Middle-East Journal of Scientific Research 14 (1): 93-106, 2013
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2013.14.1.1948

Biotechnological Studies for Improving of Stevia (*Stevia rebaudiana* Bertoni) *in vitro* Plantlets

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Abstract: *Stevia rebaudiana* Bertoni is an economically important medicinal plant act as a sugar substitute for diabetic and obese people. In the present 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 2.0 mg/l N6-benzyl amino purine (BAP) recorded the maximum number of shoots of 43.9 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 acid2-2-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 (7.3 roots/shoot), root length (2.64 cm) and plant height (4.29 cm) were obtained on MS medium supplemented with 1.0 mg/l IBA and 0.5 mg/l Alar. *Stevia rebaudiana* Bertoni plantlets were acclimatized and grown well in planting medium containing sand, peatmoss and vermiculite, at equal volumes. The best recorded survival percentage in greenhouse was 93.3%. Analysis of SDS-PAGE of soluble protein and eleven isozymes systems revealed complete similarity (100%) among the three samples of stevia (mother plant, plants treated with growth retardant and non treated plants). The level of polymorphism detected among the three samples of stevia was 44.70% using randomly amplified polymorphic DNA (RAPD), 24.00% using inter simple sequence repeat (ISSR) and 22.03% using amplified fragment length polymorphisms (AFLP). With an average of 30.24% polymorphism between the three samples obtained by all used biochemical and molecular markers. From previous results, 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 plants and for the use of *Stevia rabodiana* medicinally and commercially Bertoni.

Key words: *Stevia rebaudiana* · Microprogration · *In vitro* pre acclimatization · Plant growth retardants · Alar · Cycocel · SDS-PAGE · RAPD-PCR · AFLP · ISSR-PCR

INTRODUCTION

Stevia rebaudiana Bertoni is a small, herbaceous, semi-bushy, tropical perennial shrub belongs to Asteraceae family. It is native to Paraguay and Brazil [1]. 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. [2]. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste, non caloric and chemical stability [3]. Consequently, stevia does not have any effects on blood sugar and therefore it is friendly to human health [4]. Moreover, it has therapeutic values such as obesity, hypertension, heartburn, hypoglycemia, anticancer and to lower the uric acid levels [5].

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The seeds of stevia show a very low germination percentage and genetic variability [6]. Vegetative propagation is slow and limited by the low number of individuals obtained from single plants [7]. 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 [8-11] but a considerable effort is still required to make it more practical. The terms growth retardants is 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 [12]. 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 [13, 14]. Isozymes are used as biochemical markers for genetic uniformity of regenerated plants and monitoring the genetic purity and fidelity [15]. Random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) have been employed to assess the genomic stability in regenerated plants [16]. Moreover, the common practice to use AFLP markers to identify clonal fidelity of plants [16, 17] gives sensitive and accurate results. This provides better chances to identify the genetic variations and similarity in *in vitro* plants.

The present study aimed to find an efficient protocol for *in vitro* mass propagation of *Stevia rebaudiana* with an efficient pre- acclimatization treatments to improve survival of plantlets in greenhouse and to assess the genetic fidelity through biochemical and molecular markers analyses to observe the effect of growth retardants on improving survival of plantlets in greenhouse, which is important for promising newly introduced non caloric sweetener plant in Egypt.

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. Plants grown and maintained in the greenhouse of private farm at 68 km Cairo- Alexandria desert road. The explants were cut into small pieces (about 1.5 cm long) and then were treated with a few drop 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% HgCl2 (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 [18] medium (MS) salts and vitamins. The medium containing 3% (w/v) sucrose was solidified with 0.8% (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/cm^2 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, 0.5, 1.0, 1.5 and 2.0 mg/l). The medium was dispensed in the culture tube (150 x 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 or Kin (0.5, 1.0, 1.5 and 2.0 mg/l), MS medium without growth regulators was used for control. 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 six 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 regulators 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* Acclimatization (Pre-Acclimatization) of Micropropagated Plantlets:** Mainly this experiment was conducted to study the effect of growth retardants alar and cycocel at different concentrations (0.25, 0.5 and 1.0 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) 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.

Statistically Analysis: Data all results 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 [19].

Molecular Marker

Extraction of Total Protein: Bulked leaf sample (0.25) g of each sample was ground with liquid nitrogen and mixed with extraction buffer (pH7.5, 50 mM tris, 5% glycerol and 14 mMB-mercaptoethanol) in a mortar with pestle, left overnight then vortexed for 15 sec and centrifuged at 10.000 rpm at 4°C for 10 min. The supernatants were transferred to new eppendorf tubes and kept at 20°C until used for electrophoresis analysis according to Laemmli [20].

