	0.058 ^e	0.097 ^g
0.5 mg/l ET	0.034 ^c	0.088 ^c	0.179 ^a	1.814 ^a
1.0 mg/l ET	0.042 ^b	0.129 ^b	0.059 ^e	1.068 ^c
2.0 mg/l ET	0.062 ^a	0.171 ^a	0.082 ^d	0.670 ^d

Means followed by the same letter within a column are not significantly different at $p > 0.05$.

Acclimatization stage

A major limitation in large scale application of micropropagation technology is high mortality experienced by *in vitro* raised plants during laboratory to land transfer. Micropropagated plants on being transferred to *ex vitro* conditions are exposed to altered temperature, light intensity and water stress conditions, so need acclimatization for successful establishment and survival of plantlets (Chandra *et al.*, 2010). It is evaluated by percentage of the surviving plants, which was clearly affected by the different treatments of PBZ. The results in Table 4 indicated that acclimatization of rooted shoots in the new environment in the greenhouse ranged between 40 and 85%. The highest percentage of survived plants with new leaves was achieved 85%, when cultured on medium with PBZ at 2.0 mg/l (Figure 3), followed by 1.0 mg/l, which gave 70% survival percentage. On the other hand, the survival percentage of plants was the lowest (40%), when shoots were cultured on MS medium without supplementation of PBZ (control).

Table 4.Effect of PBZ and ET on survival percentage of Steviaplantlets during acclimatization stage.

Treatment	Survival percentage%
control	40
0.1mg/l PBZ	55
0.5 mg/l PBZ	65
1.0 mg/l PBZ	70
2.0 mg/l PBZ	85
0.1mg/l ET	55
0.5 mg/l ET	60
1.0 mg/l ET	70
2.0 mg/l ET	75



Figure (3).Hardened Stevia plant under greenhouse conditions.

According to many sources, the growth retardants increase the ability of plants to overcome acclimatization phase. Eliassonet *al.* (1994) noted that survival of *Prunusserotirina* four weeks after transfer to the soil was significantly improved by PBZ. Similarly, survival of *Philodendron* that had been treated with PBZ during proliferation was much better (100%), compared with control (78%) (Ziv, 1990). Robert and Matthews (1995) reported that *Chrysanthemum* “Pennile Reel” treated with PBZ in rooting medium had greater resistance

to desiccation. Fernandes *et al.* (2004) reported that to improve survival rate of the *in vitro* plants to soil or field conditions, the use of chemicals; such as plant growth regulators are of great interest. Moreover, the recent development of highly active growth retardants enhances the potential uses of chemical regulators. Among them, PBZ is widely used. It could be noticed that the survival frequency from the plantlets hardened was gradually increased with increasing the concentration from 0.1 to 2.0 mg/l PBZ. Regarding the effect of ET treatments on plant growth in the acclimatization stage, the survival percentage after transplantation in the greenhouse was different for the different pretreatments in rooting stage (Table 4). Inexpressive survival was ranged from 40 to 75%. The highest rate (75%) in acclimatization occurred when the plantlets were treated with ET at 2.0 mg/l during the *in vitro* rooting stage. However, the lowest rate (40%) was observed in untreated plantlets (control). This result is similar to that of Scagel and Linderman (2001) who concluded that ET treatment induced a reduction in seedling height, an increase in stem caplier, and growth of new roots in the nursery with a significant positive correlation with survival after planting. An increase of ET spray formulation reduced overall stem growth and increased cell elongation of the aboveground plant tissues. These morphological changes are expected during seedling hardening according to Jacobs and Landes (2009). Joao *et al.* (2013) reported that the use of ET reduced aboveground growth and significantly increase the root biomass of *Parchystromalongifolium*, validating its use to promote the morphological changes expected during seedling in nurseries. Based on the production of above ground tissue and increase root biomass, *Parchystromalongifolium* seedling root system may act as a storage organ in response to ET application, the increments express growth rate as a function of time and reflect growth vigor (Joao *et al.*, 2013). It should be noted that increased levels of ET are promoting a quadratic increase in the percentage of surviving plants during the acclimatization stage.

IV. Conclusion

In conclusion, PBZ and ET significantly enhanced the vegetative and reproductive characteristics of *Stevia* both *in vitro* and *ex vitro*. The results also indicated that the application of PBZ had the greatest effect on the development of *Stevia* shoot and root *in vitro* and improvement of the survival during acclimatization. According to the results of the present study, it is effective to use such treatments in the micropropagation procedure to increase the ability of *in vitro* produced plantlets after their transfer to the soil to withstand the acclimatization stress.

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