Isozyme Analysis: Isozymes were used: α-and β-esteras (Est.), acid phosphatase (Acph.), alcohol dehydrogenase (Adh.), aldehyde oxidase (Ao.), malic enzyme (Ma.), malate dehydrogenase (Mdh.), catalase (CAT), superoxidedismutase(SOD), polyphenoloxidas (Pod.) and peroxidase (Prx). Isozymes were separated according to Stegemann *et al.* [21] In gels staining, protocols of Scandalios [22] were used for α and β-Est.; Wendel and Weeden [23] for Ao, Acph and Adh; Jonathan and Wendell, [24] for Mal and Mdh; Heldt [25] for Prx. Gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours; and photographed.

DNA Extraction: Genomic DNA was extracted from fresh young leaves of ten plants chosen randomly for each population by CTAB method of Doyle and Doyle [26]. Leaves of three plants from a single population were bulked prior to extraction. DNA was quantitated by spectrophotometer and gel electrophoresis.

RAPD for DNA Amplification: RAPD reactions were conducted according to Williams *et al.* [27] by using twenty arbitrary 10-mer primers synthesized at Operon Technologies Inc. The most reproducible primers were OPB1, OPB2, OPB5, OPB6, OPD1, OPA3.The nucleotide sequences of these primers are present in Table 10. PCR conditions were carried out in a reaction volume of 25 ul containing 5U/ul Taq DNA polymerase, 10X buffer with 15mM MgC12, 200 iM of each dNTP, 10 mM of primer (Operon Technologies Inc.), 50 ng of genomic DNA. Amplification was carried out in Strategene Robocycler Gradient 96 with initial denaturation cycle at 94°C for 4 min. followed by 45 cycles consisting of 94 °C for 1.5 min. 37.5°C for 1.5 min. 72°C for 2.5 min. and a final extension cycle at 72°C for 7 min. Amplification products were separated in 1.2 % agarose gels in TBE buffer, stained with ethidium bromide and photographed with Bio-Rad Gel Doc. System.

ISSR for DNA Amplification: ISSR-PCR reactions were conducted using 10 specific primers, for three samples of stevia. The reaction conditions were the same of RAPD reaction conditions but extension time for 50 second because of the long ISSR primers and 15 µl of PCR- products were resolved in 1.5 % agarose gel electrophoresis with 1x TAE running buffer. The gel was stained with ethidium bromide. Molecular bands were detected on UV-transilluminator and photographed by Gel documentation system

AFLP Reactions: The AFLP method was carried out following the standard procedure described by Vos *et al.* [28]. High- quality genomic DNA (0.5g) was digested with a pair of restriction enzymes (Pst1/Mse1) then ligated to double stranded Pst1and Mse1 adaptors. The ligate was preamplified with nonselective primers and selective amplification carried out using pairs of 2 bp and 3bp selective primers.The products were separated on polyacrylamide gels using an M13 sequencing ladder as a size standard.

RESULTS AND DISCUSSION

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 52 to 92% for both shoot tips and stem segments, respectively. Percentage of growing explants ranged from 69.2% to 91.3% and 42.8% to 86.4 % for shoot tips and stem node segments, respectively. Data obtained after six weeks of culture revealed that shoot tips gave the highest growth percentage (91.3%) and highest average length (3.8 cm) on MS medium supplemented with 0.5 mg/l BAP, followed by MS medium containing 1.0 mg/l BAP which gave 85.7% growth percentage and average shoot length of 3.1 cm. While, the same medium gave the highest growth percentage 86.4% for stem segments, but the average length was 2.1cm. However, MS medium containing 0.5 mg/l BAP gave average length of 2.8cm. 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 concentration. From the previous results, it is clear that MS medium containing 0.5 mg/l BAP gave rise to best result for shoot tips. These results are in agreement with that obtained by Ibrahim *et al.* [9] 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.* [3] and Das et al. [29] on stevia. Whereas, in contrary to the present account, Laribi *et al.* [11] 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/explant, *in vitro* regenerated shoots were cultured on MS medium supplemented with BAP or Kin at 0.5, 1.0, 1.5 and 2.0 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 affected by BAP concentrations. Increasing BAP concentration increased the number of shoots. Shoot multiplication rate was significantly high and ranged from 7.7 to 43.9 shoots /explant due to BAP application, which increased multiplication rate. The maximum number of shoots (43.9 shoots/explant) was recorded on MS medium supplemented with 2.0 mg/l BAP compared to the other BAP treatments. Whereas, the same concentration of kinetin (2.0 mg/l) gave 17.7 shoots/explant. On the other hand, shoot length decreased by increasing BAP concentration, average shoot length ranged from 0.74 to 3.84 cm. Also, increasing Kin concentrations to 2.0 mg/l decreased the average shoot length; it was ranged from 4.96 to 5.87 cm. MS basal medium without plant growth regulators was found the best medium to improve the length of *Stevia rebaudiana* Bertoni, which reached 6.81 cm. Average number of leaves ranged from 2.42 to 7.69 and it was significantly affected among the different treatments. The highest number of leaves (7.69) 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.* [9] 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 2.0 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.* [29] 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.

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 (4.5) and average root length (3.34 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 were 80% 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 1.0 to 2.4 roots per explant. However, the average of roots length was ranged from 0.92 to 1.8 cm. On the other hand, MS basal medium without plant growth regulators recorded 70% rooting percentage, 3.4 roots number and 2.73 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*

Table 1: Effect of BAP concentrations and explant type on shoot initiation of Stevia rebaudiana Bertoni cultured in vitro after six weeks from culture.

	Explant type					
	Shoot tip			Stem node segment		
BAP Concentrations (mg/l)	Survival %	Growth to survival %	Shoot length (cm)	Survival%	Growthto survival %	Shoot length (cm)
0.0	52c	69.2c	1.15c	56e	42.8d	0.7c
0.5	92a	91.3a	3.80a	80b	75.0b	2.8a
1.0	84b	85.7b	3.10b	88a	86.4a	2.1a
1.5	80b	80.0b	2.90b	72d	72.2b	1.7b
2.0	80b	75.0c	1.92c	76c	68.4c	1.5b

Means followed by the same letter within a column are not significantly different at P ≤ 0.05.

Table 2: Effect of different concentrations of cytokinins (BAP or Kin) on shoot multiplication of Stevia rebaudiana Bertoni after 6 weeks from culture.

Concentrations (mg/l)	Average number of shoots/ explants	Average shoot length (cm)	Average number of leaves/ shoot
0.0	7.7 h	6.81 a	7.69 a
	BAP		
0.5	27.4 d	3.84 e	4.32 f
1.0	31.4 c	2.38 f	3.73 g
1.5	38.1 b	1.91 g	3.29 h
2.0	43.9 a	0.74 h	2.42 i
	Kin		
0.5	9.8 g	5.87 c	6.92 b
1.0	13.4 f	5.71 c	6.67 c
1.5	16.3 e	5.58 b	6.04 d
2.0	17.7 e	4.96 d	5.34 e

Means followed by the same letter within a column are not significantly different at P≤ 0.05.

Table 3: Effect of different concentrations of auxins (IBA, IAA and NAA) on in vitro root formation of Stevia rebaudiana Bertoni after 6 weeks from culture.

Treatments (mg/l)	Rooted shoots (%)	Average number of roots/ shoot	Average root length (cm)
0.0	70 c	3.4 b	2.73 c
	IBA		
0.5	100 a	3.7 b	2.93 b
1.0	100 a	4.5 a	3.34 a
1.5	70 c	3.4 b	2.60 c
2.0	60 d	3.1 b	2.10 d
	IAA		
0.5	80 b	1.7 f	1.22 g
1.0	80 b	2.2 d	1.60 f
1.5	50 e	1.4 f	1.03 h
2.0	30 f	1.0 g	0.92 i
	NAA		
0.5	80 b	1.9 e	1.55 f
1.0	80 b	2.4 d	1.80 e
1.5	70 c	1.7 f	1.21 g
2.0	50 e	1.3 g	1.00 h

Means followed by the same letter within a column are not significantly different at P ≤ 0.05.

Table 4: Effect of growth retardants (B 9 and CCC) in MS medium enriched with IBA (1.0 mg/l) on rooting improvement of Stevia rebaudiana Bertoni after 4 weeks of culture.

Growth retardants concentrations(mg/l)	Average number of roots/ shoot	Average roots length (cm)	Average plant height (cm)
0.0	4.7 e	3.28 a	5.11 a
	Alar (B 9)		
0.25	5.8 c	2.83 b	4.47 c
0.5	7.3 a	2.64 c	4.29 d
1.0	6.1 b	2.15 d	4.10 e
	Cycocel (CCC)		
0.25	4.9 e	2.07 d	5.00 b
0.5	5.7 c	1.72 e	4.00 e
1.0	5.2 d	1.68 e	3.96 e

Means followed by the same letter within a column are not significantly different at P = 0.05.

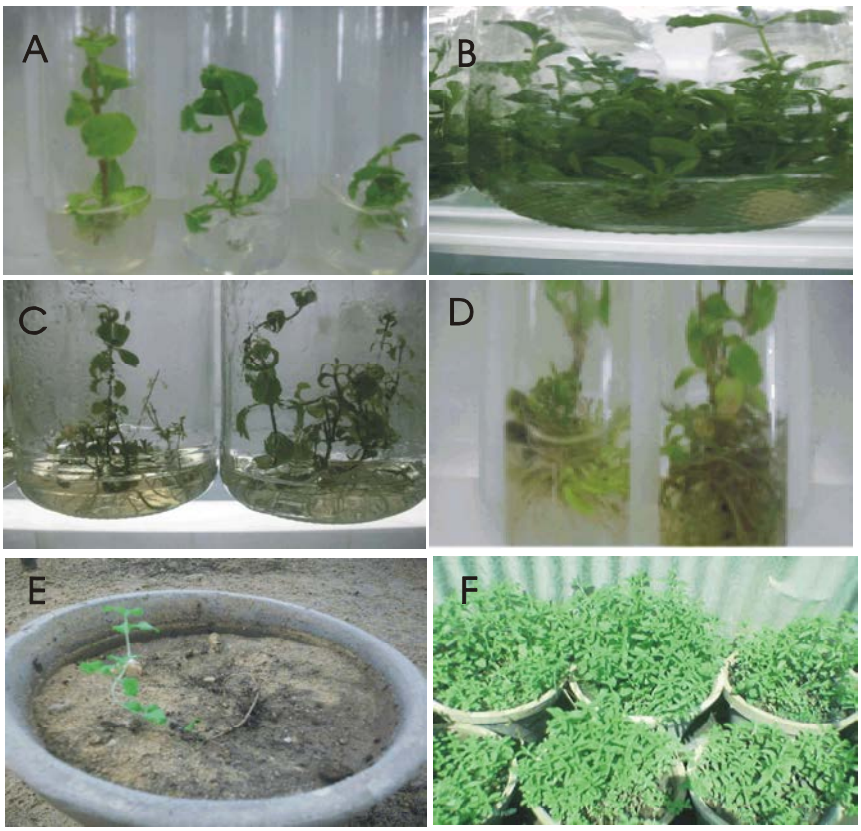


Fig. 1: Micropropagation of stevia (Stevia rabaudiana): (A) Starting stage (B) Multiplication of shoots, (C) Rooting without growth retardant (D) Rooting with growth retardant, (E) Acclimatization plantlets which non treated with growth retardant. Acclimatization plantlets treated with growth retardant : (F) After one month.(G) After four months.

rebaudiana Bertoni [3, 30, 31]. IBA treatments proved to be better than NAA and IAA. These results are in agreement with those obtained by Ibrahim *et al.* [9], Satpathy and Das [32] who stated that IBA was better than NAA and IAA for shoot and root formation of stevia. Also, Jitendra *et al.* [33] 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 [10, 34]. On the contrary, Laribi et al. [11] 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 *In vitro* Rooting Improvement: Data in Table 4 and Fig. (1D) presented the effect of alar (B9) and cycocel (CCC) separately, at different concentrations (0.25, 0.5 and 1.0 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 0.5 and 1.0 mg/l B9 compared to the control and other levels of CCC. MS medium supplemented with 1.0 mg/l IBA alone (control) and without any growth retardants, gave the highest mean length of roots and plant height (3.28 and 5.11 cm, respectively). However, the average of roots length was ranged from 1.68 to 2.83 cm for both retardants at concentrations from 0.25 to 1.0 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 [13], EL-Mokadem and Heikal [14]. 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 [35] 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.

So, this experiment revealed that growth retardants specially 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 [36]. Alar still has many uses for ornamental plants, where it is used to control vegetative growth, reduce plant height (induce dwarfism) [14] 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 [37]. Alar and cycocel prevent cell elongation and inhibit cell division due to their effect as anti gibberellins [38]. Moreover, retarded stem elongation by preventing the formation of kaurence, a precursor of gibberellins biosynthesis, subsequently inhibiting or reducing only elongation of stem cells [13]. These results are in agreement with those reported by Porwal *et al.* [39] on Rosa damascene. Also, alar treated chrysanthemum has a more branched root system [40]. 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).

***Ex vitro* Establishment:** After attaining the rapid *in vitro* acclimatization rate, successful acclimatization or establishment of tissue culture raised propagules in the greenhouse, is the key parameter of a micropropagation protocol [5]. 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 0.5 mg/l. As seen in Table 5, with respect of treated plantlets, 93.3% survival percentage was assured. While, its reduced for non-treated plantlets to 10%, because plantlets through tissue culture are heterotrophic, lack cuticle on their epidermis [20] as well as having non- functional stomata [18]. 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 (0.5 mg/l), reduced the plant height and number of leaves considerably (Table 5 Fig 1F-G). Alar indirectly inhibits cell elongation and division through blockage of ent-kaurene synthetase activity in the gibberellins biosynthetic pathway [41]. This result is in agreement with those reported by Meera and Sathyanarayana [42] 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.

Molecular Markers

SDS-PAGE: Data presented in Fig. 2 and Table 6 summarized the SDS-PAGE of soluble proteins for the three different samples of stevia. Analysis of gel revealed that molecular weight of protein sub units ranged between 11.99 to 104.33 kDa. Five bands at molecular weight of 104.33, 32.24, 25.75, 23.67 and 11.99 were obtained,

Table 5: Response of in vitro treated and non - treated plantlets with growth retardant (Alar at 0.5 mg/l) on ex vitro acclimatization of Stevia rebaudiana Bertoni after 4 weeks.

Plantlets after ex vitro transfer	Plantlets survival %	Plant height (cm)	No. of leaves	Plant health remark
non- treated (control)	10	7.92	11.84	+
Treated	93.3	4.86	8.43	+++

+++ Very good, ++ good, + fair.

Means followed by the same letter within a column are not significantly different at P≤= 0.05.

Table 6: SDS-PAGE patterns of total soluble proteins extracted from three stevia samples.

MW	Mother plant	Control	Treatment	Polymorphism
104.334	1	1	1	Monomorphic
32.242	1	1	1	Monomorphic
25.752	1	1	1	Monomorphic
23.670	1	1	1	Monomorphic
11.993	1	1	1	Monomorphic

Table 7: Polymorphism percentages generated by eleven isozyme systems among three stevia samples.

NO	Name of isozyme	Total bands	No of mono-morphic bands	% of polymorphism
1	Acp	2	2	0
2	Adh	4	4	0
3	a-est	2	2	0
4	ß-est	5	5	0
5	Ao	2	2	0
6	M a	3	3	0
7	Mdh	2	2	0
8	Prx	2	2	0
9	POD	1	1	0
10	SOD	2	2	0
11	CAT	1	1	0
Total	26	26	0	

Table 8: Number of monomorphic bands, number of polymorphic bands, total bands and polymorphism percentages of RAPD primers among stevia three samples.

No	RAPD primers	Primer sequence	Total bands	No. of momomorphic bands	No. of polymorphic bands	Unique bands	% of polymorphism
1	OPZ1	5'-TCTGTGCCAC-3'	14	12	1	1	7.10
2	OPZ4	5'-AGGCTGTGCT-3'	6	1	5	0	83.30
3	OPH13	5'-GACGCCACAC -3'	11	6	5	0	45.40
4	OPM3	5'-GGGGGATGAG -3'	4	2	2	0	50.00
5	OPM12	5'-GGGACGTTGG -3'	4	2	2	0	50.00
6	OPG7	5'-GAACCTGCGG-3'	4	2	2	0	50.00
Total			43	25	17	1	39.00

Table 9: Number of monomorphic bands, number of polymorphic bands, total bands and polymorphism percentages of ISSR primers among stevia three samples.

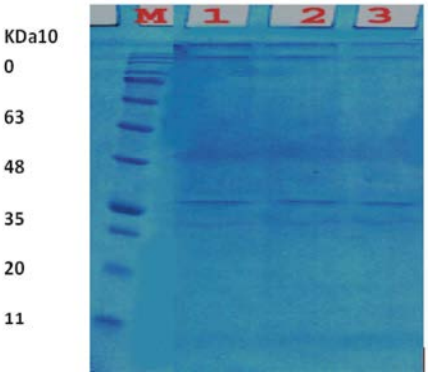
No	RAPD primers	Primer sequence	Total bands	No. of momomorphic bands	No. of polymorphic bands	Unique bands	% of polymorphism
1	17898A	(CA)6 AC	11	10	1	1	9
2	17898B	(CA)6 GT	7	5	2	0	28
3	17899A	(CA)6 AG	10	5	4	0	40
4	17899B	(CA)6 GG	7	6	1	0	14
5	HB9	(GT)6 GG	13	12	0	1	0
6	HB10	(GA)6 CC	14	3	8	1	57
7	HB11	(GT)6 CC	11	4	2	5	18
8	HB15	(GTG)3 GC	7	7	0	0	0
9	844A	(CT)8 AC	10	5	3	2	30
10	814	(CT)8 TG	7	4	3	0	42
Total	97	61	24	10	24		

Table 10: Polymorphism percentage of AFLP primers EcoR1/Mse1.

	Selective nucleotides		Number of bands		
Primer comb.	EcoRI	MseI	Total	Polymorphic	% of polymorphism
	AGG	CTC	59	13	22.03

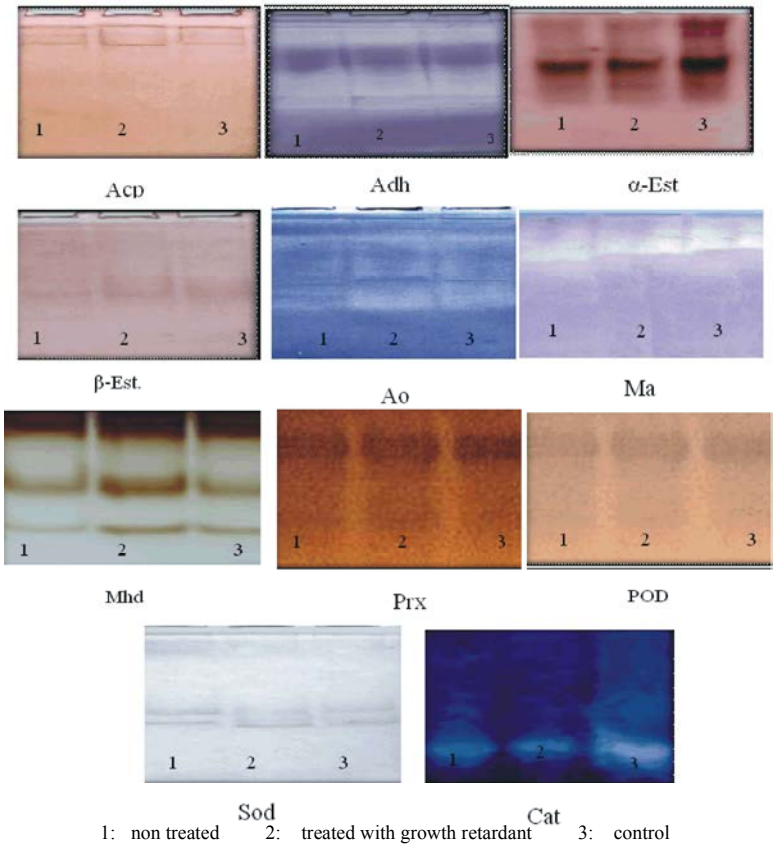
Table 11: Similarity matrix combined data of RAPD, ISSR and AFLP markers of three stevia samples.

--	1	2	3
1	100	--	--
2	80	100	--
3	79	95	100



M: protein marker 1: non treated 2: treated with growth retardant 3: control

Fig. (2): SDS-PAGE profiles of soluble proteins extracted from three stevia samples



1: non treated 2: treated with growth retardant 3: control

Fig. 3: Eleven isozymes banding patterns among the three stevia samples

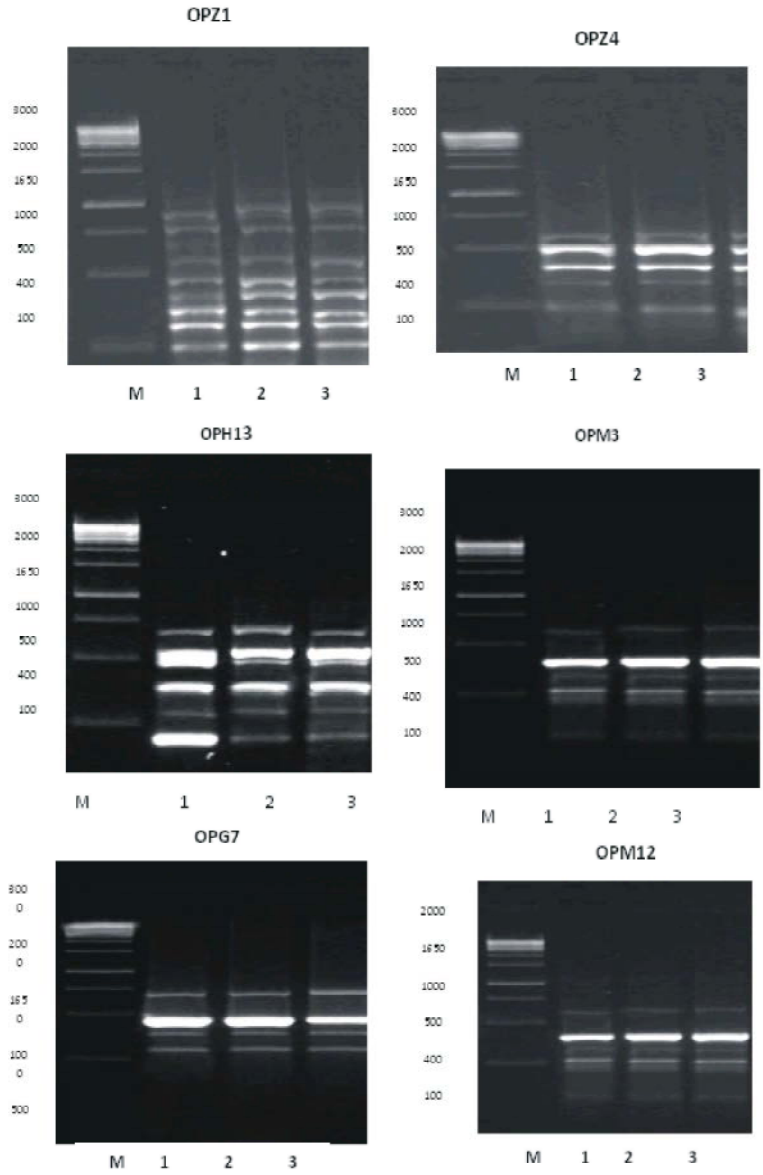
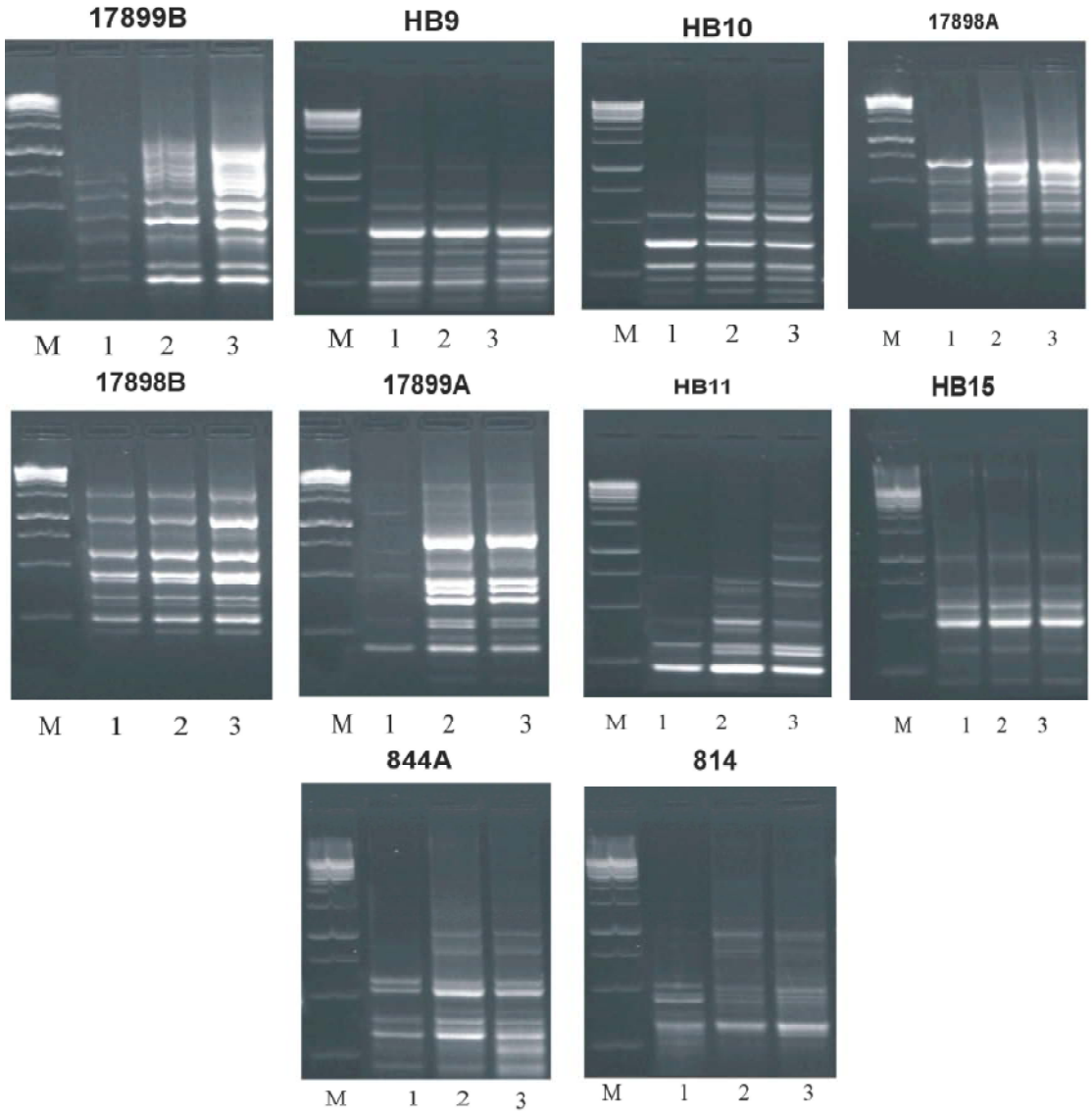


Fig. 4: RAPD analysis using six primers of three stevia samples.
M: DNA marker 1: non treated 2: treated with growth retardant

they are monomorphic bands (common bands) indicating the complete similarity in protein banding pattern among the three samples of stevia. The Hundred percentage similarity was detected among the three samples means that tissue culture treatments with growth retardants have no influence on stevia plantlets genotype.

Isozyme Analysis: Eleven isozyme systems including Acp (acid phosphatase), Adh (alcohol dehydrogenase), α and β Est (esterases), Ao (Alkaline phosphate), Ma (Malic acid), Mdh *(*malate dehydrogenase), Prx *(*peroxidase), POD (poly phenol oxidase), SOD (Superoxide dismutase) and CAT (catalase), were used to test the isozyme polymorphism among the studied stevia samples (Fig 3 and Table 7). The results showed a total of 26 bands using the eleven isozymes system; they were monomrphic and successfully confirmed genetic fidelity. Moreover, β-est gave five bands; it is the highest number of total bands comparing to the others isozymes. The results are agree with Regla *et al.* [43] who found no polymorphism in the isoenzyme profiles of *Bletia purpurea* (Lam.).



M: DNA marker 1: non treate 2: treatment with growth retardent 3: control

Fig. 5: ISSR primers of 17899B, HB9, HB10, 17898A, 17898B 17899A, HB11, HB15, 844A and 814

Identification of RAPD Marker: Six RAPD primers were used in the present study to identify the three stevia samples as shown in Fig 4 and Table 8. Twenty five bands were monomorphic and 17 were polymorphic (39%). Primer OPZ1 gave the lowest polymorphism (7.1%), while primer OPZ4 gave the highest polymorphism of 83.3%. In general, the results indicated that RAPD markers gave low polymorphism (39%), it could be ignored among three *stevia* samples. The obtained results from RAPD marker is in agreement with those reported by Esther *et al.* [44], who used RAPD for genetic evaluation of *Bletia purpurea* micropropagated plantlets. Comparison of RAPD genetic profiles for mother plants and regenerated plantlets showed little changes in the genome.

ISSR Analysis: Ten ISSR primers were used in the present study to differentiate between the three samples of stevia, as shown in Fig 5 and Table 9. Fifty five monomorphic bands and 24 polymorphic distinct fragments (24% polymorphism). The results showed that primers HB9 and HB15 gave no polymorphism, with 100% similarity. While, primers HB10 gave the highest polymorphism (57%) and lowest similarity.

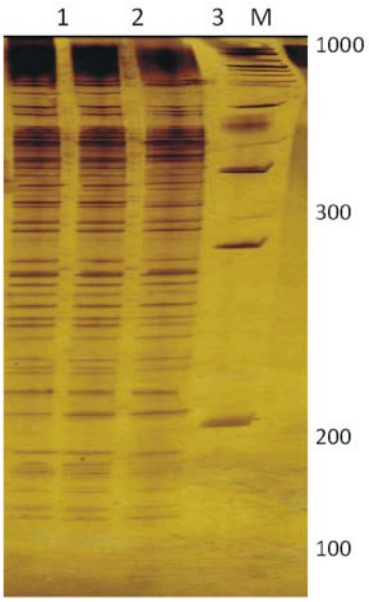


Fig. 6: Showing the AFLP fingerprints of three samples of stevia using one primer combination.
M: DNA marker 1: non treated 2: treatment with growth retardant 3: control

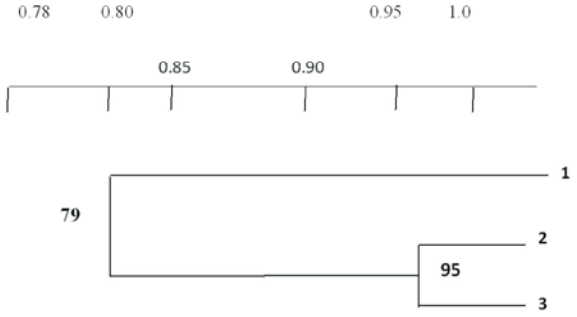


Fig. 7: Dendrogram of the three stevia samples from combined data of RAPD, ISSR and AFLP analyses.

This result is in agreement with those reported by Chandrika and Ravishankar [45], who employed ISSR markers to determine the genetic fidelity of *Ochreinauclea missionis* plantlets with twenty nine ISSR primers generated total of 183 clear, distinct and reproducible band of 2.73% polymorphism. Also, minor morphological variations were recorded in the micropropagated Date palm plants [46].

AFLP Analysis: A total number of 59 amplified fragments were obtained by using EcoI-AGG M MseI-CTC primer pair within a fragment size range of 59 to 476 bp (Fig. 6). Table 10 shows that thirteen polymorphic bands were obtained representing 22.03%.

Combined Data of RAPD, ISSR and AFLP Markers: Similarity matrix obtained from the combined data of RAPD, ISSR and AFLP, showed the closest relationship between the control and treatment with growth retardants of 95% (Table 11), while, the mother plant and the control treatment gave the lowest relationship of 79%. The dendrogram based on combined data separated the three samples into two main clusters. The first cluster contained only the mother plant, while, the second cluster included the treatment with growth retardants and control (Fig. 7).

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 in greenhouse. Growth retardant treatments proved to be optimum for improving rooting and survival of plantlets in acclimatization. Complementary genetic studies, successfully added genetic background and confirming the genetic fidelity of *in vitro* plantlets.

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